A new era in HEMOLYTIC UREMIC SYNDROME

DIAGNOSIS & TREATMENT OF A RARE DISEASE

Kioa L. Wijnsma

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A new era in hemolytic uremic syndrome
Diagnosis and treatment of a rare disease

The research presented in this thesis was carried out within the department of Pediatric Nephrology, Radboud university medical center Amalia Children's Hospital, Radboud Institute for Molecular Life Sciences, Nijmegen, the Netherlands.

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A new era IN HEMOLYTIC UREMIC SYNDROME

DIAGNOSIS & TREATMENT OF A RARE DISEASE

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Voor mijn ouders

"Noem mij, bevestig mijn bestaan, laat mijn naam zijn als een keten.
Noem mij noem mij spreek mij aan, o noem mij bij mijn diepste naam.
Voor wie ik liefheb wil ik heten."

Neeltje Maria Min

General introduction
and outline of the thesis

General introduction and outline of the thesis

A new era has commenced for the disease group referred to as thrombotic microangiopathy (TMA). TMAs are associated with significant morbidity and mortality, including end stage renal disease. Early recognition is important to allow rapid initiation of therapy. This holds in particular for a rare but severe form of TMA, the atypical hemolytic uremic syndrome (atypical HUS; aHUS). The elucidation of the role of complement in aHUS and the introduction of anti-complement therapy has greatly improved the outcome of aHUS. However, this particular therapy is accompanied with a high burden in costs. Optimizing differential diagnosis and treatment of the TMAs poses many challenges, and controversies remain.

Thrombotic microangiopathy

TMA is a heterogeneous group of disorders, characterized by vascular occlusion of capillaries due to thrombus formation, leading to thrombocytopenia, mechanical hemolytic anemia (low hemoglobin level together with high lactate dehydrogenase, schistocytes and undetectable haptoglobin) and ischemia of end organs.[1] Due to the immense network of capillaries intertwining the kidney, the kidneys are highly susceptible for the consequences of endothelial damage and vascular occlusion as result of the TMA.[2] Despite the fact that acute renal failure due to TMA can be often found, TMA is not solely restricted to the kidneys and other organs such as central nervous system are also often affected. The term TMA is a pathological description based on various characteristics found in a renal biopsy: fibrin and platelets rich thrombi in arterioles and capillaries, thickening and inflammation of the vascular wall, swelling and detachment of endothelial cells, reduplication of the capillary wall and subendothelial widening.[2]

In recent years advances have been made in unraveling this heterogeneous group of TMA. Yet, discrimination between the different causes of TMA remains challenging. Roughly, TMA can be divided into four subcategories, of which thrombotic thrombocytopenic purpura (TTP) and shiga toxin producing *Escherichia coli* (STEC) HUS are the most prevalent conditions (**Figure 1.1**).

Yet, clear differences can be noted between TTP and HUS. TTP is, besides the classical features of hemolytic anemia and thrombocytopenia, characterized by primarily neurological involvement instead of acute kidney injury. TTP originates from a defective cleavage of ultralarge von Willebrand factor multimers by ADAMTS13.[2,3] This defective cleavage can either result from pathogenic mutations in the *ADAMTS13* gene or via acquired auto-antibodies directed against ADAMTS13. Due to this impaired cleavage, microvascular thrombi arise, mainly consistent of von Willebrand factor and aggregated (unactivated) platelets. Treatment comprises plasmatherapy in combination with immunosuppressive drugs in case of auto-antibodies against ADAMTS13.

This in contrast to STEC-HUS and aHUS, in which the cascade leading to TMA commences through endothelial injury, leading to platelet activation and fibrin deposition. Hence, kidney injury characterized by fibrin-rich thrombi is the predominant manifestation. The scope of this thesis comprises both STEC-HUS as aHUS and these diseases are extensively discussed below. Besides these relatively frequent encountered forms of TMA, TMA also occurs secondary to a broad range of systemic disorders such as malignant hypertension, pregnancy, malignancy, infections (e.g. *S.pneumoniae* associated HUS), inherited disorders (e.g. Cobalamin C deficiency HUS) or intoxication. The mainstay of treatment is to treat the underlying disease causing TMA.[4,5]

Scope of this thesis

The diagnosis and treatment of STEC-HUS and aHUS are the main scope of this thesis. The most prevalent form of HUS is STEC-HUS, seen in over 90% of pediatric patients who present with HUS.[6] Only a small proportion (<10%) of patients presenting with HUS has aHUS. Yet, increasing awareness for aHUS has been raised over the last decade. Defining the role of complement and especially complement dysregulation as the cause of aHUS, can be appointed as a watershed moment.[7] By unraveling the pathophysiology of aHUS, new complement targeted therapies are emerging, ultimately leading to the golden age for complement therapeutics, pursuing the success of the first complement inhibitor: eculizumab.[8,9] The clinical presentation of STEC-HUS and aHUS shows a high degree of resemblance. Moreover, since aHUS is a diagnosis *per exclusionem* but treatment comprises the highly expensive orphan drug eculizumab, establishing STEC infections, as well as other causes of TMA, with high accuracy is indispensible.[10] As treatment of STEC-HUS is still supportive, these divergent treatment options underline the need for differentiation.

The thesis is divided into two parts, both new aspects of pathophysiology and diagnosis of STEC-HUS as well as treatment of aHUS will be discussed within this thesis. In **Part I** of this thesis we focus on the clinical conundrum of differentiating between STEC-HUS and other forms of TMA like aHUS. Although STEC-HUS is one of the most common causes of acute kidney injury in children, the exact pathogenesis leading to TMA and the high diversity in clinical severity is still only partially understood. Besides endothelial injury caused by among others Shiga toxins derived from STEC, other factors could contribute or enhance the process leading to TMA. Secondly, the current gold standard to detect STEC infection consists of fecal diagnostics. However, sensitivity of fecal diagnostics is limited due to the natural course of the disease. The aim of this part of the thesis is to further elucidate pathophysiology and improve diagnostics of STEC-HUS.

In **Part II** we focus on strategies to personalize treatment in aHUS patients. After the introduction of eculizumab in 2011, a worldwide debate arose regarding the optimal treatment dose and duration. Initial guidelines suggested lifelong treatment with a fixed dose for all patients. However, there is no evidence to support this strategy, raising the question if one could taper or even discontinue eculizumab therapy in aHUS patients. Furthermore, little is known regarding the pharmacokinetic and pharmacodynamic properties of eculizumab, indicating a gap in the knowledge to optimize eculizumab treatment in patients with aHUS. The aim of this part of the thesis is to study the ability to taper and withdraw eculizumab therapy in aHUS patients. Furthermore, pharmacological data are gathered to individualize treatment, ultimately achieving tools for tailored patient care and hereby personalize treatment in patients with aHUS.

Figure 1.1 Differential diagnosis of thrombotic microangiopathy

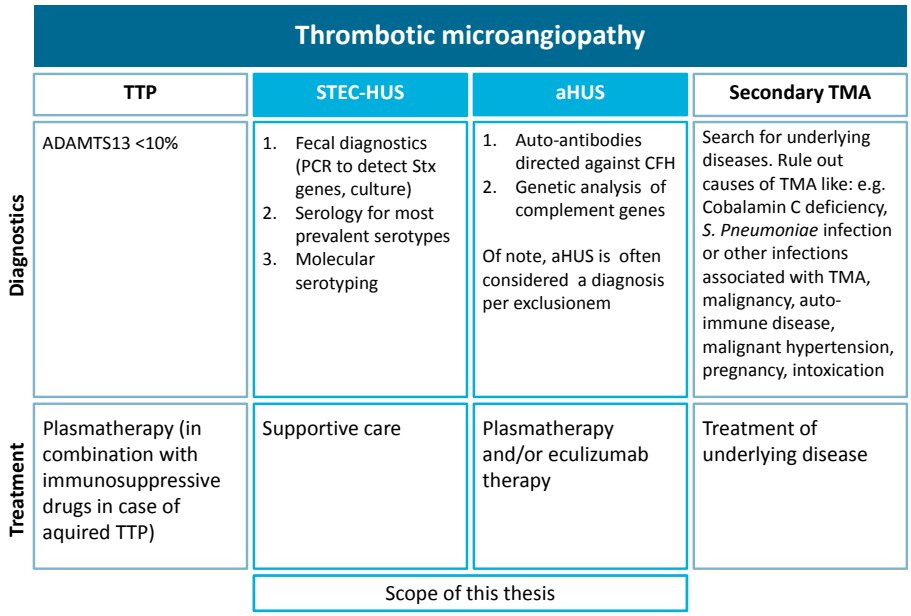


Figure 1.1 A heterogeneous group of diseases can lead to thrombotic microangiopathy (TMA). Thrombotic thrombocytopenic purpura (TTP) and Shiga toxin producing Escherichia coli (STEC) hemolytic uremic syndrome (STEC-HUS) are the most prevalent conditions associated with TMA. It is highly important to differentiate between different causes of TMA to start the appropriate treatment. ADAMTS13; a disintegrin and metalloproteinase with thrombospondin type 1 motif member 13, aHUS; atypical hemolytic uremic syndrome, CFH; complement factor H, STEC; Shiga toxin producing E.Coli, Stx; shiga toxin

Shiga toxin producing E. coli-HUS (STEC-HUS)

Epidemiology

Transmission of STEC occurs via a wide variety of contaminated food (among others undercooked beef and raw products), contaminated water and directly via person-to-person transmission. In particular, cattle is a well known STEC resevoir. STEC is a non-invasive pathogen. Gastro-intestinal infections caused by STEC are usually sporadic cases, although large outbreaks have been reported, with the German outbreak in 2011 as most noted example.[11,12] Overall, the incidence is about 0.6-0.8/100,000 in children and adolescents.[5,6] In the Netherlands, this accounts for approximately 20 new cases of STEC-HUS annually.[13] There are many STEC serotypes known to cause HUS. Although the distribution of STEC serotypes causing HUS has changed considerably, O157 remains the most prevalent serotype and accounts for 50% of the STEC-HUS cases. Common non-O157 serotypes are: O26, O103, O111 and O145.[14]

Clinical presentation

In all children presenting with HUS, over 90% can be related to STEC infection. Typically, young children (boys and girls equally affected) between six months to five years of age present with STEC-HUS (50% of all STEC-HUS cases).[15,16] After ingestion of the STEC bacteria, with an incubation period of three days (ranging from 2-12 days), patients start to develop symptoms (**Figure 1.2**).[17] The prodomal phase usually begins with abdominal cramping and non-bloody diarrhea (~90%), often accompanied by vomiting (~50%) and fever (~30%).[6,18] Typically, after a couple of days, the diarrhea becomes bloody (>80%) after which patients seek medical attention. Approximately 15% of the patients with an STEC infection will develop TMA (5-10% of sporadic cases and about 20% during outbreaks), usually 3-13 days after the start of the symptoms.[6] Diarrhea stops usually within one week. Moreover, 10% of the patients with STEC-HUS does not report diarrhea as preceding symptom. Besides the typical features of hemorrhagic colitis and renal failure, the central nervous system is often involved (~20%). Furthermore, extra-renal manifestations such as pancreas involvement resulting in diabetes mellitus, cardiac involvement and in rare cases skin, eye, or pulmonary involvement have been reported.[6,18]

Diagnosis

Since aHUS is a diagnosis *per exclusionem,* making the correct diagnosis of an STEC infection has major implications for treatment, the more after the introduction of the orphan drug eculizumab as treatment for aHUS. A rapid and accurate diagnosis of STEC infection in patients with HUS is highly important considering appropriate public health response, treatment and outcome.

Currently, fecal diagnostics is the gold standard to diagnose STEC infection, which comprises both polymerase chain reaction (PCR,) to detect genes encoding for Shiga toxins (*Stx*) and in addition the attaching and effacing protein, intimin, and feces culture using a Sorbital MacConkey agar plate.[19] To improve STEC detection, an enrichment step is often needed.[20] However, in up to 30% of the patients with strong clinical suspicion of STEC-HUS, fecal diagnostics remains negative.[21-23] Hence, the discrimination between STEC-HUS and other causes of TMA like aHUS, based on clinical course of the disease can be challenging.

Various factors could explain this high number of negative fecal diagnostics, most having to do with the interval between presentation and the onset of symptoms (**Figure 1.2**). Most patients (and/or caregivers) seek medical attention when the bloody diarrhea starts, usually two to three days after the onset of symptoms. However, the bacterial load decreases quickly after a few days and some STEC bacteria could even lose their Stx during some stage of infection or upon subcultivation.[24,25] Moreover, only a low inoculum is needed to cause disease, making detecting of STEC in feces by either PCR or culture more difficult even in the first few days. Collection of feces could also be difficult since the diarrhea prodrome has often ceased at time of presenting with HUS. Even new techniques like whole genome sequencing depend on culture of the strain, hereby limiting the diagnostic yield.[20] Other diagnostic assays have been described, such as the detection of Stx concentration in blood, either bound or free, yet need to be further validated.[26-28]

To complement diagnostics and to enhance detection of STEC infection, serology against different STEC serotypes has been recommended.[29-31] By determining the presence of Immunoglobulin M (IgM) antibodies against different STEC serotypes, STEC-HUS can be verified. IgM can be detected after 3-5 days up till two months after start of the symptoms and hereby broadens the time window to detect STEC infection (**Figure 1.2**).

In **CHAPTER 2** we study the added value of serology to detect antibodies against STEC serotype O157 in combination with fecal diagnostics. Yet, this lipopolysaccharide enzyme-linked immunosorbent assay (LPS-ELISA) appeared to have various drawbacks. The most prominent one is the potential cross-reactivity between different gram negative bacteria and between different STEC serotypes. Recently, a new serological antibody assay (glyco-iELISA) has been developed, tackling various limitations of the old assay.[32] In **CHAPTER 3** we discuss the advantages of this new sensitive serological method to detect STEC-HUS. We compare the LPS-ELISA with glyco-iELISA in a single center cohort comprising pediatric patients with STEC-HUS, as well as in nationwide cohort of patients with TMA. Yet, even with the introduction of serology, only a limited amount of serotypes can be detected. Fecal diagnostics with molecular methods, which takes advantage of whole genome sequencing to detect STEC isolates, are increasingly performed and provide additional data regarding sporadic and outbreak isolates.[20,33]

Due to improved diagnostic assays, more knowledge is gathered the non-O157 STEC serotypes causing HUS. In **CHAPTER 4** we describe a case of a 16 month-old boy with STEC-HUS, caused by an unusual *Stx*2d producing STEC O80:H2 which resulted in an unusual and highly severe disease course. Our case emphasizes the importance of adequate use of STEC diagnostic tests in all children with HUS, including those with an atypical presentation, and emphasizes the importance of molecular and serotyping assays to estimate the virulence of an STEC strain.

Pathophysiology

After oral ingestion, STEC colonizes the gut by translocation of their own receptor, Tir, into the gut epithelium. Subsequently, the formation of attaching and effacing lesions is stimulated.[34] Within a few days, STEC will express and release various virulence factors. One of the most important virulence factors is the Stx, a toxin composed of a single A subunit and a pentameric B subunit. The intact Stx is translocated across the epithelium into the blood via multiple routes including transcellular and paracellular transport.[35] There are two types of Stx, Stx1 and 2 respectively, each with various subtypes (Stx1a, Stx1c, Stx1d, Stx2a, Stx2b, Stx2c Stx2d, Stx2e, Stx2f, Stx2g).[36] However, in the evolvement of STEC infection to HUS, Stx2 plays a prominent role due to its higher degree of pathogenicity.

When Stx is released into the bloodstream, it can bind to several circulating cells (e.g., monocytes, erythrocytes and platelets), serving as Stx carriers to target organs. Upon binding, cells are activated and shed microvesicles which are pro-inflammatory and pro-thrombotic.[37] With its B subunit, Stx can bind to target cells via the globotriaosyl ceramide (Gb3) receptor on the cell membrane. Depending the density of Gb3 on cells, various tissues have different degrees of susceptibility. Especially the endothelium of the kidney, but also intestine and brain express the Gb3 receptors, explaining the characteristic symptoms of STEC-HUS with acute renal failure, hemorrhagic colitis and neurological involvement in some patients.[6] After binding to its receptor, retrograde transport of the A subunit through the Golgi apparatus to the endoplasmic reticulum occurs. Subsequently leading to inhibition of protein synthesis in the ribosome, resulting in the end in cell death, as such initiating the cascade leading to TMA.[35,37]

Figure 1.2 Timeline of development of STEC-HUS

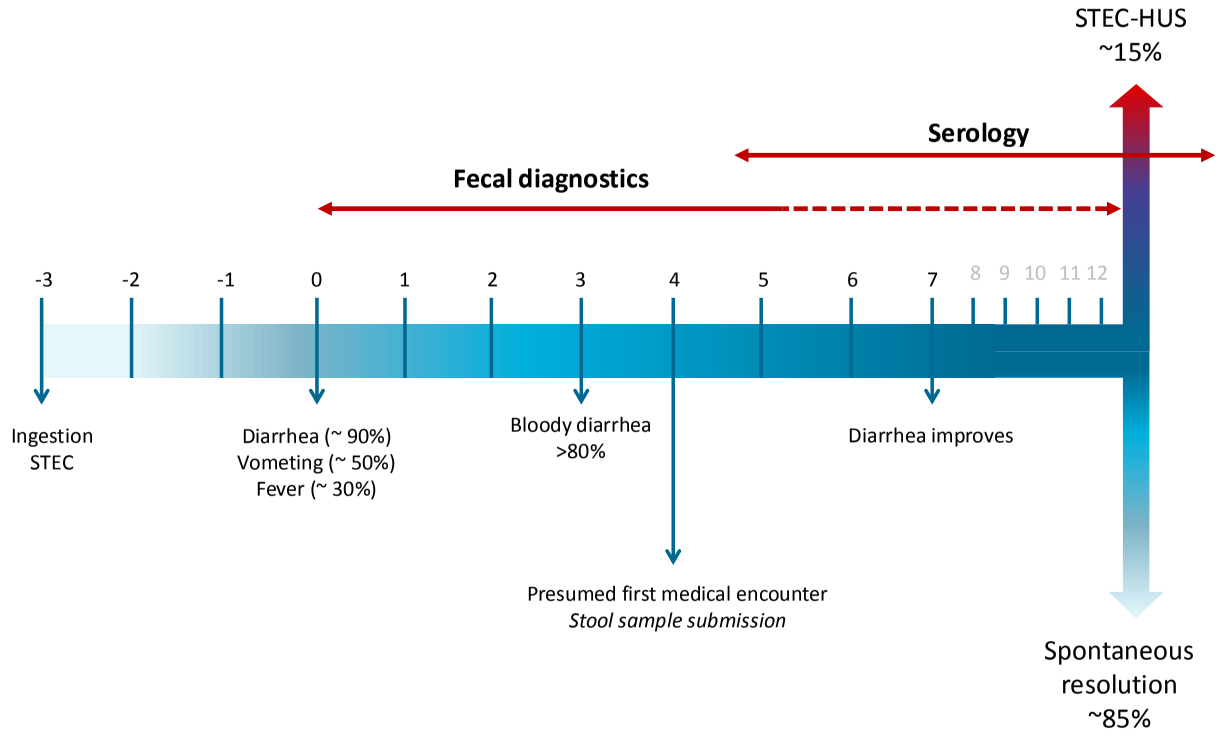


Figure 1.2 The time from ingestion of STEC to development of HUS is shown. Gastro-intestinal symptoms start 2-3 days after ingestion of STEC and diarrhea become bloody after an additional 1-3 days. At the time of bloody diarrhea most patients seek medical attention, and the first stool samples are collected. However, the chance of finding STEC in feces declines quickly after the start of the symptoms and becomes increasingly difficult 5-7 days after the start of the symptoms. When fecal diagnostics is combined with serology, the chance of finding STEC increases. Especially seven days or more after the start of the symptoms, serology has an clear added value. Adapted from Tarr et al.[17] (Lancet 2005; 365:1073-1086)Copyright Elsevier. STEC; Shiga toxin producing E. coli, HUS; hemolytic uremic syndrome

Only 15% of all patients infected with STEC will develop HUS, which can most likely be explained by a complex interplay between pathogen, host and environment, ultimately resulting in TMA. Various contributing pathogenic mechanisms have been described influencing the cascade leading to TMA. Enhancement of the effect of Stx release leading to TMA possibly occurs via various pathways like the interaction of Stx with von willebrand factor, hereby decreasing ADAMTS13 mediated cleavage resulting in a pro-thrombotic state.[38] Also, signs indicative of increased complement activation have been described in STEC-HUS. However, the contribution of complement activation in the pathophysiology of STEC-HUS remains controversial and a topic of discussion.[6,39-42] We hypothesized that the noxious molecule heme, as released during extensive hemolysis in the acute phase of disease, could enhance TMA in patients with STEC-HUS. In **CHAPTER 5** we describe the contribution of systemic heme levels in the evolvement of STEC-HUS.

Treatment & outcome

STEC-HUS is considered a relative benign disease when compared to aHUS, and is self-limiting. Patients are merely treated symptomatically. Nevertheless, 30% of the patients have chronic sequelae such as proteinuria, hypertension and/or diminished estimated glomerular filtration rate at five years follow up.[23] Overall, only a small proportion (<5%) of patients will progress to end stage renal disease and death is reported in up till 3% of the patients in the acute phase.[23]

Atypical HUS

Clinical presentation

Atypical HUS is an orphan disease, with an estimated incidence of 1-2 cases per million.[10,43] Unlike STEC-HUS, aHUS is known for its relapsing and hereby chronic character. The first onset of aHUS appears slightly more often during childhood (60%). Within the pediatric patients, half of the patients experience their first episode before the age of seven years. This corresponds with the typical age to develop STEC-HUS, making differentiation based on age less favorable.[6,44]

Atypical HUS is usually preceded by a triggering event leading to complement dysregulation. In 84% of the children with onset of aHUS a trigger could be identified. Gastro-intestinal symptoms were reported most frequently in 74% of the patients, with diarrhea in 25% of the patients. Respiratory tract infections were reported in 45% of the patients.[44] Pregnancy is a frequently reported trigger during adulthood. Moreover, clinical differentiation between different etiologies of TMA can be highly difficult, since aHUS can also be triggered by among others infections, malignant hypertension, vaccinations, malignancy, drugs, each also associated with secondary TMA (**Figure 1.1**).

Extra-renal manifestations are also seen in aHUS. Neurological involvement is frequently observed in 10-48% of the patients and usually comprises seizures and unconsciousness.[45] Pancreatitis (8%) is a common complications and elevated liver enzymes are observed frequently (>40%). Cardiovascular (10%) and respiratory involvement (7%) are less common.[46] In rare cases, gangrenous lesions of fingers and toes have been reported.[45]

Diagnosis

In 5-10% of the aHUS cases serological auto-antibodies against complement factor H (CFH) could be detected to diagnose aHUS.[47] These auto-antibodies can bind to circulating CFH and are mainly directed against the C-terminal part of CFH hereby inhibiting cell surface protection against complement activation. The first onset is usually at 5-15 years of age and mimicks the typical presentation of aHUS.[47] Furthermore, genetic analysis should be performed (**Table 1.1**). With the use of more comprehensive genetic screening tools, new mutations associated with development of aHUS are still discovered. For example, with the use of multiplex ligation-dependent probe amplification (MLPA) various hybrid genes of *CFH* have been discovered, associated with aHUS.[48,49] Still, at present in only 60-70% of the patients with aHUS, a genetic cause or presence of auto-antibodies can be found explanatory for aHUS. Most aHUS cases are sporadic, but in approximately 10-15% of the patients presenting with aHUS, there is a family history of aHUS.[10] Moreover, incomplete penetrance of mutations has been reported, indicating the need for additional triggers (either environmental or genetic) to develop disease manifestation. Patients who have

both a pathogenic mutations and are homozygous for one of the known haplotypes described in *CFH* or membrane cofactor protein (*MCP*), have an increased penetrance of approximately 50% to 70%.[50]

However, genetic analysis to screen for pathogenic mutations in complement genes can take weeks and consequently cannot inform therapeutic decisions. More research to discover potential biomarkers to diagnose and monitor disease activity in patients with aHUS is needed. C3 levels in plasma can be both normal as decreased during the acute phase of disease. The C3d/C3 ratio could help to differentiate between STEC-HUS and aHUS, yet needs to be further evaluated.[42] Therefore, despite the sharp increase in knowledge regarding the complement system, the mainstay to diagnose aHUS is to exclude other forms of TMA.

Pathophysiology

With over 40 soluble and membrane bound proteins, the complement system is an important part of innate immunity and facilitates the elimination of pathogens.[51,52] Via respectively the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP), the complement system exerts its function (**Figure 1.3**).

Following a specific trigger (e.g. antibodies and mannose residues in resp. CP and LP), complement proteins are activated. All pathways converge at complement component C3. With help of C3 and C5 convertases, splicing products of C3 (C3a and C3b) and C5 (C5a and C5b) are generated.[51,52] Ultimately leading to three major functions of the complement system: opsonisation of pathogens/cells (via C3b), chemotaxis (C3a and C5a) and cell lysis (via assembly of C5b, C6, C7, C8 and C9 to form the lytic pore C5b-C9). In contrast to the other two pathways, the AP is permanently active at low levels. It is mainly the dysregulation of this latter pathway which is the culprit in aHUS.[51,52]

Due to spontaneous hydrolysis of C3 (known as the tick-over) resulting in formation of C3 convertases, the AP has the ability to create an amplification loop and produce an excess of C3 convertases. To prevent complement overactivation or dysregulation, the complement system is tightly controlled by various regulators (**Figure 1.3**). Most regulators have a role in the AP, and exists both in fluid phase as well as membrane bound to host cells.[51,52] Various mutations in (regulator) complement proteins of the AP are associated with development of aHUS (**Table 1.1**).

Treatment

Once diagnostic bloodsamples have been gathered, it is highly important to start treatment as soon as possible to reduce chronic sequelae. Plasma exchange (PE) was considered the cornerstone of treatment in aHUS since the early nineties. The complement balance could be restored with PE by removal of auto-antibodies or mutant overactive proteins

and delivery of nonmutant complement proteins via plasma infusion.[6,57,58] Nowadays, PE in combination with immunosuppressive drugs is only recommended for aHUS patients with proven auto-antibodies directed against CFH to reduce the titer of antibodies.[57]

With the introduction of the first complement inhibitor eculizumab in 2011, the prognosis of aHUS changed dramatically. Based upon two prospective, open label studies, eculizumab became the fist-line treatment for aHUS.[8]

Eculizumab is a monoclonal antibody directed against complement component C5, blocking the splicing of C5 into C5a and C5b, and subsequently the formation of the terminal complement complex C5b-C9 (**Figure 1.3**). Eculizumab is administrated intravenously every two weeks to maintain complement blockade. However, the necessity of a fully blocked complement, maintained with lifelong treatment to prevent aHUS relapse, need yet to be established. Furthermore, surprisingly little is known regarding pharmacological characteristics of eculizumab. In **CHAPTER 6** we describe serum eculizumab trough levels in relation to complement blockade in 11 aHUS patients during standard therapy and tapering. In **CHAPTER 7** we review current knowledge on pharmacokinetics and pharmacodynamics of eculizumab and we discussed the potential of therapeutic drug monitoring to improve treatment and reduce costs.

Of note, eculizumab is one of the most expensive orphan drugs on the market. Following the introduction of eculizumab, a worldwide debate arose regarding the optimal treatment duration, particularly in view of the high costs and potential side effects of eculizumab. Some case reports suggested that a restrictive treatment regimen was feasible. In **CHAPTER 8** we describe a cohort of 20 aHUS patients, treated with a restrictive eculizumab regimen. In **CHAPTER 9** we review the current literature and focus on the safety, efficacy and feasibility of restrictive use of eculizumab.

Figure 1.3 Schematic overview of the complement system

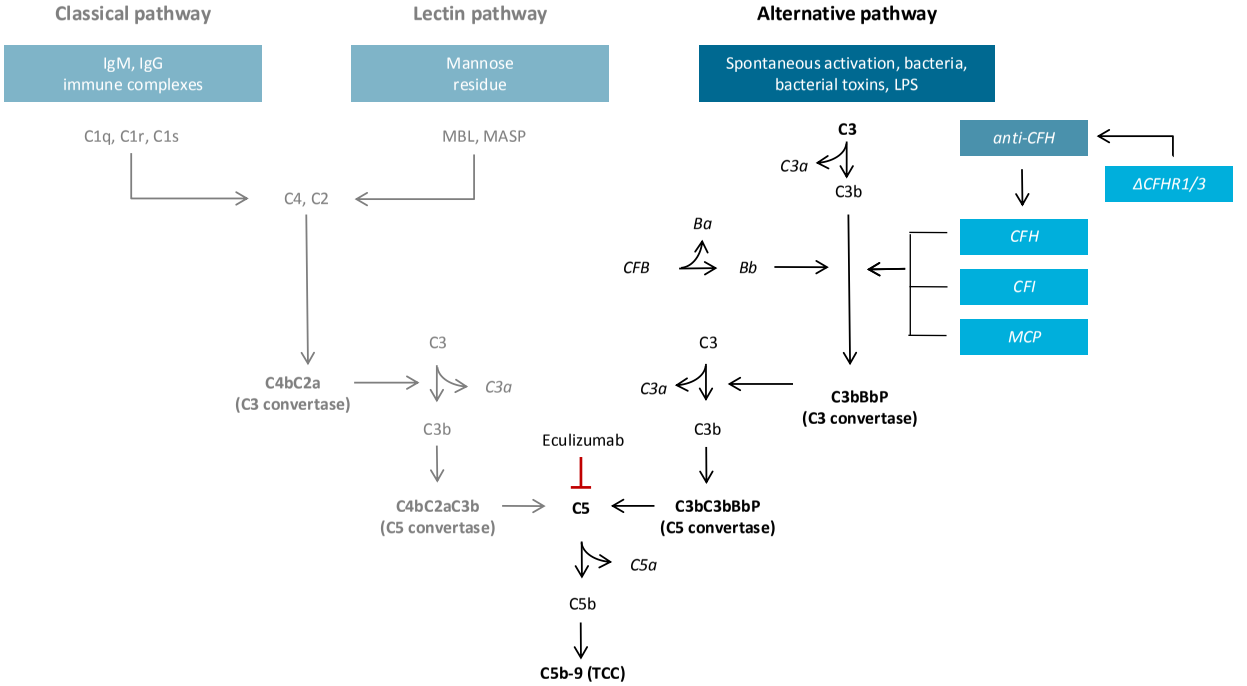


Figure 1.3 The complement system consists of three pathways. The culprit in aHUS is a dysregulation of the alternative pathway. The alternative pathway can be activated by a specific trigger, but can also be spontaneously activated. Upon activation C3 convertases and C5 covertases are formed, ultimately leading to the formation of the terminal complement complex, the lytic pore C5b-C9. To regulate the complement system, various complement regulatory genes are involved such as CFH, CFI and MCP. Eculizumab is a monoclonal antibody that binds to C5, hereby blocks the splicing of C5 into C5a and C5b, consequently inhibiting the formation of C5b-C9.
Adapted from Westra et al.[59] (Neth J Med 2012; 70:121-129) Copyright MacCahin.
anti-CFH; auto-antibodies against factor H, CFB; complement factor B, CFH; complement factor H, CFHR; complement factor H related, CFI; complement factor I, IgG; Immunoglobulin G, IgM; Immunoglobulin M, MCP; membrane cofactor protein, LPS; lipopolysaccharide, TCC; terminal complement complex

Table 1.1 Mutations in complement (regulator) proteins involved in development of aHUS

Mutation in gene encoding for*	Type of mutation
Factor H (CFH)	Loss-of-function
Factor H related proteins 1-5 (CFHR1-5)	
Homozygous CFHR3-1 deletion	Deficiency of CFHR 3 and 1
Homozygous CFHR1-4 deletion	Deficiency of CFHR 4 and 1
Factor H/CFHR1 hybrid	Genetic rearrangement
Factor H/CFHR3 hybrid	Genetic rearrangement
Membrane cofactor protein (MCP; CD46)	Loss-of-function
Factor I (CFI)	Loss-of-function
C3	Gain-of-function
Factor B (CFB)	Gain-of-function

NA; Not applicable, Ref; Reference

** Besides these mutations in complement genes, other mutations have been described in relation to aHUS.*

	Function of protein	Prevalence in aHUS	Typical characteristrics clinical presentation	Ref
	· Competes with CFB for binding to C3b (to form C3 convertase; C3bBb) · Cofactor for CFI to inactivate C3b · Accelerates decay of C3 convertase	20-30%	-	53,54
	Complement regulatory activities	Unknown		
	-	3-10%	87% of patients with auto-antibodies against CFH has this deletion	49,55
	-	Unknown	Associated with auto-antibodies against CFH	49
	Due to formation CFH/CFHR1 hybrid, normal CFH binding to cell surface is inhibited	3-5%	-	6,49
	Due to formation CFH/CFHR3 hybrid, normal CFH binding to cell surface is inhibited	Unknown	-	49
	Cofactor protein for CFH to inactivate C3b	5-15%	Usually first presentation during childhood with median age of 5 years	53,54,56
	Inactivation of C3b which is no longer able to bind CFB	4-10%	-	53,54,56
	Central complement component, needed to build convertases and produce C3a and C3b	2-10%	-	53,54
	By binding of CFB (Bb part) to C3b, C3 convertases are build	1-2%	Almost always low serum C3	53,54

However, these mutations in the genes DGKε, trombomodulin, plasminogen and inverted formin 2 are not described here since there role within complement dysregulation is not yet known.

Dutch guideline regarding diagnosis and treatment of TMA

In the Netherlands, a unique initiative was undertaken by a group of experts assembled in the national aHUS working group. In 2016, a new national guideline regarding diagnosis and treatment of patients who present with TMA, funded on the same principles as described in chapter 9, was implemented by this working group.[4] This working group consists of one nephrologist and one pediatric nephrologist from every university medical center in the Netherlands. Together they discuss all patients treated with eculizumab and explore options to taper or withdraw therapy. Simultaneously with the implementation of this new guideline, which advocates a restrictive treatment regimen, eculizumab had to be reimbursed by the National Healthcare Institute. After careful consideration and reviewing all data present, the Ministry of Health, Welfare and Sport decided to no longer reimburse eculizumab when administered following the pharmaceutical treatment scheme; every fortnight as lifelong treatment. Yet, they decided to temporary reimburse eculizumab for the indication of aHUS when administered according the new Dutch guideline, for another four years.

In the Netherlands several criteria must be met before orphan drugs can be (re) imbursed. A so called orphan drug arrangement was implemented with the aim to guarantee healthcare access for everyone, promote efficient and purposeful usage of orphan drugs, and to optimize

(cost-)effectiveness of orphan drugs like eculizumab. The first criteria is the presence of an indication committee, such as the national working group aHUS. Secondly, there have to be clear start and stop criteria, as described in the new guideline. Finally, these patients have to be monitored in a registry to evaluate efficacy of the drug and to perform a cost-effectiveness analysis.[60]

In **CHAPTER 10** we describe the protocol of the national observational study to monitor and evaluate this new guideline concerning treatment of aHUS, called CUREiHUS. With this study, all aHUS patients treated according to the new guideline are monitored in a database. Furthermore a cost effectiveness analysis and budget impact analysis will be conducted at end of the study. The study estimated end date is December 2020. Consequently, treatment with eculizumab is reimbursed by the National Healthcare Institute for four years, with the restriction that all patients (when possible and after informed consent) are included in the CUREiHUS study.

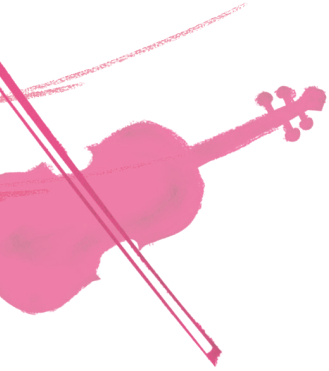
Finally, **CHAPTER 11** provides a summarizing discussion together with future perspectives regarding the research described in this thesis.

Part I

NEW ASPECTS OF PATHOPHYSIOLOGY AND DIAGNOSIS IN STEC-HUS

"It is far more important to know what person the disease had,
than which disease the person had"

Hippocrates



Chapter 2

Fecal diagnostics in combination with serology: best test to establish STEC-HUS

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Abstract

Background

In the majority of pediatric patients, the hemolytic uremic syndrome (HUS) is caused by an infection with Shiga toxin-producing *Escherichia coli* (STEC), mostly serotype O157. It is important to discriminate between HUS caused by STEC and complement-mediated HUS (atypical HUS) due to differences in treatment and outcome. As STEC and its toxins can only be detected in the patients stool for a short period of time after disease onset, the infectious agent may go undetected using only fecal diagnostic tests. Serum antibodies to lipopolysaccharide (LPS) of STEC persist for several weeks and may therefore be of added value in the diagnosis of STEC

Method

All patients with clinical STEC-HUS who were treated at Radboud university medical enter between 1990 and 2014 were included in this retrospective single-center study. Clinical and diagnostic microbiological data were collected. Immunoglobulin M (IgM) antibodies against LPS of STEC serotype O157 were detected by a serological assay (ELISA).

Results

Data from 65 patients were available for analysis. Fecal diagnostic testing found evidence of an STEC infection in 34/63 patients (54%). Serological evidence of STEC O157 was obtained in an additional 16 patients. This is an added value of 23 % ($p < 0.0001$) when the serological antibody assay is used in addition to standard fecal diagnostic tests to confirm the diagnosis STEC-HUS. This added value becomes especially apparent when the tests are performed more than seven days after the initial manifestation of the gastrointestinal symptoms

Conclusion

The serological anti-O157 LPS assay clearly makes a positive contribution when used in combination with standard fecal diagnostic tests to diagnose STEC-HUS and should be incorporated in clinical practice.

Introduction

Hemolytic uremic syndrome (HUS) is diagnosed when the characteristics of hemolytic anemia, thrombocytopenia, and acute renal failure are present.[17] There are different etiologies leading to HUS, with the Shiga toxin-producing *Escherichia coli* (STEC) HUS and complement-mediated atypical HUS (aHUS) being the most prominent ones. In more than 90% of cases, HUS follows a gastrointestinal infection with STEC. Additionally, over 50% of STEC-HUS cases are due to STEC serotype O157, although other serotypes, such as O26, O103, O145, and O111, are increasingly associated with HUS as well.[6,30,61,62] With symptomatic treatment, patients with STEC-HUS often recover spontaneously, with only a small number progressing to end-stage renal disease (ESRD) In contrast, aHUS generally has a poor outcome, with 2–10% mortality among patients in the acute phase of the disease and up to 50% of patients progressing to ESRD.[43]

It is therefore vital to be able to distinguish between STEC-HUS and aHUS, as this has major consequences in terms of treatment possibilities (for example, the use of the expensive orphan drug eculizumab, which is currently the standard treatment for aHUS) and outcome.[63] However, it can be challenging to differentiate clinically between these two entities due to their similar symptoms. For example, in 6–10% of children with STEC-HUS there is no (bloody) diarrhea, whereas aHUS is preceded by diarrhea in 25% of the cases.[43,44] As aHUS is often diagnosed *per exclusionum* providing proof for the presence of STEC forms the basis for differentiation between aHUS and STEC-HUS.[6] In most laboratories, an STEC infection can only be confirmed by an examination of fecal material, mostly through simultaneous testing of stool culture, shiga toxins immunoassays, and/or polymerase chain reaction (PCR) assays for the detection of shiga toxin (*Stx*) genes.[19] With stool cultures, the presence of an STEC can only be established in a limited number of patients (approx. 30–69%) with clinical signs and symptoms of HUS.[30,64] The additional use of PCR for detection of *Stx* genes increases the odds of finding evidence of an STEC infection up to 70%.[21,22] However, the presence of STEC in the intestines declines rapidly during the first week of the illness[19,65], whereas the average time between the first day of diarrhea and the development of HUS is 5–13 days.[6,17]

Over two decades ago, Chart et al. described the use of serological assays to detect antibodies against, among others, serotype O157 lipopolysaccharide (LPS) as a diagnostic tool to establish an STEC infection.[29,66-68] A few studies have subsequently shown that in patients with clinical signs and symptoms of HUS, for whom only a limited number of the stool cultures were positive, the results of serological testing for STEC were positive in 60–94% of the patients.[30,64,69] The most important explanation for this low number of positive stool cultures next to the low inoculums of the bacteria is the small time window when STEC can be detected in the feces. In con-

trast, the antibody response, composed of immunoglobulin M (IgM), can be detected from five days up to two months after the onset of the symptoms.[70,71]

Based on these characteristics, a broad application of serological assays for STEC may be expected. Additionally, in most of the microbiological studies conducted to date, various diagnostic techniques have been compared, using conventional and molecular methods; however, all of these methods depend on the presence of fecal material.[21,33] Hence, only limited data have been published on the combined use of fecal diagnostic testing and the serological antibody assay in patients with STEC-HUS. A review of the literature and discussion with colleagues suggested to us that interest in the serological antibody assay for the detection of an STEC infection has been largely neglected in past years.

In the study reported here, we assessed the added value of the serological anti-O157 antibody assay in combination with fecal diagnostic testing in pediatric patients with a clinical STEC-HUS. We also examined the time window between the onset of symptoms and testing for STEC using fecal diagnostic tests and a serological antibody assay.

Material and Method

This was a retrospective single-center study which included all patients who presented with a clinical pattern of STEC-HUS between 1990 and 2014 to the Pediatric Nephrology department of the Radboud University Medical Center Amalia Children's Hospital. All available clinical and diagnostic data were collected. The diagnostic data included the results of all fecal diagnostic tests [stool cultures, cell cytotoxicity assays [free fecal Stx test (FStx), used in our center until June 2011] and PCR analyses for *Stx* genes (from June 2011 onwards). An enzyme-linked immunosorbent assay (ELISA) was used as the serological assay for IgM antibodies against O157 LPS.[66]

A clinical pattern of STEC-HUS was defined as signs of a thrombotic microangiopathy, hemoglobin level below the lower limit of normal for a specific age, signs indicative of hemolysis, acute renal failure, thrombocytopenia of <150 × 109/l, and (bloody) diarrhea or family members with diarrhea. Clinical data at presentation and follow-up were collected from the medical records. Hypertension was defined as repeated blood pressure measurements above the 95th percentile for sex, height, and age.[72] Anuria was defined as a urine production of <0.1 ml/kg/h for at least 12 h, and oliguria was defined as a urine output of <0.5 ml/kg/h. Neurological involvement was indicated by apathy, irritability, reduced consciousness, seizures, paralysis, encephalopathy, and coma, and signs of pancreatic involvement included pancreatitis and diabetes mellitus. The estimated glomerular filtration rate (eGFR) was calculated with the Schwartz formula (k-coefficient = 36.5).[73] Extent of renal impairment was based on guidelines from Kidney Disease: Improving Global Outcome.[74] The onset of disease was defined as the first day of diarrhea. The onset of disease was defined as the first day of diarrhea. The time window of the fecal diagnostic tests and serological antibody assay was defined as the time between the onset of the symptoms and the collection of feces and/or serum.

Fecal diagnostics

Feces were collected as soon as possible after admission to the hospital. In cases where feces could not be obtained, a rectal swab was done. Fecal material was plated on Sorbitol MacConkey agar containing 1% sorbitol and on blood agar. After 24 hours of incubation, non-sorbitol fermenting colorless colonies were tested for agglutination with anti-O157 O-antigen serum. Until 2011, fecal samples were also tested for the presence of FStx using cell cytotoxicity assays as previously described by Karmali et al.[75,76] In June 2011, the PCR assay for the detection of *Stx*1, *Stx*2 and virulence genes, E. coli attaching and effacing gene (*eae*), and enterohemorrhagic E. coli hemolysin (*hly*) in feces replaced the time-consuming cell cytotoxicity assays as a standard test, in addition to the stool cultures. Nowadays, when a patient is suspected of HUS the feces is first tested for the presence of STEC with a PCR assay. When the assay results are positive, indicating STEC infection, a stool culture

on Sorbitol MacConkey agar plates is performed. To further determine all strains, we send all isolates to the Dutch National Institute for Public Health and Environment (RIVM).

Anti-O157 LPS assay

Serum was obtained from all patients and stored at −80 °C until analysis. Sera were screened for antibodies against the LPS serotype O157 with an ELISA as previously described by Chart.[66] In brief, the ELISA plates were coated with LPS from E. coli O157:H7 [List Biological Laboratories Inc., Campbell, CA; product code 206, diluted in carbonate buffer (pH 9.6) to a concentration of 20 ug/ml]. After incubation overnight at 4 °C, the plates were blocked, and diluted serum was added to the plate together with predetermined positive and negative control sera. After addition of the antibody goat anti-human IgM (Sigma-Aldrich, St. Louis, MO; product code A0420, diluted 1/500 with phosphate buffered saline with bovine serum albumin) and substrate (p-nitrophenyl phosphate tablets in diethanolaminebuffer), the absorbance was measured at 405 nm. A positive IgM reaction was defined by a mean absorbance of >0.800; values of <0.400 were to be considered negative and values between 0.400 and 0.800 were considered to be dubious and as such taken to be negative for the purpose of this study.

Statistics

All available clinical variables for each patient were included in the analysis. Clinical values were expressed as valid percentages for categorical variables and as the mean and standard deviation or median and 25–75 percentile (interquartile range; IQR) for continuous variables, as appropriate. Values for the serological assays, stool cultures, FStx, or PCR that were inconclusive or missing were classified as a negative test result. The Chi-square test was performed to compare categorical data and the two-sided t test or Mann–Whitney U test was used to compare continuous data. To compare positive and negative test results in relation to the time since onset of disease, we used binary logistic regression analyses, and dummies were computed for the categorical variables. P values of <0.05 were considered to be statistically significant.

Results

Patient characteristics

During the period between 1990 and 2014, 72 children with a clinical pattern of STEC-HUS were seen in the Radboud University Medical Center Amalia Children's Hospital. Seven patients were excluded because no data on feces and serology were available for further analysis. The patient characteristics of the 65 children with a clinical pattern of STEC-HUS are described in **Table 2.1**. The majority of patients (79%) presented with STEC-HUS before the age of six years. All but two children had diarrhea at presentation (97%), which was generally bloody diarrhea (79%). One patient died in the acute phase of the disease from a systemic inflammatory response syndrome combined with severe STEC-HUS, as proven by positive results for both the fecal diagnostic tests and the serological assay. Another patient, with proven STEC-HUS based on fecal and serological tests, did not show any recovery of renal function and subsequently was placed on hemodialysis before undergoing kidney transplantation.

Serological assays in addition to fecal diagnostic tests

Evidence of an STEC infection was found in 50 patients (77%) of the 65 patients with a clinical pattern of STEC-HUS. Fecal diagnostic tests identified STEC in 34/63 patients (54%); in 26 of these 34 patients a STEC strain could be isolated with culture. Serological evidence of an STEC O157 infection was found in an additional 16 patients, which is an added value of 23% ($p < 0.0001$) when fecal diagnostic tests are combined with the serological antibody assay to confirm the diagnosis of STEC-HUS (**Figure 2.1, Table 2.2**). Among those patients with negative stool cultures, the PCR assay was positive for STEC-HUS in three patients and the cell cytotoxicity assay (FStx) was positive for STEC-HUS in five patients. Since implementation of the PCR assay in 2011, *Stx* genes have been detected in nine (69%) of the 13 patients who presented with HUS. Of these nine STEC infections, seven were also detected by serological assays. Three additional patients who tested negative for *Stx* genes in repeated PCR assays showed serological evidence of an STEC infection (**Table 2.2**).

Two additional serotypes, serotypes O26 ($n = 3$) and O5, were detected in fecal specimens from three and one patient, respectively, sent to the RIVM for further testing.

Table 2.1 Patient characteristics of 65 pediatric patients with clinical STEC-HUS

Parameter	All patients
Male	52%
Age of onset in years	2 (1 - 4)
0-24 months (n=18)	17 (10 - 20)
2-6 Years (n=33)	3 (2 - 4)
≥ 6 Years (n=14)	11 (9 - 12)
Symptoms at presentation	
Fever	27%
Diarrhea, total	97%
Of which bloody	79%
Oliguria	29%
Duration in days (n=5)	5 (3 - 8)
Anuria	59%
Duration in days (n=26)	8 (7 - 10)
Blood pressure	
< p95	34%
≥ p95	66%
Neurological involvement	34%
Pancreas involvement	3%
Biochemical evaluation at presentation (reference range)	
Hemoglobin *(6.0 – 9.0 mmol/l)*	5.8 (4.8 - 6.7)
White blood cells *(5.0 - 13.0 x 10^9/l)*	15.3 (10.9 - 22.3)
Platelet count *(210 - 430 x 10^9/l)*	47 (30 - 73.5)
Haptoglobin *(0,3-1,6 g/l)*	<0.08 (0.05 - 0.08)
LDH *(<250U/l)*	3720 (2325 - 5809)
eGFR *(>90 ml/min.1.73m^2)*	15 (10 - 24)
Treatment	
Dialysis	63%
Duration of dialysis in days	10 (7 - 15)
Erythrocytes transfusion	80%
≥ 3 transfusions	11%
Follow up	
eGFR at discharge *(ml/min.1.73m^2)*	55 (43 - 66)
eGFR<60 after 5 years follow up (n=8) (*ml/min.1.73m)*	4 (6%)
Received Kidney transplantation	1 (2%)
Diseased	1 (2%)

Categorical values are expressed as absolute number and percentage of total and for continuous variables the median with interquartile range (IQR) is expressed. The numbers of patients for whom data were available are reported in parentheses. P95: percentile for age and height.

Table 2.2 Proportion of positive serology for serotype O157 in relation to the fecal diagnostics

Diagnostics	Positive Serology	Negative Serology	Total (%)
Serology	38 (63%)	22 (37%)	60
Missing			*5*
Fecal diagnostics			63
Missing serology			*4 (6%)*
Positive	22 (65%)	12 (35%)	*34 (54%)*
Negative	15 (60%)	10 (40%)	*25 (40%)*
Feces culture			63
Missing serology			*4 (6%)*
Positive	18 (69%)	8 (31%)	*26 (41%)*
Negative	19 (58%)	14 (42%)	*33 (53%)*
FStx, until 2011			33
Missing serology			*1 (3%)*
Positive	10 (59%)	7 (41%)	*17 (52%)*
Negative	9 (60%)	6 (40%)	*15 (45%)*
PCR, since 2011			13
Missing serology			-
Positive	7 (78%)	2 (22%)	*9 (69%)*
Negative	3 (75%)	1 (25%)	*4 (31%)*

Table 2.2 Test results from all 65 patients with clinical STEC-HUS where serology and fecal diagnostics were performed to confirm an STEC infection. The percentage of patients with positive or respectively negative serology is reported in parentheses. In a few patients, only fecal diagnostics are performed, the missing serology values are presented in the table.

Time window between onset of disease and sample collection

The median time between onset of disease and the collection of feces and serum was 7 (IQR 5–9) and 8 (IQR 5–12) days, respectively. The added value of serology became more evident when the fecal and serological diagnostic tests were run on specimens collected ≥7 days after the start of the symptoms (**Table 2.3**). Within seven days after the first manifestation of the symptoms, 14 patients had negative fecal diagnostic test results; of these, 5 (36%) had positive results on the serological assay, confirming a STEC infection. However, of the 12 patients with negative fecal diagnostic test results based on fecal specimens collected ≥7 days after the start of the disease, eight (67%) were found to test positive for O157 on the serological assay.

Hence, when feces and serology specimens were collected ≥7 days after the start of the symptoms, the serological antibody assay had an added value of 33%, which is significantly higher than the 14% added value when the specimens for testing were collected within seven days ($p = 0.024$).

Discussion

Various fecal diagnostic tests are recommended in the literature to establish an STEC infection as the cause of HUS including stool cultures, Stx immunoassays, cell cytotoxicity assays, and PCR for *Stx* genes. [19] However, limited data are published on the benefits of combining fecal diagnostic with serodiagnostic testing in patients with STEC-HUS. The results of our retrospective study show that the serological anti-O157 antibody assay is of additional value to the fecal diagnostic tests used in patients with a clinical STEC-HUS. In our study, use of the standard fecal diagnostic tests resulted in a STEC infection being detected in half of the HUS patients; in comparison, evidence of an STEC infection was detected in 77% of the 65 patients when serological testing was used in combination with the standard fecal diagnostic tests. The added value of serological testing further improves when the specimens for testing were collected ≥7 days after the start of the symptoms.

Figure 2.1 Serological anti-O157 antibody assay in addition to the standard fecal diagnostics.

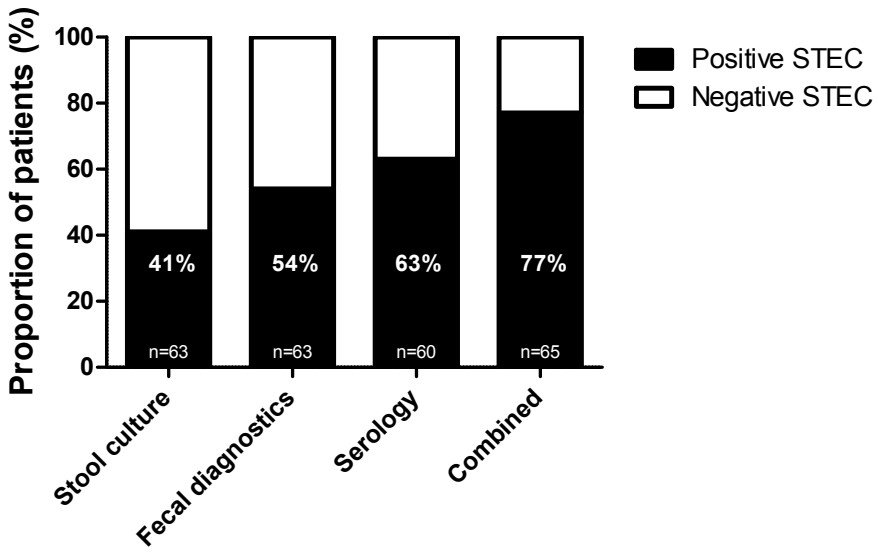


Figure 2.1 Test results from all 65 patients with clinical STEC-HUS where serology and fecal diagnostics were performed to confirm an STEC infection. When the fecal diagnostics (stool culture, cell cytotoxicity assay and/or PCR) are combined with the serological antibody assay, 77% of the patients has a confirmed STEC infection (p<0.0001).

Our results are in line with the study of Espié et al. who studied 900 children with HUS and tested for STEC by stool culture, PCR assay for *Stx* genes, and serological assays for multiple serotypes. [30] In 232 (37%) children, The presence of a STEC was confirmed in

232 (37%) children with a stool culture and in 37 patients *Stx* was detected with the PCR assay. STEC infection was confirmed in 518 (60%) patients based on positive serological results, of which 85% of patients had serotype O157. The combined use of fecal diagnostics and serology provided evidence of a STEC infection in 590 (66%) patients. Unfortunately, no further information on the time window was provided by the authors.

Table 2.3 Added value of serology in relation to the time window between onset of symptoms and collecting material

Diagnostics	**Proven STEC infection**		
	Total	*<7 days*	*≥ 7 days*
Fecal diagnostics	54% (34/63)	44% (11/25)	62% (20/32)
Serology	62% (38/61)	45% (9/20)	69% (25/36)
Combined: Fecal diagnostics & Serology	77% (50/65)	58% (7/12)	95% (20/21)
Added value of Serology	**23%**	**14%**	**33%**

Table 2.3 Collection of diagnostics in the first six days after the start of the symptoms are compared with seven days or more since onset and the influence on the detection of STEC infection is compared. The added value of serology becomes more evident as of seven days (p=0.024).

The fecal diagnostic tests (stool culture, cell cytotoxicity assay, and/or PCR) detected STEC in only half of our patients with a clinical pattern of STEC-HUS. A few factors may explain this low number of positive stool cultures, all of which are linked to the time of presenting in relation to the development of the disease. First, the isolation rate of STEC in feces declines quickly after the first manifestation of the gastrointestinal symptoms, and most parents seek medical attention after the first signs of bloody diarrhea (which is after approx. three days). Secondly, there is a low inoculum whereby the odds of finding a STEC in the feces is also limited during the first seven days. The third and final factor is that the diarrheal prodrome has often ceased before the onset of HUS, making detection of the pathogen or its toxins rather difficult when the fecal speciment is collected at the time of presenting with a HUS.[17] Frequent stool collections may be considered one option to increase the chance to detect a STEC infection. Furthermore, new and promising techniques have been developed for the detection of STEC infection since the start of this retrospective study, as illustrated by the PCR assay for *Stx* genes, but also more recently by the molecular approach to assess the virulence profile and serotyping of STEC strains. In comparison to the other standard fecal diagnostic tests (e.g. stool culture and PCR), molecular testing is generally only performed in reference laboratories, as this approach is mostly used for epidemiological analysis.[19,20] The PCR assay for *Stx* genes was introduced only a few years ago in our hospital, and data on this assay were only available for a minority of patients in our study, although results are promising. Even though these techniques are more precise

in terms of detecting the presence of STEC, they still require fecal material for testing. Therefore, in cases without stool production, besides a rectal swab that is highly recommended, it may still be difficult to detect STEC.

In 15 (23%) of the 65 patients enrolled in our study, the results of the serological assay and the fecal diagnostic tests were negative for a STEC infection. One explanation may be that the techniques used were not sufficiently sensitive, especially because the majority of the specimens were tested before the implementation of the PCR assay. Another possible explanation is the timing of sampling, even though the time between the onset of symptoms and the collection of fecal specimens in our cohort is comparable with those of other studies.[30,77] In our study, only a serological antibody response against O157 was tested. O157 currently accounts for 30–80% of STEC-HUS cases in the Netherlands, and our serological assay could not detect other important serotypes.[15,23,30] Also, the serum samples may have been collected too early in the disease course—that is, prior to seroconversion. When these explanations appear to be unlikely, other causes of HUS, such as aHUS, should be taken into account. However, during follow up, none of the 15 patients with negative fecal diagnostic and serological test results presented with a relapse indicative of aHUS or showed signs of permanent complement dysregulation.

Our results clearly show that a patient with HUS in the absence of positive fecal diagnostic test results is not equivalent to one with aHUS. Moreover, it may very well be a STEC infection that is causing the HUS despite the negative fecal diagnostic test results. The need for regular serological antibody assays in patients with HUS becomes even more important with the introduction of the new and very expensive complement inhibitor eculizumab as a treatment for aHUS. We recommend that this assay be performed centrally in specialized laboratories per country to guarantee the quality and reliability of the assay and thereby ensure its feasibility and affordability in general clinical use.

The added value of the serological assay in relation to the time window has not been studied previously. Our results show that this added value becomes more evident (14 vs. 33% before and after seven days, respectively) when the specimens are collected >7 days after the first manifestation of symptoms. The number of patients in Table 2.3 is quite small due to missing data on the time window. In addition, feces were collected within seven days in 11 patients, whereas the serum was collected >7 days after the start of the symptoms, presumably due to negative fecal diagnostic test results. We anticipated the rise in positive serological assay results after seven days, as IgM is detectable approximately five days after the onset of the symptoms.[70] In contrast, the rise in positive fecal diagnostic test results after seven days was not expected considering the awaited rapid decrease of STEC in the feces. The high percentage of positive results for the fecal diagnostic tests is partly explained by the inclusion of the cell cytotoxicity tests (FStx) and PCR assays, both of which are highly sensitive tests, even after seven days, as compared to stool culture techniques.[33] However, these techniques all rely on the availability of fecal material. We

recommend testing for the presence of STEC with fecal diagnostic tests and serological test concurrently in HUS patients at admission because the time course of the disease at presentation is often unclear. In the case of negative fecal diagnostic and serological test results for specimens tested within seven days after the start of the symptoms, our advice would be to retest for antibodies after ≥7 days since the onset of disease.

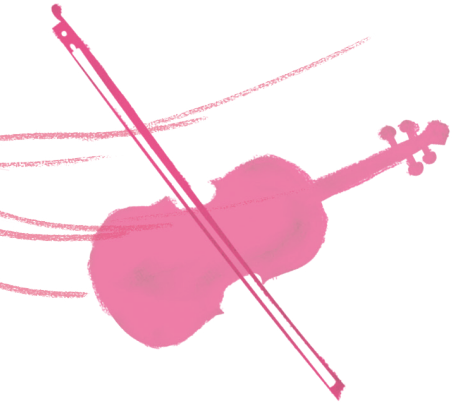
Based on our results, the serological antibody assay seems to be indispensable to establish a diagnosis of STEC-HUS; however, the role of serological antibody detection in healthy controls remains unclear and needs further exploration. In one study, 22 of the 606 tested healthy controls with high exposure to cattle carrying STEC had positive serological test results for O157. However the antibodies found were mainly IgG, which could be indicative of repeated exposure. Presumably, the IgM antibody response we test in this study is more indicative of an acute infection.[7,8] There is one important limitation of the serological anti-O157 antibody assay—namely, the possibility of cross-reaction with subsequent false negative results. Cross-reaction has been reported for pathogens such as as Brucella abortus, Yersinia enterocolitica, Vibrio cholera, Escherichia hermanni, Citrobacter freundii, Citrobacter sedlakii, and Salmonella.[29] However, these pathogens are rarely associated with the onset of HUS.

One limitation of our study is its retrospective nature. All information had to be gathered from medical records, thereby increasing the odds that the information would be difficult to interpret or even be missing. Furthermore, serological data from the antibody assay were available only on serotype O157; although serotype O157 is still the main serotype associated with HUS in the Netherlands, other STEC serotypes associated with HUS are being increasingly detected. However, it was not the purpose of this study to investigate the epidemiology of STEC, rather to evaluate the added value of the serological assay, which has been perform in our hospital since 1990, in addition to the fecal diagnostic tests to determine a STEC. STEC serotype O157 is still the cause of HUS in over 50 % of the cases in the Netherlands. The remaining serotypes are more scattered; for example, the second most prevalent serotype associated with HUS, serotype O26, accounts for <20% of the HUS cases.[15,23,30] In our cohort, four patients had a confirmed STEC infection with a non-O157 serotype (tested by RIVM)—serotypes O26 and O5. To increase the detection of the pathogens causing HUS, we are currently updating the serological antibody assay for other important serotypes as well.

In conclusion, the serological O157 antibody assay is an important additional test for the confirmation of STEC, especially when the fecal diagnostic test results are not sufficient to establish a STEC infection. Moreover, it is essential to take the time window into account: when patients present ≥7 days after the start of the symptoms, the serological antibody assay could be indispensable to establish a diagnosis of STEC-HUS. We recommend the implementation of the serological antibody assay as standard diagnostic method in combination with the fecal diagnostic tests for all patients who present with a clinical STEC-HUS.

"Misschien zijn alle draken in ons leven eigenlijk prinsessen die alleen maar zitten te wachten op het moment waarop we eindelijk, mooi en moedig, in actie schieten. Misschien is datgene waar we bang voor zijn in zijn blootste essentie iets wat hulpeloos onze liefde zoekt."

Rainer Maria Rilke.



Chapter 3

Glyco-iELISA: a highly sensitive and unambiguous serological method to diagnose STEC-HUS caused by serotype O157

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Abstract

Background

Providing proof of the presence of Shiga toxin-producing *E. coli* (STEC) infection forms the basis for differentiation between STEC - hemolytic uremic syndrome (HUS) and atypical HUS. As the gold standard to diagnose STEC-HUS has various limitations, the use of ELISA to detect serum antibodies against the lipopolysaccharides (LPS) of STEC has proven its additional value. Yet, the conventional LPS-ELISA has its own drawbacks, with as most important limitation the presence of cross-reactivity due to the conserved lipid A part of the LPS. The newly described glyco-iELISA tackles this issue by using modified LPS with elimination of its lipid A part. In this retrospective study the incremental value of glyco-iELISA compared to LPS-ELISA is assessed.

Materials and Methods

A retrospective study was performed, which included all pediatric patients (n=51) who presented with a clinical pattern of STEC-HUS between 1990- 2014 in our hospital. Subsequently, the diagnostic value of glyco-iELISA was evaluated in a retrospective nationwide study (n=264) of patients with thrombotic microangiopathy. LPS- and glyco-iELISA were performed to detect IgM against STEC serotype O157. Both serological tests were compared with each other and with fecal diagnostics.

Results

The glyco-iELISA is highly sensitive and has no cross-reactivity. In the single center cohort, fecal diagnostics, LPS-ELISA, and glyco-iELISA identified STEC O157 infection in 43%, 65%, and 78% of patients, respectively. Combining the glyco-iELISA with fecal diagnostics STEC infection due to O157 was detected in 89% of patients. In the nationwide cohort, 19 additional patients (8%) were diagnosed with STEC-HUS with glyco-iELISA.

Conclusion

In conclusion, this study shows that the glyco-iELISA to detect IgM against STEC serotype O157 has a clear benefit compared to the conventional LPS-ELISA, contributing to optimal diagnostics in STEC-HUS.

Introduction

Providing proof of the presence of Shiga toxin (Stx) producing *Escherichia coli* (STEC) infection forms the basis for differentiation between STEC - hemolytic uremic syndrome (HUS) and atypical HUS (aHUS). Both important causes to bear in mind in patients who present with signs indicative of thrombotic microangiopathy (TMA); hemolytic anemia, thrombocytopenia and acute renal failure. The current gold standard to detect STEC infection involves fecal examination by culture and detection of *Stx* encoding genes by polymerase chain reaction (PCR).[22,30,79] However, detection of STEC in the feces is limited due to the natural course of the disease. Furthermore, due to low inoculums, detection of STEC in the feces becomes increasingly difficult as the disease progresses. Despite new and upcoming fecal diagnostic techniques, like molecular serotyping, solely use of fecal diagnostics is not yet sufficient in establishing STEC infection.[20]

As the gold standard diagnostics of STEC-HUS has various limitations, the use of enzyme-linked immunosorbent assay (ELISA) to detect serum antibodies against the lipopolysaccharides (LPS; LPS-ELISA) of STEC has proven its value.[30,31,80] As previously shown, the combination of fecal diagnostics with the LPS-ELISA to detect Immunoglobulin M (IgM) against STEC serotype O157 has clearly an added value in the detection of STEC-infection as cause of HUS.[80] Yet, the conventional LPS-ELISA, in which plates are coated with purified LPS, has various drawbacks. The most important limitation is the presence of cross-reactivity caused by the conserved lipid A part of the LPS molecule. [29,31,81] The LPS structure consists of a lipid A part, an outer and inner core and a O-antigen, of which the latter has the highest immunogenic activity. Since the lipid A is also present in other strains of STEC and even other gram negative bacteria, this could lead to cross-reactivity and ultimately lead to false positive results.[29,82,83] Although O157 antigen remains the most prevalent serotype associated with STEC-HUS, other non-O157 strains (such as O26, O103, O104, O111, O55) are increasingly detected. Differentiation between the different STEC serotypes is important considering the variation in clinical presentation, course of the disease per serotype and the epidemiological consequences.[30,32,80]

To tackle the aforementioned issues, the group of J.E. Ugalde and D.J. Comerci exploited a new ELISA technique, the indirect glycoprotein-based ELISA (glyco-iELISA) for the detection of STEC infection in HUS patients.[32,81] This glyco-iELISA takes advantage of a bacterial glycoengeneering technology to develop and produce recombinant serotype specific glycoproteins consisting of the O157 polysaccharide attached to the protein carrier acceptor AcrA (O157-Acr), in absence of the lipid A part. As shown by Melli et al. the glyco-iELISA was able to diagnose STEC in HUS patients even in patients where fecal diagnostics failed.[32] More importantly, due to the absence of the lipid A structure of LPS in the glycoprotein-constructs used in this assay, potential cross-reactivity is counteracted. Thus, glyco-iELISA appears to be a highly sensitive and specific assay.[81]

Up till now, differentiation between STEC-HUS and aHUS remains a clinical conundrum which can be tackled with the introduction of the glyco-iELISA.[84] Although STEC-HUS and aHUS require a completely different treatment approach and clinical outcome is divergent, discrimination in the acute phase remains challenging.[5,85] Whereas diarrhea is the clinical hallmark of STEC-HUS, in up to 30% of the patients with aHUS, a gastro-intestinal infection has been found.[5,85] Moreover, aHUS is treated with one of world's most expensive orphan drugs: the humanized monoclonal antibody, eculizumab.[8] In contrary, the treatment of STEC-HUS is merely symptomatic and eculizumab is not indicated for the treatment of STEC-HUS.[5,85] Hereby underlining the importance of discriminating between both types of HUS and limit the unnecessary use of eculizumab in STEC-HUS.

With this retrospective study the clinical utility of the glyco-iELISA compared to LPS-ELISA is accessed in two cohorts. The first cohort comprises pediatric patients of a single center with strong clinical suspicion of STEC-HUS, of which all clinical data were gathered. The second cohort is nationwide cohort of patients with signs indicative of TMA of which STEC infection could be a potential cause.

Materials and methods

A retrospective single-center pilot study was performed, which included all pediatric patients who presented with a clinical pattern of STEC-HUS between 1990 and 2014 to the Pediatric Nephrology department of the Radboud University medical center (Radboudumc) Amalia Children's Hospital. A clinical pattern of STEC-HUS was defined as signs of a thrombotic microangiopathy (TMA) together with (bloody) diarrhea or a family member with diarrhea. Signs of TMA were classified as: signs indicative of hemolysis (low hemoglobin, elevated LDH, depleted haptoglobin), acute renal injury and thrombocytopenia of <150 x 10^9/l. Fever at presentation was defined as body temperature above 38.2 Celsius as reported by patients and/or parents. Anuria was defined as a urine production below 0.1ml/kg/hour for at least twelve hours, The estimated glomerular filtration rate (eGFR) was calculated with the Schwartz formula (k: 36.5).[73] According to the pediatric Risk, Injury, Failure, Loss, End-stage renal disease (pRIFLE) criteria, renal injury is defined as a increased creatinine x2 or decreased eGFR > 50%. Renal failure was defined as an increased creatinine x3 or decreased eGFR>75%.[86] The first day of illness was defined as the first day of diarrhea reported by the patient and/or parents. All available clinical and diagnostic data of these patients were collected in the STEC-HUS registry – an online web based database. Residual material (serum), received during standard care, was used to detect IgM antibodies against serotype O157 with the LPS-ELISA as well as the glyco-iELISA. This single center study comprised the same patient cohort as previously described by Wijnsma et al.[80]

Subsequently, the diagnostic value of glyco-iELISA in STEC-HUS diagnosis was evaluated in a retrospective nationwide study. Since Radboudumc Amalia Children's Hospital is the expertise center for HUS patients in the Netherlands, the LPS-ELISA for serotype O157 is only performed at the Translational Metabolic Laboratory in Radboudumc. This study included all residual sera samples from both pediatric as adult patients with signs of TMA. Of note, TMA was defined as thrombocytopenia, hemolytic anemia and organ damage, yet the underlying disease leading to the development of TMA was unknown at time of sampling. Samples of these patients were sent to Radboudumc Amalia Children's Hospital, between 2007 and 2014, from other university medical centers throughout the Netherlands for the performance of LPS-ELISA to diagnose or exclude STEC-HUS. All these residual sera samples were additionally tested for the presence of IgM antibodies against STEC O157 with glyco-iELISA. In addition, serum of 19 healthy adult controls were collected to determine specificity of the assays and determine cut-of values for the assay.

This study doesn't fall within the remit of the Medical Research Involving Human Subjects Act (WMO). The study has been reviewed by the ethics committee on the basis of the Dutch Code of conduct for health research, the Dutch Code of conduct for responsible use, the Dutch Personal Data Protection Act and the Medical Treatment Agreement Act. The ethics committee has passed a positive judgment on the study.

Index test: glyco-iELISA

The glyco-iELISA was performed as described by Melli et al.[32] In brief, a microtiter plate was coated with recombinant glycoproteins (O157-AcrA) and incubated overnight at 4°C. The following day, the plate was blocked with PBS-0.1% Tween20 (PBST) + 0.5% skim milk for 1 hour at room temperature (RT). Subsequently, diluted human serum samples (dilution of 1:800) were added and incubated for 1 hour at RT. Next the plate was washed and goat anti-human IgM (HRP-conjugated) antibody added and incubated for 1 hour at RT. Hereafter, 3,3',5,5'-tetramethylbenzidine (TMB, Sigma Aldrich) reagent as a substrate for HRP was added. Finally, the enzymatic reaction was stopped with 0.16 M sulfuric acid (H_2SO_4) and the absorbance measured at 450 nanometer (nm) with a spectrometer-based microtiter plate reader. Patients were considered positive when an optical density (OD) above 0.5 was observed.

Cross-reactivity was tested by adding sera of predetermined positive patients with various STEC serotype infections (resp. O26, O55, O103, O111, O145) together with pre-determined positive and negative patients, to an ELISA plate coated with glycoprotein serotype O157.

To study the effect of multiple freeze-thawning cycles on the patient blood samples, we freeze-thawed different previously determined positive samples, taken from three STEC-HUS patients, on five subsequent days. In the end we had a sample of one up till five freeze-thawn cycles of each patient.

Reference standard: Fecal diagnostics and LPS-ELISA

Feces and serum from suspected STEC-HUS patients were collected as soon as possible after admission to the hospital. In cases where feces could not be obtained, a rectal swab was done. Serum was received from all patients during standard care and stored at -80 °C until analysis. Fecal diagnostics and LPS-ELISA were performed as previously described.[80] Fecal diagnostics were considered positive when either PCR for *Shiga toxins* 1 and/or 2, the presence of fecal free shiga toxin by using the verocell assay, or the fecal culture (using Sorbitol MacConkey agar plate) was positive for STEC. In case of dubious test results, we considered the result as negative. After 2007, it became possible to send the STEC strains, isolated from the feces, to the Dutch National Institute for Public Health and Environment (RIVM) for further determination of the serotype both O157 and non-O157. Within the LPS-ELISA patients were considered positive when an optical density (OD) above 0.8 was observed. This cut-off was previously determined in close collaboration with Chart et al.[87]

Assay characteristics

The response of both LPS-ELISA as glyco-iELISA was established by determining the lowest signal to detect positive sample. In each setup of the plate, 1 previously determined positive and negative patient for the presence of antibodies against O157 antigens were diluted with different concentrations. Positive/negative ratio's (P/N ratios) were calculated by dividing the OD's obtained for each specific concentration. Subsequently, the coating antigen concentration was also taken into account when the sensitivity of the assays was compared.

Statistics

For each assay, the mean, standard deviation (SD), and coefficient variation (CV) were calculated. Furthermore, for the glyco-iELISA assays with patient screening, cut-off values were established using the following formula: mean of optical densities of control sera ±2 times the SD.

Clinical values were expressed as valid percentages for categorical variables and as the mean and SD or median and 25-75 interquartile range (IQR) for continuous variables, as appropriate. The Chi-square test was performed to compare categorical data. *P* values of <0.05 were considered statistically significant. All graphs were performed using GraphPad Prism software version 5. For statistical analyses, SPSS software (version 22.0) was used.

Results

Patient characteristics

As previously published, during the period between 1990 and 2014, 65 patients with a clinical pattern of STEC-HUS presented in the Pediatric Nephrology Department of Radboudumc Amalia Children's Hospital. Unfortunately, of the 65 patients, 14 patients had to be excluded of this analysis due the absence of residual material to test glyco-iELISA. The patient characteristics of the 51 STEC-HUS patients are described in **Table 3.1**. One patient with a highly severe presentation of STEC-HUS died due to a systemic inflammatory response syndrome. STEC infection was proven with both fecal diagnostics as serology.

During the period between 1990 and 2017, a total of 264 serum samples were sent to the laboratory of the HUS expertise center of the Radboudumc Amalia Children's Hospital of patients with acute TMA. Of the 264 serum samples of this nationwide cohort, 212 samples were collected from 206 patients with TMA and 52 samples from 50 relatives of these patients. The median (range) age of the patients was 6 (0-73) years. The majority of the relatives was a parent (n=42), five siblings were tested and three grandparents. Unfortunately, no clinical data were available from these patients as the samples were obtained from different hospitals in the Netherlands.

Table 3.1 Characteristics of pediatric patients with STEC-HUS in a single center cohort

Parameter	All patients (n=51)
Male	47%
Age of onset in months	36 (23 – 65)
Symptoms at presentation	
Fever[a]	22%
Diarrhea, total[b]	96%
Of which bloody	78%
Anuria (defined as <0.1 ml/kg/h)	57%
Blood pressure	
< p95	44%
≥ p95	56%
Neurological involvement	12% (n=6)
Convulsions	8% (n=4)
Coma	0%
Miscellaneous[c]	10% (n=5)
Pancreas involvement	4%

Table 3.1 Continued

Parameter	All patients (n=51)
Biochemical evaluation at presentation *(reference range)*	
Hemoglobin (mmol/l) *(6.0 – 9.0)*	5.3 (4.0 - 6.3)
White blood cells (x10^9/l) *(5.0 - 13.0)*	14.5 (10.97 - 22.5)
Platelet count (x10^9/l) *(210 - 430)*	45 (32 - 76)
Haptoglobin (g/l) *(0,3-1,6)*	<0.08 (0.04 - 0.10)
LDH (U/l) *(<250)*	3929 (2525 - 5817)
Creatinine (µmol/l) *(strongly depending age and bodymass)*	307 (190.5 – 430)
eGFR (*ml/min.1.73m^2*)[d] *(>90)*	13 (8 - 23)
Treatment	
Dialysis	65%
Duration of dialysis in days	10 (7 - 14)
Erythrocytes transfusion	92%
≥ 3 transfusions	20%

Categorical values are expressed as percentage of total and for continuous variables the median with interquartile range (IQR) is expressed. Neurological involvement included areflexia, coma, epilepsy and signs indicative of encephalopathy (decreased consciousness, abnormal behavior, amnesia, desorientation for time/person/place, disturbed speak, apraxia, hyperreflexia). P95: percentile for age and height[88].

[a] Fever, defined as body temperature above 38.2 Celcius, was reported by patients and/or parents

[b] Of note, we report two patients suspected of STEC-HUS without diarrhea. In one patient, STEC infection could be established by both fecal diagnostics (with rectal swab) as well as serology. In the second patient, serology for STEC O157 was negative and PCR was repeatedly reported as dubious. STEC-HUS seemed very likely, also in the light of good clinical recovery with minimal sequelae (mild proteinuria) and no recurrence after 6 years.

[c] Either in combination with convulsion or as solo presentation. Other neurological symptoms reported were decreased consciousness (n=3), ataxia (n=1) and apathy (n=1)

[d] All patients had signs indicative of renal injury according to the pRIFLE criteria. In total, 46 patients had renal failure based on the pRIFLE criteria.

Assay characteristics of glyco-iELISA

Since some samples were stored for quite some years, we accessed the stability of antibodies in our samples. However, freeze-thawning cycles up till five times did not seem to have an effect on determined OD values in the glyco-iELISA (data not shown). Furthermore, in contrary to LPS-ELISA, glyco-iELISA shows low intra- and inter-assay variation (CV<20%).

The discrimination capacity to detect a positive and negative signal of LPS-ELISA and glyco-iELISA were determined for serological antibodies against O157 antigen and compared to each other (**Figure 3.1**). Higher Positive/Negative (P/N) ratio's were obtained with the glyco-iELISA, especially when using 1250 ng/ml glycoprotein with 800 times serum dilution (P/N ratio of 6.6) compared to LPS-ELISA. Furthermore, no cross-reactivity was observed for STEC serotypes O26, O111, O145, O103 and O55 antigens in the glyco-iELISA (**Figure 3.2**).

Sensitivity of glyco-iELISA

In total, 51 STEC-HUS patients with serology and clinical data were available and included in the analysis. Since the PCR for *Shiga toxin* (*Stx*) genes was introduced in our hospital in 2011, in only 12 patients PCR was performed to detect the presence of *Stx* genes. In total, nine patients had a proven STEC infection based on PCR for *Stx*, of which seven were also confirmed with glyco-iELISA O157. The remaining two patients had a proven STEC infection with serotypes O55 and O26, explaining the negative glyco-iELISA result (see **Table 3.2**).

Figure 3.1 Response of LPS-ELISA versus glyco-iELISA

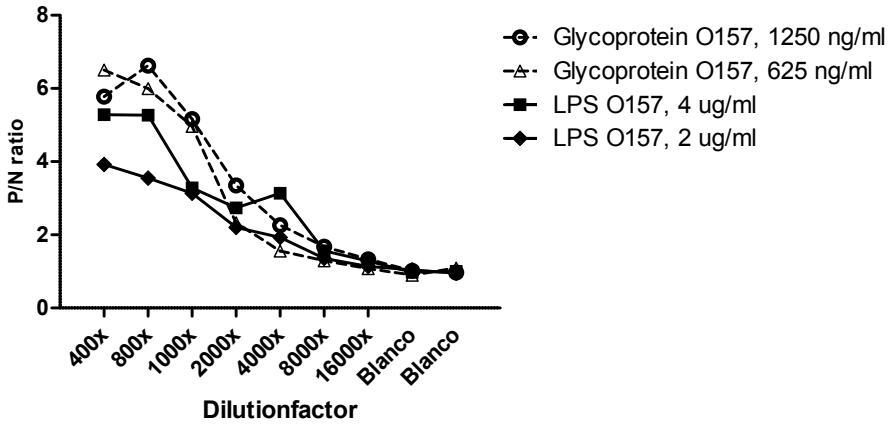


Figure 3.1 The response of both assays was accessed by determining the lowest signal at which a positive sample could still be detected for both LPS-ELISA as glyco-iELISA for STEC serotype O157. One previously determined positive and negative sample were diluted with different concentrations. Positive/negative ratio's were calculated by dividing the optical density obtained for each specific concentration. Subsequently, the coating antigen concentration was also taken into account when the accuracy of the assays was compared. LPS; lipopolysaccharide, P/N; positive negative ratio.

Figure 3.2 No cross-reactivity was observed with the glyco-iELISA for different STEC serotypes

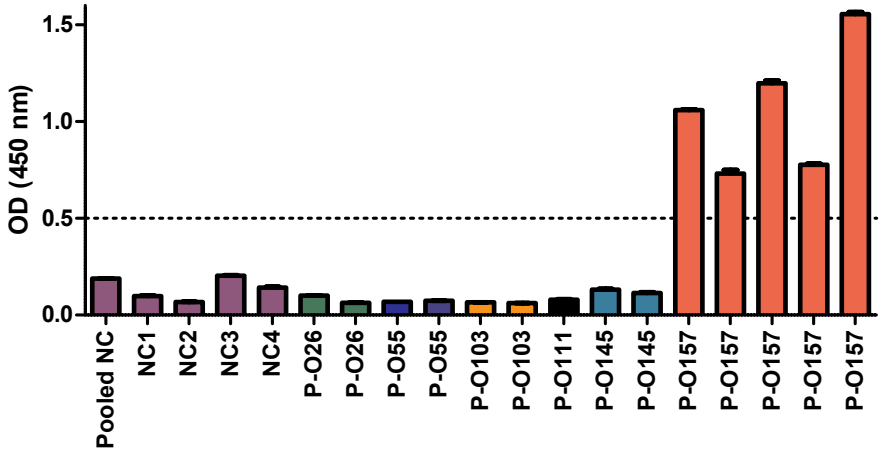


Figure 3.2 No cross-reactivity with different STEC serotypes was observed with the O157 glyco-iELISA. After coating with glycoprotein O157,pooled sera of various healthy negative controls (NC) and 4 separate NC together with sera of patients (P) with predetermined STEC infection with resp. STEC serotypes O26, O55, O103, O111, O145 and O157 were added. Every bar represents one patient with STEC-HUS due to the serotype as indicated. The dotted bar represents the cut-of value of 0.5 optimal density (OD). Only the patients with STEC-HUS with serotype O157 were determined as positive, indicating no cross-reactivity.

Furthermore, in 17 patients further determination of STEC serotype took place based on fecal diagnostics and yielded 14 STEC infections with isolated strain serotype O157. All these patients had also serology for O157 tested with the glyco-iELISA. This compared to serology tested by LPS-iELISA in which two patients tested negative.

Time window to perform glyco-iELISA

In eight patients of our single center cohort, serum was collected on various time points after the onset of diarrhea. These samples were used to examine the production of serum IgM antibodies (tested with the glyco-iELISA) during the disease course of STEC-HUS (**Figure 3.3**). A clear positive result for STEC O157 was observed after already one day after the start of diarrhea. However, there is a strong variation between individuals. Patient 3 had no antibodies against STEC O157 on day 1, however when tested on day eight and 14 antibodies could be detected. Patient 4 (with negative serology on day 1) had a positive antibody response up to 23 days after which the signal dropped significantly. Patient 5 had even persisting antibodies until 51 days after the start of diarrhea.

Table 3.2 Comparison between fecal diagnostics and glyco-iELISA for antibodies against STEC O157 antigen in patients with STEC-HUS in single center cohort

Assays	Positive glyco-iELISA O157	Negative glyco-ELISA O157	Total number of patients
Positive fecal diagnostics*	18	4	**22**
Feces culture	*15*	*2*	*17*
Free fecal toxin (verocell assay)	*8*	*3*	*11*
PCR	*7*	*2*	*9*
Negative fecal diagnostics	22	7	**29**
Feces culture	*9*	*8*	*17*
Free fecal toxin (verocell assay)	*11*	*5*	*16*
PCR	*2*	*1*	*3*
Total number of patients	**40**	**11**	**51**

** Patients can be positive for each fecal diagnostic assay separate as well as all combined.*
PCR; Polymerare chain reaction

Figure 3.3 Time window to perform glyco-iELISA to detect IgM against STEC O157

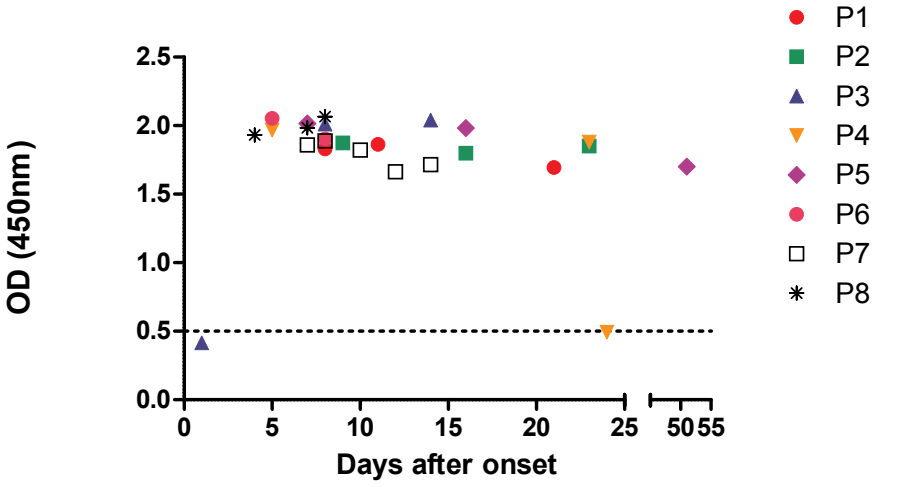


Figure 3.3 From eight STEC-HUS patients multiple serum samples collected after the onset of diarrhea on different days during the course of their disease, have been tested by using the glyco-iELISA for the presence of antibodies against serotype O157. The dotted line represents the cut-off value of 0.5 optical density (OD) after which samples are categories as positive for antibodies against serotype O157in the glyco-iELISA.

Single center cohort of STEC-HUS patients

Of the 51 STEC-HUS patients, 22 (43%) had a positive fecal diagnosis for STEC, 33 (65%) had positive serology based on the LPS-ELISA against O157, and 40 (78%) had positive serology based on the glyco-iELISA (see **Table 3.3** and **Figure 3.4**). In total, three patients appeared positive in O157 LPS-ELISA whereas they were negative with glyco-iELISA. In two of these patients fecal diagnostics revealed STEC infection with serotype O26 and O55, explanatory for the negative glyco-iELISA result and indicating a false (due to cross reactivity) positive result in the LPS-ELISA. In the third patient, the STEC strain was not further determined. Overall, the glyco-iELISA yielded 10 patients that were previously negative with LPS-ELISA. In conclusion, glyco-iELISA yielded significantly more patients positive for the presence of STEC when compared to fecal diagnostics ($p<0.0001$) and LPS-ELISA ($p=0.04$). Moreover, when combining fecal diagnostics with glyco-iELISA, STEC was detected in significantly (86% , $p=0.03$) more patients than when combined with LPS-ELISA (73%).

Table 3.3 Comparison between LPS-ELISA and glyco-iELISA for antibodies against STEC O157 antigen in single center cohort

Assays	Positive glyco-iELISA O157	Negative glyco-iELISA O157	Total
Positive LPS-ELISA O157	30	3*	33
Negative LPS-ELISA O157	10	8	18
Total amount of STEC-HUS patients	40	11	51

HUS; hemolytic uremic syndrome, LPS; lipopolysaccharide, STEC; Shiga toxin producing Escherichia coli

** Fecal diagnostics revealed: O26 strain (n=1), O55 strain (n=1), and no further determination of serotype was performed (n=1)*

Nationwide cohort of patients with TMA

The 264 serum samples of the nationwide cohort of patients with TMA, send in for STEC O157 serology, were tested with both LPS-ELISA and glyco-iELISA and outcome differed significantly ($p<0.0001$). When the 212 samples from the patients with TMA suspicion were tested using LPS-ELISA, 48 (23%) samples were diagnosed as positive, and 164 (77%) as negative (**Table 3.4**). When tested with glyco-iELISA, 60 (38%) samples of patients with TMA were confirmative for STEC O157 infection.

Subsequently, the 52 serum samples from the relatives of patients with TMA were tested with LPS and glyco-iELISA as well (**Table 3.4**). Interestingly, 10 were positive with the glyco-iELISA indicative of STEC infection. Of these relatives, in all but one the index patient tested positive for STEC infection as well. In this patient, mother tested positive for STEC O157, however the patients tested negative with both glyco-iELISA as well as LPS-ELISA.

In total, of all patients with clinical suspicion of STEC-HUS (n=206 with 212 samples), 56 (27%) patients tested positive for STEC O157 infection with glyco-iELISA. Overall, using the glyco-iELISA, 19 (7.2%) patients with TMA that were previously diagnosed as negative with LPS-ELISA could be diagnosed as STEC O157 positive (p<0.001).

Figure 3.4 Proportion of pediatric HUS patients from single center cohort tested positive for STEC-infection with different diagnostic tools

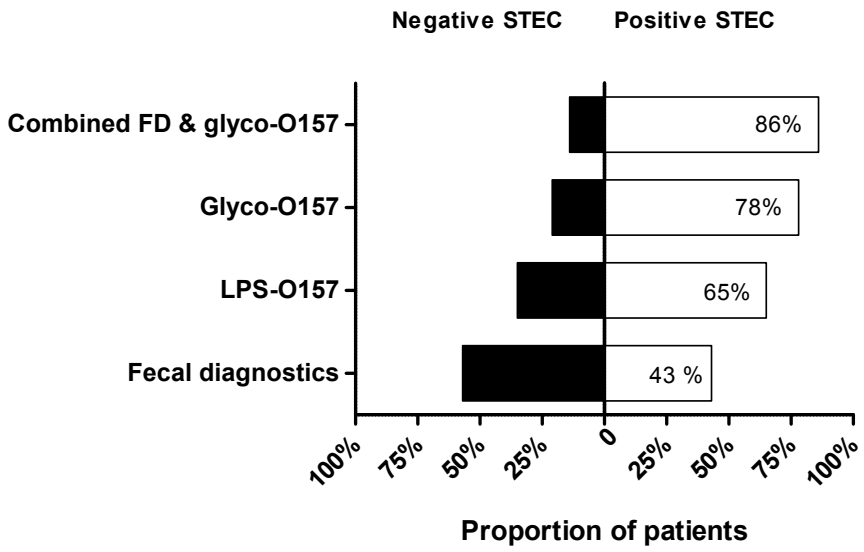


Figure 3.4 The percentage of STEC-HUS patients in the single center cohort per diagnostic test is described, given in percentages from the total of 51 patients. The proportion of respectively positive (white bar) and negative patients (black bar) are depicted for fecal diagnostics(STEC detection by stool culture, free fecal shiga toxin by verocell assay, PCR for shiga toxin genes),LPS-ELISA and glyco-iELISA for STEC serotype O157 (respectively LPS-O157 and Glyco-O157) and combined. When glyco-iELISA is combined with fecal diagnostics the percentage of positive STEC patients increased up till 86%.FD; fecal diagnostics, LPS; lipopolysaccharide, STEC; Shiga toxin producing Escherichia coli

Table 3.4 Comparison between LPS-ELISA and glyco-iELISA in nationwide cohort of patients with TMA of unknown etiology

Assays	Positive glyco-iELISA O157	Negative glyco-iELISA O157	Total
Total number of patients/relatives with positive LPS-ELISA O157	51	0	51
Patients	*48*	*0*	*48*
Relatives	*3*	*0*	*3*
Total number of patients/relatives with negative LPS-ELISA O157	19	194	213
Patients	*12*	*152*	*164*
Relatives	*7*	*42*	*49*
Total number of patients/relatives	70	194	264

HUS; hemolytic uremic syndrome, LPS; lipopolysaccharide, STEC; Shiga toxin producing Escherichia coli

Discussion

Differentiation between different etiologies of HUS is highly important regarding treatment and outcome. Since aHUS is a diagnosis *per exclusionem*, proving proof of STEC infection in a 'typical' STEC-HUS is essential. However, fecal diagnostics, the gold standard to diagnose STEC-HUS has some major drawbacks, most importantly due to the natural course of disease and low inoculums. Serological diagnostics like anti-O157 LPS antibodies has proven its added value to fecal diagnostics although this diagnostic assay has a different bottleneck; potential cross-reactivity and limited sensitivity. Here we show that the novel glyco-iELISA, to detect anti-O157 antibodies, is highly sensitive and specific and its use in STEC diagnostics led to more patients displaying positive STEC-O157 infections causing HUS. More importantly, using glyco-iELISA STEC O157-infections could be detected for a long period of time after start of the disease.

With this study the clinical utility of the glyco-iELISA was assessed. Melli et al. were the first to publish their findings regarding this novel glyco-iELISA using bacterial engineered glycoproteins for serotype O157, O145 and O121.[32] In a cohort of 71 samples taken from pediatric patients (comprising both STEC positive patients and STEC negative patients with clinical suspicion of STEC-HUS), they showed that the glyco-iELISA was highly sensitive and specific. Furthermore, no cross-reactivity between the previous serotypes was observed, confirming our results. The same group published a second article in 2017 by Castillo et al. where they further investigated cross-reactivity between different STEC serotypes (resp. O111, O103, O45, O26, O104) and other gram-negative bacteria (salmonella, *Brucella abortus*, *Yersinia enterocolitica O9*). Again no cross-reactivity was observed.[81] This in contrast to LPS-ELISA, where clear cross-reactivity between different serotypes has been reported.[29] Overall, with access to the glycoproteins, the glyco-iELISA is an easily implemented and performed assay with stable results.

Ideally, one would calculate sensitivity (proportion of patients with STEC-HUS in which the glyco-iELISA is positive) and specificity (proportion of patients with TMA caused by other conditions than STEC in which the glyco-iELISA remains negative) for an assay like glyco-iELISA. However, various problems arised when attempting to do so. The most prominent one has to do with the accuracy of the gold standard to diagnose STEC-HUS. Since fecal diagnostics are not sufficient to diagnose all STEC-HUS patients, no optimal gold standard is present to calculate sensitivity and specificity. Moreover, serology should not replace fecal diagnostics, but should be used in addition, to complement the microbial diagnostics and broaden the time window to detect STEC. Hence, accurate estimation of sensitivity and specificity is not feasible. Although, in our cohort, all patients with proven O157 in the feces were positive with glyco-iELISA in contrast to LPS-iELISA, indicating high sensitivity (100% in our cohort). Furthermore, in patients

with proven STEC infection with non-O157 serotype, glyco-iELISA for O157 remained negative in contrast to LPS-ELISA for O157, indicating high specificity.

As stated previously, cross-reactivity between LPS of different Gram-negative bacteria is a known problem due to the conserved lipid A part of the LPS molecule. We hypothesized that cross-reactivity, as observed in LPS-based ELISAs, can present as a false positive test result in the O157 LPS-ELISA. This may be due to the presence of antibodies against other non-O157 STEC serotypes or even other gram-negative bacteria, which to some extent are able to bind to the lipid A part of STEC serotypes. Interestingly, using the highly specific and sensitive glyco-iELISA, we observed an increase in the detection of STEC infections, rather than a decrease due to false positive results. Different explanations could explain this better performance. Primarily, STEC serotype O157 is still a highly prevalent serotype causing HUS in the Netherlands. Therefore not much cross-reactivity could be found, since non-O157 serotypes causing HUS are less common. Furthermore, as shown in our single center cohort, the glyco-iELISA is able to detect all patients with confirmed O157 serotype in the feces, and remains negative in patients with a confirmed infection with other serotypes. Hence, the negative result obtained with the glyco-iELISA seemed accurate indicating that the LPS-ELISA is probably a false positive result due to cross-reactivity between different STEC serotypes, as this was found to be the case in two of our patients. Other methods to detect serology have been reported such as line blot immunoassay and immunoblotting, however all use purified LPS to detect antibodies, hence potential cross-reactivity remains present.[29]

Interestingly, of the 52 samples of relatives without clinical HUS features, 10 (19.2%) had antibodies detected with the glyco-iELISA, indicating STEC O157 transmission person by person or intake of same contaminated food. In all except one, the index patient tested positive for STEC infection. Although we have no clinical information about relatives in our study, we could show that family members of STEC-HUS patients with no or mild signs of gastro-intestinal infection can develop antibodies against O157. These results are in line with Ludwig et al. who reported that 17% of the household contacts (symptomatic as well as asymptomatic) of STEC-HUS patients had LPS IgM antibodies against STEC serotype O157.[71] The exact rate of IgM antibodies against STEC serotypes in healthy population is still unknown. However, to exclude potential false positive results, one could consider to only test for presence of IgM and not include IgG, since IgG can be present for years after infection. Yet, we would recommend to test family members of patient with STEC (with fecal diagnostics and serology), especially in patients who tested negative for STEC infection. By providing proof of STEC infection in household contact, the diagnosis STEC-HUS in the index patient despite negative diagnostics becomes more likely.

We found IgM antibodies against STEC O157 up to 55 days after onset of the disease. These results are in line with previous reported kinetics of IgM (LPS based assay) against STEC by Chart et al.[70] Hence, in contrast to fecal diagnostics in which the isolation rate declines

quickly after the initial symptoms (within one week), serology (both LPS- and glyco-iELISA) broadens the time window to diagnose STEC infections. Furthermore, when serum is collected too early in the course of the disease, serology could be negative due to yet incomplete seroconversion, as was the case in two of our patients. In case of a negative serology result tested in serum collected within seven days after disease onset, the advice would be to collect and test serum again after seven days for a re-evaluation. As described previously, the added value of serology increased even more seven days or more after the start of the symptoms.[80]

Limitations of this study are the retrospective nature and the lack of clinical data of the national TMA cohort. Serological detection of STEC infection by detection of anti-O157 antibodies in serumis advised in the national guideline of diagnostic work-up for TMA at presentation. Presumably, quite some patients in the national cohort had a different diagnosis that not only comprised STEC-HUS , but also aHUS or other causes of TMA. Concerning the single center cohort of pediatric patients, STEC infection could not be detected in seven patients with clinical suspicion of HUS. Yet, aHUS as diagnosis is highly unlikely regarding the clinical presentation with bloody diarrhea in all seven patients Although, aHUS can present in 30% of the cases with gastro-intestinal infection, bloody diarrhea is seldom reported in aHUS. Also, follow up data showed no disease recurrence, making aHUS highly unlikely in this single center cohort. In three patients, genetic analysis was performed and showed no pathogenic mutations in complement genes associated with aHUS. Furthermore, in most patients serology was only tested at one timepoint. Seroconversion takes 3-5 days, hence patients who were seen early in the course of disease could be false negative. Furthermore, we focused on the still most prevalent STEC serotype O157 causing HUS in our country, however nowadays non-O157 serotypes are increasingly detected as cause of HUS. The relatively high number of patients with STEC O157 infection in our cohort could be explained by the substantial amount of STEC-HUS patients who were included in the late nineties, when serotype O157 was then and still now the main serotype to cause STEC-HUS. Nowadays, the amount of non-O157 serotypes causing HUS are increasingly detected, partly explained due to the new and improved diagnostic assays. In this study, non-O157 STEC serotypes were not detected.[20,30] It would be very worthwhile to examine the 14% of clinical STEC-HUS patients who were negative in fecal and serological diagnostics with glyco-iELISA for other STEC serotypes in the near future. Hence, future plans are to expand the glyco-iELISA to detect multiple serotypes. Melli et al. described already the use of glyco-iELISA for STEC serotype O145 and O121 with comparable results regarding absence of cross-reactivity and sensitivity of the assays.[32] Furthermore, to it is highly important differentiate between STEC-HUS and aHUS as soon as possible, to start appropriate treatment. Since the current glyco-iELISA takes at least 24 hours to perform, future studies should focus on improving this assay for bedside use. For example, with by using lateral flow technology one could develop a point of care test for patients presenting with TMA.

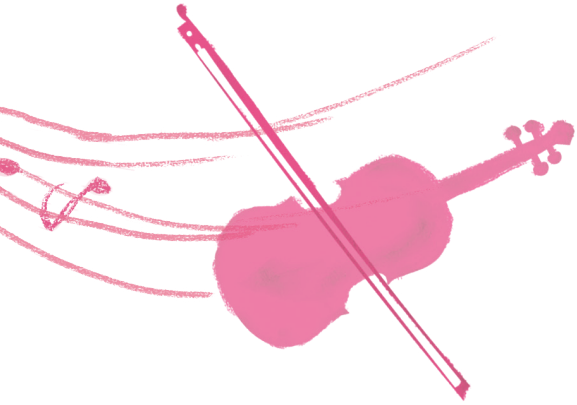
In conclusion, serological assays for STEC O-antigens have a place in the diagnostic work up plan of patients with TMA. Moreover, since aHUS is a diagnosis *per exclusionem* it is highly important to diagnose STEC-HUS. Therefore, we advocate to always combine fecal diagnostics together with serological diagnostics to achieve optimal diagnostics and prevent unnecessary use of the highly expensive orphan drug eculizumab. The optimal assay to determine serological antibodies against STEC serotype O157 is the glyco-iELISA.

Acknowledgement

We thank all our colleagues in pediatric nephrology and nephrology within the Netherlands who have send sera of their patients over the years to perform serology against O157 STEC.

"In your arms, where all my journeys end"

The Promise – Tracy Chapman



Chapter 4

Unusual severe case of hemolytic uremic syndrome due to Shiga toxin 2d producing E.coli O80:H2

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Abstract

Hemolytic uremic syndrome (HUS) is one of the most common causes of acute renal failure in children, with the majority of cases caused by an infection with Shiga toxin-producing *Escherichia coli* (STEC). Whereas O157 is still the predominant STEC serotype, non-O157 serotypes are increasingly associated with STEC-HUS. However, little is known about this emerging and highly diverse group of non-O157 serotypes. With supportive therapy, STEC-HUS is often self-limiting, with occurrence of chronic sequelae in just a small proportion of patients. In this case report we describe a 16-month old boy with a highly severe and atypical presentation of STEC-HUS. Despite the presentation with multi organ failure and extensive involvement of central nervous system due to extensive thrombotic microangiopathy (suggestive of atypical HUS), fecal diagnostics revealed an infection with the rare serotype: shiga toxin 2d producing STEC O80:H2. This report underlines the importance of STEC diagnostic tests in all children with HUS, including those with an atypical presentation, and emphasizes the importance of molecular and serotyping assays to estimate the virulence of an STEC strain.

Background

Hemolytic uremic syndrome (HUS) is the most common cause of acute renal failure in children and diagnosed when the features of hemolytic anemia and thrombocytopenia are present simultaneously.[6] In over 90% of pediatric cases, HUS follows a gastro-intestinal infection with Shiga toxin-producing *Escherichia coli* (STEC), previously known by the name of post-diarrheal or typical HUS, because of its main characteristic of (bloody) diarrhea.[6] Whereas O157 is still the predominant STEC serotype, non-O157 serotypes are increasingly associated with STEC-HUS.[30] However, in contrast to STEC O157, little is known about this emerging and highly diverse group of non-O157 serotypes. Thrombotic microangiopathy (TMA) causing HUS predominantly affects the renal vasculature. Nevertheless, especially in non-O157 STEC serotypes, extra renal involvement such as central nervous system involvement is described.[89] If symptomatic treatment is started in time, the recovery of STEC-HUS is often spontaneously with minimal chronic sequelae.[90]

Here we present a case of a 16-months-old boy with an unusual presentation and rare STEC serotype resulting in a very severe course of STEC-HUS.

Case report

A previously healthy 16-month old boy presented at the emergency department of a general hospital with convulsions and impaired consciousness. He had a two-day history of coughing, vomiting and lethargy, without signs of fever. Three months prior, he had a period of non-bloody diarrhea for over a week after visiting a biological farmer. A detailed medical history of the patient and family revealed no ingestion of unpasteurized milk or cheese nor a visit to a foreign country. On physical examination he exhibited fluctuating levels of alertness. Respiratory rate was 29/minute with oxygen saturation of 97%. Body temperature (36.4°C) and blood pressure were normal (98/60 mmHg) with mildly elevated pulse rate (138 beats per minute). Examination of the heart, lungs and abdomen was unremarkable. Skin examination revealed a pale looking patient with petechiae at lower limbs.

Based on clinical suspicion of meningitis or non-convulsive status epilepticus, ceftriaxone and midazolam were administered and the patient was transferred to our hospital. Initial laboratory evaluation showed hyperglycemia and the triad of HUS (**Table 4.1**). In the first hours, consciousness further decreased and the patient was transferred to the pediatric intensive care unit (ICU), where peritoneal dialysis was initiated due to persistent anuria. Blood and feces samples to determine the presence of an STEC infection were obtained. Blood cultures were performed repeatedly and remained negative during admission. Thrombotic thrombocytopenic purpura (TTP) was excluded with a normal ADAMTS13 activity (**Table 4.1**). Atypical HUS (aHUS), caused by complement dysregulation, was considered because of severity of the presentation, mainly neurological presentation, no recent history of (bloody) diarrhea, and young age of the patient. Consequently, the patient received 600mg of eculizumab. Later incoming results of additional complement and DNA diagnostics showed no abnormalities (**Table 4.1**).

The following day, fecal diagnostics revealed an STEC infection, indicating STEC-HUS; real-time polymerase chain reaction (PCR) was positive for *Shiga toxin* 2 (*Stx2*) and attaching and effacing (*eae)* genes. In addition, molecular serotyping using whole genome sequencing revealed the rare STEC serotype O80:H2, which contained the *Stx*2d gene and the rarely in human seen eae ξ gene variant (**Table 4.1**).[91]

In the course of admission, the patient developed multiple signs of severe, extra renal manifestations of TMA. Due to the severe neurological symptoms, with convulsions and decreased consciousness, brain magnetic resonance imaging (MRI) was performed, showing diffusion restriction of the deep white matter (**Table 4.1**).

Table 4.1 Multi-organ involvement

	Test	At admission	Most abnormal value	At discharge	Normal value or range
Differential diagnosis	Blood culture	Negative		Negative	
TTP	ADAMTS13 activity (%)	91%			>65%
STEC-HUS	Serology				
	O157 IgM / G / A	Negative			
	O26 IgM / G / A	Negative			
	PCR *Stx1 gene*	Negative		Negative	
	Stx2 gene	Positive		Negative	
	eae gene	Positive		Negative	
	WGS Serotype	O80:H2			
	Isolate	Stx2d, *eae* ξ			
	Sequence type	variant ST301			
	Virulence factors				
	Stx1gene				
	Stx2gene	Negative			
	eae	Positive			
	aggR	Positive			
	aatA	Negative			
		Negative			
aHUS	Complement				
	C3 (mg/l)	874	NA	NA	900-1800
	C4 (mg/l)	71	NA	NA	150-400
	Anti factor H autoantibodies	Negative			
	DNA analysis				
	Factor H	No pathogenic variation			
	Factor I	No pathogenic variation			

Table 4.1 Continued

	Test	At admission	Most abnormal value	At discharge	Normal value or range
aHUS (Continued)	*Factor B*	No pathogenic variation			
	C3	No pathogenic variation			
	MCP	No pathogenic variation			
	CFHR 1-5	No pathogenic variation			
	DGKε	No pathogenic variation			
	THBD	No pathogenic variation			
	MLPA *Factor H operon*	No aberrations			
Hematological	Hemoglobin (mmol/l)	4.5	3.3	7.3	6.8-8.6
	Platelet count (x10^9/l)	22	18	872	210-430
	WBC count (x10^9/l)	15.5	29.0	12.9	5.0-17.0
	Schizocytes (%)	>5	>5	NA	<0,5
	Haptoglobin (g/l)	Hemolytic	Hemolytic	1.85[a]	0.3-1.6
	LDH (U/l)	2285	6521	908	<250
Kidney	Creatinine (µmol/l)	167	444 (PD)	470 (PD)	15-45
	eGFR(ml/min/1.73 m^2)	17	anuria	anuria	80-120
	BUN (mmol/l)	43.9	44.2	22.4	2.5-7.0
Brain	MRI	Diffusion restriction of the deep white matter consistent with metabolic encephalopathy	NA	NA	
	EEG	No epileptic activity	NA	NA	
Heart	CK (U/l)	2743	8390	139[a]	<170
	Ntpro-BNP (pg/ml)	NA	>180,000	NA	<320
	Troponin T levels (ng/l)	557	23,444	508 [a]	<14
	Echo	NA	Left ventricular dysfunction	Normal left ventricular function[a]	

Table 4.1 Continued

	Test	At admission	Most abnormal value	At discharge	Normal value or range
Pancreas	Amylase (U/l)	NA	1933	42 [a]	<105
	Glucose (mmol/l)	9.9	30.7	6.2	4.0-5.6
	Triglycerides (mmol/l)	NA	14.17	4.92	0.8-2.0
Liver	AST (U/l)	163	1020	114	<35
	ALT (U/l)	62	480	144	<45
	Gamma-GT (U/l)	6	1810	824	<55
	Alkaline phosphatase (U/l)	205	1529	401	<115
	Direct bilirubin (µmol/l)	4	326	35	<5
	Ultrasound		Edema around and sludge inside gallbladder		
	Biopsy		Cholestasis		

aatA; necessary for translocation of dispersin (Aap), ADAMTS13; a disintegrin and metalloproteinase with a thrombospondin type 1 motif; member 13, aggR; transcriptional regulator aggR, aHUS; atypical hemolytic uremic syndrome, ALT; alanine transaminase, AST; aspartate transaminase, BUN; blood urea nitrogen, CFHR; complement factor H related proteins, CK; creatine kinase, DGKε; diacylglycerol kinase epsilon, eae; E. coli attachment effacement gene (intimin), EEG; electroencephalogram, eGFR; estimated glomerular filtration rate based on Schwartz estimation with k-value of 36.5, gammaGT; gamma-glutamyl transferase, IgA; immunoglobulin A, IgG; immunoglobulin G, IgM; immunoglobulin M, LDH; lactate dehydrogenase, MCP; membrane cofactor protein, MRI; magnetic resonance imaging, NA; not available, Ntpro-BNP; N-terminal of the prohormone brain natriuretic peptide, PCR; polymerase chain reaction, PD; peritoneal dialysis; STEC; Shiga toxin producing Escherichia coli, Stx1; Shiga toxin 1, Stx2; Shiga toxin 2, THBD; thrombomodulin, TTP; thrombotic thrombocytopenic purpura, WBC; white blood cell count, WGS; whole genome sequencing. [a] Two months after presentation

On day four of admission, the patient was resuscitated twice due to post-intubation hypotension and bradycardia, with rapid recovery of cardiac output. Cardiac biomarkers were elevated, and retrospectively elevated troponin T levels and creatine kinase were already present. Echocardiogram revealed a mildly dilated and dysfunctional left ventricle. Eventually, follow up demonstrated a significant improvement in cardiac biomarkers and function.

Pancreas involvement was noticed based on a gradual rise in serum glucose and elevation of serum amylase and triglyceride concentrations without clinical signs of pancreatitis. Insulin treatment was needed for two weeks.

After two weeks, jaundice was observed in combination with elevated bilirubin levels, without signs of ongoing hemolysis. Progressive elevation of transaminases was measured, with normal levels of clotting factors but low serum albumin (**Table 4.1**). Liver biopsy showed extensive signs of cholestasis without microthrombi, most likely due to sludging or drug related effects (**Figure 4.1**).

Figure 4.1 Liver biopsy

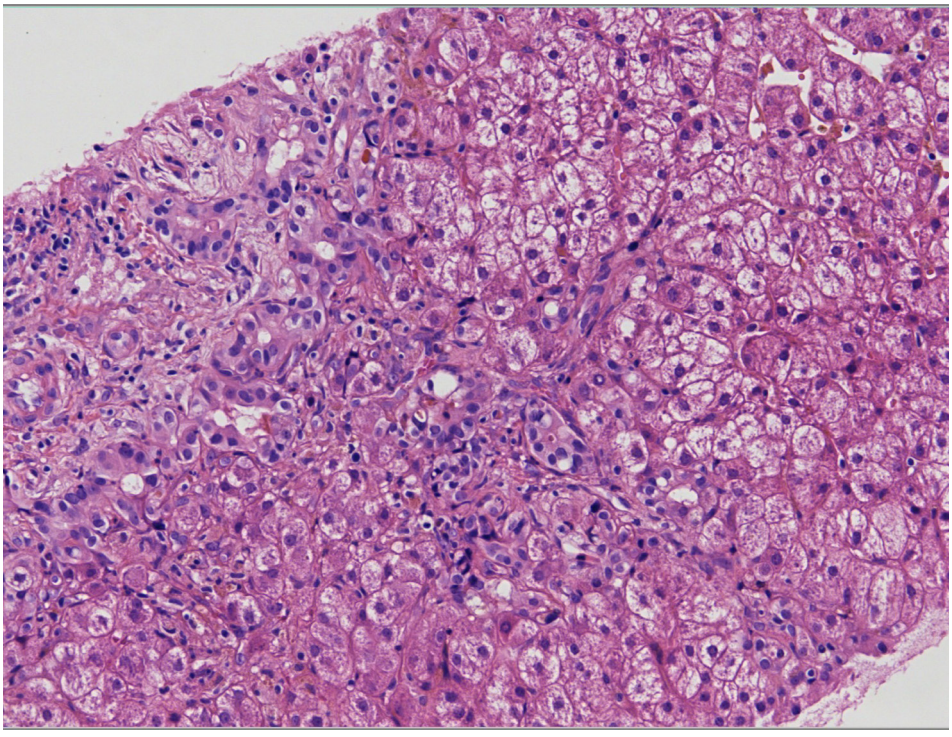


Figure 4.1 Light microscopy on the liver biopsy (with hematoxylin and eosin staining) revealed hepatocyte swelling and extensive signs of cholestasis.

Eventually, our patient was hospitalized for over three months. Only minimal signs of neurological improvement were observed and over time the patient developed spastic dystonia. Furthermore, he exhibited persistently decreased levels of consciousness with frequent periods of severe agitation. The patient remained anuric for which dialysis was continued. Nine months after initial presentation the patient died due to peritoneal- and hemodialysis failure combined with progressive liver failure and further neurological impairment.

Discussion

In this report we describe a 16-month-old boy with an unusual, severe presentation and course of STEC-HUS due to the rare *Stx*2d producing *E.coli* O80:H2. STEC-HUS is a common cause of HUS in childhood and in more than half of the cases results from an STEC infection with serotype O157. However, non-O157 serotypes are increasingly associated with HUS in recent years, most likely due to improvement of diagnostics.[6,30] Usually, the majority of STEC-HUS resolves with no or minimal chronic sequelae.

STEC infection with serotype O80:H2 resulting in HUS was rarely described up to recently.[30,92-94] To our knowledge, up till now this serotype was only found in patients with HUS in France and this report is the first describing a case in the Netherlands. In addition, one study from Spain already described the finding of this serotype in cattle over ten years ago. Interesting to note is that there is no direct geographic connection between the different areas. Hence, the source of the O80 serotype remains of unknown origin.[94]

STEC strains can produce different shiga toxins, respectively *Stx1* and *Stx2*. Within these genotypes, especially the *Stx2 gene*, there is a wide variety of *Stx* subtypes, such as *Stx2d* which we describe in this case report.[20] Moreover, the *Stx2*d gene is known to cause a more severe course of disease. This together with the rare *eae* ξ and the plasmid of the O80:H2 STEC, which showed high resemblance with the previous described plasmid associated with extra intestinal virulence, resulted in an increased association with HUS.[94] Only recently, Soysal et al. described the STEC serotype O80 as a new therapeutic challenge in patients with STEC-HUS due to its capacity to cause a bacteremia. Furthermore, Espié et al. described an outbreak of STEC serotypes O26 and O80 co-infection related to unpasteurized cheese.[92] However, our patient had no bacteremia and no indication of co-infection, tested with both fecal diagnostics and serological antibody assays against both O157 and O26 lipopolysaccharide (**Table 4.1**)

Mariani-Kurkdjian et al. described the case of an adult male with a severe episode of HUS caused by STEC O80:H2 containing both *Stx*2d and the rare *eae* ξ variant like the strain described above.[93] Similar to our case, the adult male patient presented with afebrile convulsions followed by coma and, after a few weeks, he developed bacteremia. This raised the question if the O80 serotype may not be cleared rapidly, in contrast to most other serotypes. Such a slow clearance would make it more likely that the STEC found in our patient could be the consequence of the gastro-intestinal infection three months prior to presentation.[94,95]

Nowadays, in the majority of laboratories, STEC diagnostics comprises PCR, culture and/or enzyme immune-assays. Obviously, these approaches could have missed the identification of the O80:H2 strain. Using additional molecular and serotyping assays allows the detection of virulence factors and typing of the *Stx* genes, both important to estimate the virulence of the strain. Such additional diagnostic tests are relevant both

for individual patients as for public health to monitor new or rare serotypes that cause severe HUS and emerge into the population.[20]

It can be challenging to clinically differentiate between STEC-HUS and aHUS due to similarity of symptoms. Similar as in our patient, in 6-10% of the children with STEC-HUS there is no (bloody) diarrhea, whereas aHUS is preceded by diarrhea in 25% of cases.[44] Since aHUS is merely a diagnosis *per exclusionem,* it is essential to proof the absence of an STEC infection.[6,80] Even in cases without (bloody) diarrhea and atypical presentations such as young age of the patient, it is highly recommended to perform both fecal and serological diagnostics in every HUS patient to exclude STEC-HUS.[80]

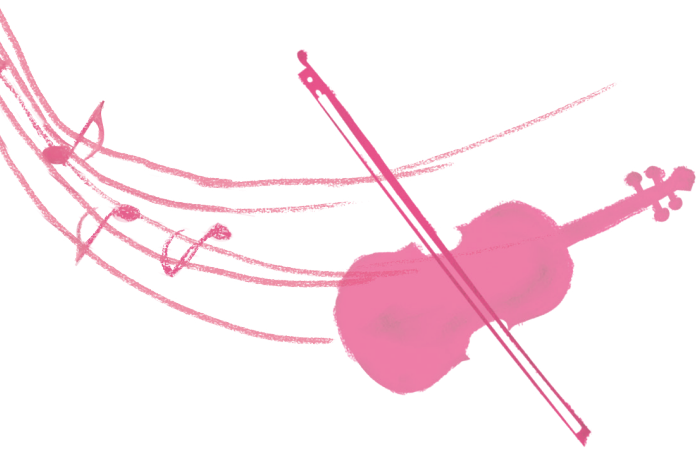
The treatment of STEC-HUS is merely symptomatic. A topic of discussion remains the use of antibiotics to eradicate STEC infection. Increased transcription, production and release of shiga toxins, possibly aggravating HUS, can be induced by antibiotics.[96] Soysal et al. studied in vitro effects of different antibiotics on *Stx* production. Ceftriaxone, which our patient received at presentation, seems to have no effect on *Stx* production.[94] Another controversial topic in patients with STEC-HUS is the use of eculizumab. Eculizumab, a monoclonal antibody directed against complement C5, is nowadays the standard treatment in patients with aHUS.[97] Some case series, among others Pape et al., reported better outcome of STEC-HUS after eculizumab administration, particularly in neurologically affected STEC-HUS patients.[89,98] However, Pape et al. also described the use of eculizumab in patients with multi-organ failure, where it seems to be associated with a less favorable outcome.[98] Well-designed and well-powered randomized controlled trials are needed to shed light on the effect of eculizumab in STEC-HUS.

Extra-renal manifestations of TMA are not uncommon in HUS. In 25% of HUS cases neurological involvement is noted, other organs are affected less frequently.[6,89] Although severe multi-organ failure and especially liver failure is rarely described in STEC-HUS, the highly severe disease presentation in our patient could partially be explained by the (extraintestinal) virulence factors of this O80 strain. However, in our patient jaundice and liver failure evolved two weeks after one dose of eculizumab possibly indicating hepatotoxicity. This was recently described in five pediatric aHUS patients.[99] Transient liver enzyme derangement was noticed in these children 10-29 days after the first dose of eculizumab with spontaneous resolution. The time pattern in our patient as described above, cannot rule out the hypothesis that administration of eculizumab could have contributed to liver failure in our patient.

In conclusion, we describe a severe case of STEC-HUS caused, by an unusual *Stx*2d producing STEC O80:H2. This resulted in an unusual and severe disease course, complicated by multi-organ failure and central nervous system involvement. This case emphasizes the importance of molecular and serotyping assays to estimate the virulence of an STEC strain.

"A person who never made a mistake never tried anything new."

Albert Einstein



Chapter 5

Heme as contributing factor in the evolvement of Shiga toxin Escherichia coli induced hemolytic uremic syndrome

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[*] Contributed equally

Submitted

Abstract

Although Shiga toxin (Stx)-producing *Escherichia coli* hemolytic uremic syndrome (STEC-HUS) is one of the most common causes of acute kidney injury in children, the exact pathogenesis still remains elusive. Stx-mediated endothelial injury initiates the cascade leading to thrombotic microangiopathy (TMA). Yet, there is wide variability in clinical presentation and outcome. One explanation for this could be the enhancement of TMA through other factors. We hypothesize that heme, as released during extensive hemolysis, contributes to the development of TMA. Plasma levels of heme and its scavenger hemopexin and degrading enzyme heme-oxygenase 1 (HO-1) were measured in 48 STEC-HUS patients. Next, the effect of these disease specific heme concentrations, in combination with Stx, were assessed on human primary glomerular microvascular endothelial cells (HGMVECs).

Significantly elevated heme levels up to 21.2 μM were found in STEC-HUS patients compared to controls (median of 1.8 μM). These elevated heme levels inversely correlated with plasma hemopexin levels (R^2-0.74). Furthermore, heme is internalized and led to a significant increase in reactive oxygen species production. We observed heme-induced nuclear translocation of NFκB together with increased cell surface tissue factor expression. Interestingly, upregulation of the cytoprotective enzyme HO-1 was impaired by Stx.

In conclusion, we are the first to show elevated heme levels in patients with STEC-HUS. These increased heme levels promote endothelial injury, thus may contribute to the cascade leading to TMA. Moreover, the impaired upregulation of HO-1 by Stx in endothelial cells leads to a decreased protection against injurious heme and contributes to Stx-mediated TMA.

Introduction

Typically, the cascade leading to thrombotic microangiopathy (TMA) is explained as a cause-effect relationship. This cascade commence via a specific trigger causing endothelial injury, leading to the formation of thrombi in the small vessels, subsequently leading to thrombocytopenia, hemolytic anemia and acute kidney injury.[2,5] The most common cause of TMA in children is a gastro-intestinal infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC). In the majority of cases STEC hemolytic uremic syndrome (STEC-HUS) has a prodromal phase characterized by (bloody) diarrhea.[5,6] Stx produced by STEC is known to be the most important factor leading to HUS. However, only approximately 15% of all patients with STEC infection develops HUS, suggesting other factors may be involved as well.[17]

The exact pathogenesis of STEC-HUS is only partially understood.[85] It is thought that endothelial cell injury is caused by the Stx, an AB toxin, secreted from the STEC bacteria. Stx can enter the bloodstream and binds to its receptor, the glycolipid globotriaosylceramide (Gb_3) present on endothelial cell surfaces. After the transport of the A subunit of Stx intracellularly, cellular activation and damage is caused together with the promotion of a pro-inflammatory and pro-thrombotic state.[89] Yet, there is wide variability in clinical presentation and outcome in STEC-HUS ranging from no chronic sequelae to hypertension, proteinuria, end stage renal disease or even death.[5] One possible explanation for this broad range of clinical outcome could be the enhancement of TMA through other additional factors.

One of these additional factors could be heme, as released during extensive hemolysis such as present in STEC-HUS patients.[100] Heme is known for its noxious effects and primarily causes oxidative stress, together with the release of pro-inflammatory and pro-thrombotic molecules.[100-103] Furthermore, it has the unique ability to intercalate in the membrane of red blood cells, thereby enhancing hemolysis.[104] To prevent the accumulation of heme and limit the heme-mediated toxicity, heme is normally directly scavenged extracellularly by hemopexin, and degraded intracellularly by heme-oxygenase 1 (HO-1).[102,104,105] When the extracellular heme scavenger hemopexin is saturated, heme will bind to less potent scavengers present in the blood like albumin or lipoproteins, hence remaining (partly) bioreactive.[106,107] In addition, heme proteins could get trapped in the kidney where they can release their free heme group.[108] Even more interestingly, Bitzan et al. described the ability of Stx to inhibit HO-1 expression in human renal carcinoma-derived tubular epithelial cells. To our knowledge, no studies have been published regarding the effect of heme and Stx2 on HO-1 regulation in primary glomerular endothelial cells.[109]

Unfortunately, very little is known about the (patho)physiological levels of extracellular heme in case of hemolysis in STEC-HUS patients. Knowledge regarding accurate and representative heme levels as present during hemolysis *in vivo* is necessary to study the possible noxious effect of heme levels *in vitro*. We hypothesize that extracellular accumulation of bioreactive heme can initiate and amplify the cascade leading to TMA. In this study we measured plasma levels of heme in STEC-HUS patients, during the acute phase and assessed *in vitro* the effects of these disease specific heme concentration, in combination with Stx2, on primary human glomerular microvascular endothelial cells.

Methods

Patient cohort

All patients who presented in the Radboud University Medical Center (Radboudumc) Amalia Children's hospital between 1990-2016 with signs indicative of STEC-HUS were included. A clinical pattern of STEC-HUS was defined as; hemoglobin level below the lower limit of normal for the specific age and signs indicative of hemolysis, acute renal failure, thrombocytopenia < 150 x 10^9/l, and (bloody) diarrhea or family members with diarrhea. STEC-HUS was diagnosed by fecal diagnostics and serology. Both plasma and serum were collected on admission (acute phase) and stored at -80°C. Clinical data were collected retrospectively from medical records. In addition, 24 plasma samples of healthy adult controls and 26 samples of age matched controls were included after informed consent was obtained. Exclusion criteria for this control group were: fever, bacterial/viral infection in the past few weeks, chronic illness, inborn or acquired immune disorders and the use of immunosuppressive drugs. The ethics committee (CMO Arnhem-Nijmegen) has passed a positive judgment on the study (2017-3490).

Methods in supplemental data

Hemopexin and HO-1 were measured with enzyme-linked immunosorbent assay (ELISA), the method is described in the supplemental data. For detailed description of flow cytometric apoptosis assay, reactive oxygen species (ROS) measurement, quantitative polymerase chain reaction (qPCR), western blot and statistic analysis, see supplemental data.

Colorimetric heme quantification assay

Plasma heme was measured using 1-Step™ Turbo 3,3',5,5'-Tetramethylbenzidine (TMB)-ELISA Substrate (Thermofisher scientific). This method is based on the oxidation of TMB by the pseudoperoxidase activity of heme. For this assay, hemin chloride porcine (designated as heme, Sigma-Aldrich) was diluted in 0.1M NaOH (Merck) and the pH was adjusted to 7.8-8.2.[110] Subsequently, this solution was filtered through 0.2 µm pore size membrane (Whatman). Hereafter, serial dilutions (4.0 - 2.0 – 1.5 – 1 – 0.5 – 0.25 – 0 µM) of an 2 mM heme stock were made in 20 mM HEPES (Sigma-Aldrich) + 1% bovine serum albumin (BSA, Millipore), pH 7.4. Next, plasma samples (patients 10x, controls 2.5x) were diluted in 20 mM HEPES + 1% BSA, pH 7.4, where after 20 µL of every sample and 80 µL of the turbo TMB substrate was added to a microtiter plate (Greiner Bio-One™) and incubated for 10 minutes in the dark. The reaction was stopped with 100 µL 2 M sulfuric acid (Sigma-Aldrich) and absorbance was read at 450 nanometer (nm) with Victor 3 V multilabel plate reader (Perkin elmer).

Cell culture

Human glomerular microvascular endothelial cells (HGMVECs) were isolated and cultured as described previously by van Setten et. al.[111] Cells were cultured 24 hours prior to the experiments in serum free conditions to avoid the effect of serum in experiments.

Immunofluorescence imaging

Confluent monolayer of HGMVECs cultured on 1% gelatin (Fluka) coated round glass coverslips Ø 1 cm (VWR) were stimulated with 25 or 50 µM heme and/or Zn(II) Mesoporphyrin IX (ZnMP; Frontier scientific) for 6 hours. After stimulation, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 15 minutes at RT and subsequently stained with appropriate antibodies (CD31(mouse IgG1; Sanquin, monoclonal mouse α-human NF-kB p65 (F-6; Santa Cruz)). Subsequently, secondary antibody alexa fluor goat α-mouse IgG (Thermofisher scientific) and DAPI was added. Finally, stained cells on the coverslips were mounted on microscopic slides (Thermofisher scientific). Slides were viewed with a Zeiss fluorescence microscope or by Olympus FV1000 confocal microcopy.

Tissue factor (TF) measurement

With a cell-based ELISA, TF expression on the surface of HGMVECs were assessed. Confluent monolayer of HGMVECs on gelatin coated 96-well tissue culture plate (Corning ® Costar ®) were stimulated with 25 and 50 µM heme for 6 hours. Subsequently, the cells were fixed with 0.025 % glutaraldehyde (Merck), where after cells were incubated with biotinylated goat anti-human coagulation factor III detection antibody (DuoSet ELISA, R&D systems) diluted in M199-10% FCS. Next, diluted streptavidin-HRP (DuoSet ELISA, R&D systems) was added. Signal was visualized with TMB (Sigma-Aldrich)substrate at 450 nm using Viktor 3 V multilabel plate reader.

Results

Patient characteristics

In total, 48 patients were diagnosed with STEC-HUS from 1990 to 2016 in the department of Pediatric Nephrology of Amalia Children's Hospital Radboudumc of which medical information was present. Patients characteristics on admission are shown in **Table 5.1**. Patients showed signs of (mechanical) hemolytic anemia, based on low hemoglobin and increased lactate dehydrogenase (LDH) levels with decreased and often depleted haptoglobin levels. Dialyses was started in majority of the patients.

Increased plasma heme levels

Plasma levels of heme in STEC-HUS patients in acute phase were significantly higher ($p<0.0001$) when compared to healthy controls. A median (range) of 3.2 (0.5-21.2) μM heme was found in STEC-HUS patients compared to 1.8 (0.6-3.8) μM in healthy controls (**Figure 5.1A**). Of note, no difference between pediatric and adults controls was observed. In total, 12 patients had heme levels above the 75 quartile range (6.3 μM) up till 21.2 μM. Median (IQR) time between hospital admission and sample collection was 3 (1-11.3) days. Furthermore, when samples were collected later on in the disease course, the risk of measuring lower heme levels was OR of 0.79 (95% CI 0.64 – 0.99, $p=0.49$).

Plasma levels of heme neutralizing proteins: hemopexin and HO-1

STEC-HUS patients in the acute phase had significantly lower plasma hemopexin levels compared to healthy controls with median (range) of 0.4 (0.01-1.2) g/L and 0.9 (0.2-1.3) g/L respectively ($p<0.0001$, **Figure 5.1B**). Hemopexin was depleted (<0.1 g/L) in 15 patients with a heme median (IQR) of 11 (6.5-12.8) μM. Moreover, a strong inverse correlation between measured heme levels and hemopexin levels was observed with R^2 of -0.74 (**Figure 5.1B**). Of the 12 patients with high heme levels, nine had depleted hemopexin (<0.01 g/L), and the remaining three patients had hemopexin levels below 0.1 g/L.

HO-1 levels in plasma were significantly elevated in STEC-HUS patients with median (range) of 163 (2.57 – 789) ng/ml compared to 23.7 (6 - 70) ng/ml in healthy controls ($p<0.001$). Interestingly, especially patients with high heme levels (n=12, heme levels above 75 quartile range) had high plasma HO-1 levels with median of 332.5 (86-720) ng/ml ($p=0.008$, **Figure 5.1C**).

Clinical outcome in relation to plasma heme levels

As heme is known for its toxic effect, we hypothesized that high heme levels would be associated with a more severe clinical presentation (**Figure 5.2**). Therefore we performed a binary logistic regression to look at the risk of developing various clinical parameters in light of increasing heme levels (by 1 μM). The risk for the need of erythrocyte infusion

seems to increase (OR 1.8 with 95% CI 0.86-3.8, p=0.116) with higher plasma heme levels, although the results were not significant. This corresponds with the fact that heme is mainly released from red blood cells. Surprisingly, the risk for intensive care admission, dialysis and multiorgan involvement decreased when higher heme levels were observed.

Table 5.1 Patient characteristics

Parameters	**STEC-HUS (n=48)**
Age at disease onset, in years	3 (1-4)
Female	50%
Time between first day of illness and day of admission at academic hospital, in days	7 (5-8)
Clinical presentation	
Diarrhea	98%
Of which bloody diarrhea	75%
Fever defined as >38.2 Celsius	29%
Blood pressure ≥ 95 percentile for age and height	55%
Multi organ involvement	31%
Duration hospital admission, in days	16 (11-21)
Biochemical evaluation at presentation *(reference range)*	
Hemoglobin (mmol/l) *(6.0 – 9.0)*	5.5 (4.6-6.0)
Platelets (x10^9/l) *(210 - 430)*	47 (32-69)
Haptoglobin (g/l) *(0,3-1,6)*	0.1 (0.1-0.14)
Leukocytes (x10^9/l) *(5.0 - 13.0)*	15 (11-22)
eGFR (ml/min/1.73m^2) *(>90)*	11 (8-19)
LDH (U/l) *(<250)*	4255 (2609-6290)
Treatment	
Dialysis	75%
Duration dialysis, in days	10 (7-15)
Need for erythrocyte transfusion	86%
Proven STEC infection	85%

Results show percentages or median with interquartile range (IQR). LDH; lactate dehydrogenase, eGFR; estimated glomerular filtration rate, n; number of patients of which data was available, STEC; Shiga toxin producing Escherichia coli.

Endothelial cells internalize heme

As heme levels are elevated in STEC-HUS patients, we next aimed to characterize the functional consequences of disease-relevant heme levels on primary HGMVECs. Exposure of HGMVEC to 25 and 50 μM auto fluorescent heme analog, ZnMP caused a detectable red fluorescent signal in the cells, with no reciprocal difference between both concentrations (**Figure 5.3A** middle panels). To confirm whether heme and not only its analogue ZnMP is internalized by endothelial cells, endothelial cells were next exposed to 25 and 50 μM ZnMP and heme simultaneously. A decreased ZnMP signal in endothelial cells was observed due to competitive uptake of ZnMP and heme (**Figure 5.3A**, right panels). Finally, to confirm that ZnMP was truly internalized by the endothelial cells rather than sticking to its outer membrane, the ZnMP signal in endothelial cells by confocal microscopy was established. By co-staining the outer membrane with CD31 (green fluorescence), we confirmed that the ZnMP signal was indeed within the HGMVEC (**Figure 5.3B**). In sum, these data indicate that human glomerular endothelial cells have the capacity to internalize heme.

Heme causes oxidative and inflammatory stress

To assess whether the exposure of endothelial cells to disease-relevant heme levels causes endothelial cell activation and cell stress, we analyzed reactive oxygen species (ROS) production by HGMVECs either in the absence or presence of heme. Treatment of HGMVECs with heme (25 or 50 μM) resulted in significantly increased ROS generation after 1 hour (**Figure 5.4A**). Furthermore, ROS generation led to a profound nuclear translocation of the pro-inflammatory transcription factor NFκB (**Figure 5.4B**). HGMVECs exposed to heme (25 or 50 μM) for 24 hours showed increased necrosis, yet this was not observed after 48 hours (**Figure 5.4C**). Despite NFκB translocation and increased ROS production, exposure to heme did not result in apoptosis (**Figure 5.4C**).

Heme promotes a pro-thrombotic state

As the pro-thrombotic state, present during TMA caused by STEC infection, is an important feature of STEC-HUS, we assessed the effect of heme on tissue factor, the key initiator of the coagulation cascade. A significantly increased cell surface tissue factor expression was observed on HGMVECs following incubation with 25 or 50 μM heme, indicating that heme may enhance the pro-thrombotic state in STEC-HUS patients (**Figure 5.4D**).

Figure 5.1 Plasma levels of heme, hemopexin and HO-1 in STEC-HUS patients

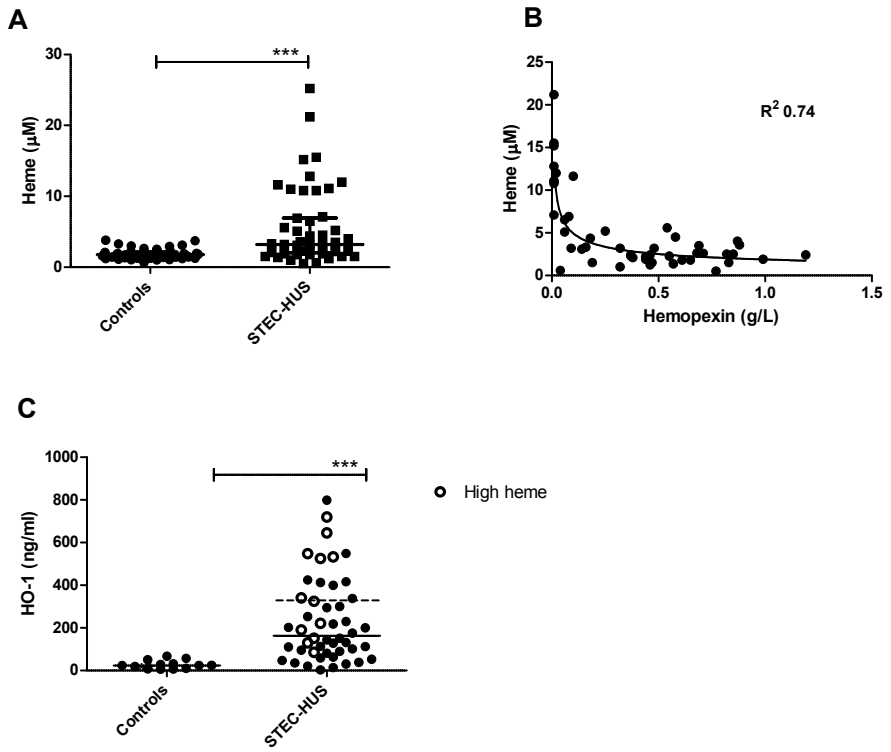

Figure 5.1 A. Significant higher heme levels were observed in STEC-HUS patients compared to and healthy controls ($p<0.001$) B. Heme levels in STEC-HUS patients inversely correlated with plasma hemopexin levels with (R^2 -0.74) C. HO-1 levels in plasma were significantly elevated in STEC-HUS patients compared healthy controls ($p<0.001$). Interestingly, especially patients with high heme levels (n=12, heme levels above 75 quartile range) had high plasma HO-1 levels ($p=0.008$) HO-1; Heme oxygenase 1, STEC-HUS; Shiga toxin producing Escherichia coli hemolytic uremic syndrome

Stx2 inhibits the expression of heme degrading enzyme HO-1

HO-1 is the most important intracellular heme degrading enzyme and provides resistance to heme-induced oxidative stress and cell injury. Stx2 has previously been shown to enter endothelial cells and interfere with protein translation. We therefore assessed HO-1 regulation in endothelial cells in response to heme in the presence or absence of Stx2 both on mRNA and protein level. HO-1 mRNA expression was found upregulated in HGMVECs in response to 25 or 50 μM heme after 6 hours (**Figure 5.5A**). Interestingly, in the co-presence of Stx2, HO-1 mRNA upregulation was even more pronounced. In contrast, protein levels of HO-1 were severely reduced in the co-presence of Stx2 compared to conditions in which Stx2 was absent (**Figure 5.5B**). Altogether, these data indicate that HO-1 mRNA expression is upregulated in response to heme, but that its translation into heme-degrading proteins is impaired by Stx2.

Figure 5.2 Correlation between clinical severity and plasma heme levels

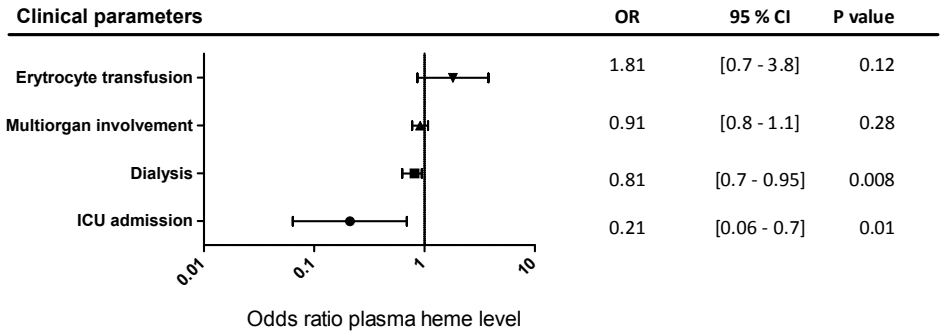

Figure 5.2 Odd ratios for different clinical parameters in relation with rise of plasma heme levels is depicted. With every rise in plasma heme of 1 μM the estimated risk of erythrocyte transfusion, development of multiorgan involvement, dialysis and ICU admission is calculated with binary logistic regression. CI; confidence interval, ICU; intensive care unit, OR; odds ratio

Figure 5.3 Endothelial cells internalize heme

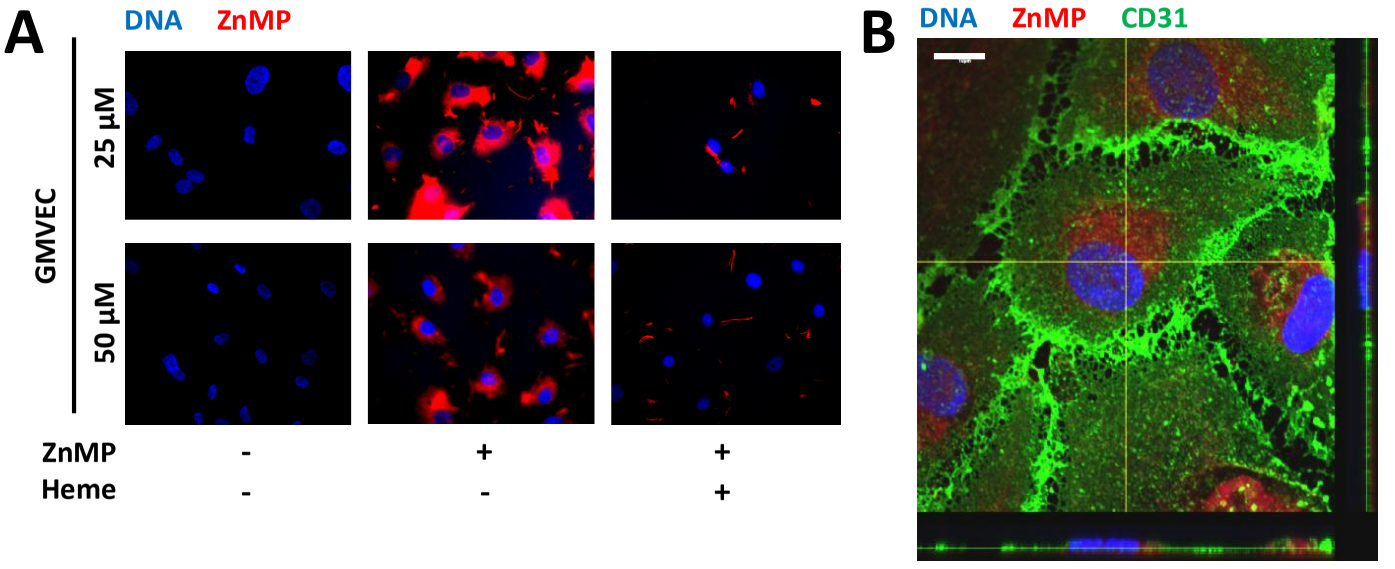

Figure 5.3 A.Exposure of HGMVEC to 25 or 50 µM of red-fluorescent ZnMP for 4 hours yields increased signal intensity (middle panels), which is decreased when same concentrations of heme is simultaneously added to cell culture (right panels). B. Confocal microscopy confirms endothelial uptake of red-fluorescent ZnMP. Scale bars: 10 µm. HGMVEC; human glomerular microvascular endothelial cell, ZnMP; zinc mesoporphyrin, CD31; endothelial cell marker.

Figure 5.4 Heme causes endothelial cell injury and promotes a pro-thrombotic state

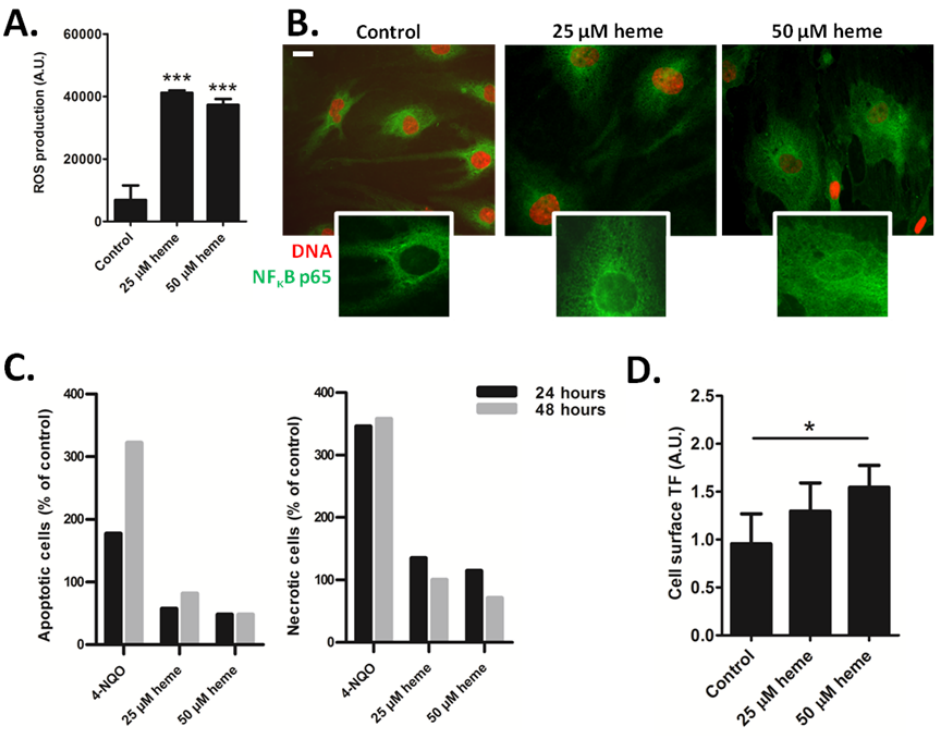

Figure 5.4 A. Exposure of HGMVEC to 25 or 50 µM heme resulted in increased reactive oxygen species generation (ROS) (n=3). B. HGMVEC exposed to 25 or 50 µM heme showed nuclear translocation of NFκB p65 (200x magnification). C.HGMVEC exposed to 25 or 50 µM heme for 24 and 48 hours showed no apoptosis, however necrosis was increased after 24 hours (n=3). D. HGMVEC expressed membrane-bound tissue factor in response to 25 and 50 µM heme after 6 hours (n=3).

****$p < 0.001$, *$p < 0.05$, compared to control unless specified otherwise. Scale bars: 10 µm. HGMVEC; human glomerular microvascular endothelial cell, ROS; reactive oxygen species, A.U; arbitrary units, 4-NQO; 4-nitroquinoline 1-oxide (positive control for apoptotic cell death).*

Discussion

Although STEC-HUS is one of the most common causes of acute kidney injury in children, the exact pathogenesis and the high diversity in clinical severity is only partially understood. In general, extensive hemolysis leads to release of the noxious molecule heme. To our knowledge, we are the first to show elevated heme levels in patients with STEC-HUS. These increased levels of extracellular heme are associated with depleted hemopexin and increased levels of HO-1 in plasma. Furthermore, when applied *in vitro* on primary HGMVECs, these heme concentrations caused ROS production and stimulated both a pro-inflammatory (e.g. nuclear translocation of NFκB) and pro-thrombotic state (e.g. increased TF expression). Moreover, the cytoprotective effects of HO-1 against the noxious effects of heme are largely abrogated by the HO-1 inhibiting effects of Stx2. Consequently, heme released during mechanical hemolysis could contribute, amplify and even drive the detrimental cascade leading to TMA. This heme-mediated effect is even more profound in STEC-HUS since Stx2 inhibits the induction of the protective HO-1 enzyme.

Previous literature regarding pathogenic heme levels refer mostly to one article of Muller-Eberhard et al. published in 1968.[112] As one of the first, they described free heme levels, determined with the use of pyridine hemochromagen assay, in patients with various hemolytic diseases in correlation with hemopexin levels.[112] From 6 μg/ml (~10 μM) of heme onwards hemopexin levels were depleted, and heme levels up to 30 μg/ml (~50μM) were measured.[112]

Since then, accumulating studies demonstrate that heme can contribute to the etiology of a wide variety of diseases as exemplified below.[108,113] Some studies report heme levels, mainly measured in patients with sickle cell disease, where heme levels around 4 (up to 20) μM were measured.[114] Heme was found to strongly contribute to the pathogenesis of sickle cell disease, whereas hemopexin and HO-1 protect against heme-induced detrimental effects.[103,115,116] Reported heme levels in patients with severe malaria are similar with median of 10 (IQR 4-22) μM and strongly contribute to its pathogenesis.[117,118] Similarly, heme has been shown to augment the severity of sepsis.[119] These previously reported heme values correspond well with the values we found in STEC-HUS patients.

Measurement of free heme in polar solvents is considered challenging due to its poor stability. Furthermore, heme easily reacts with various proteins, with the highest affinity for hemopexin, but is also known to bind to other proteins like albumin and lipoproteins.[104,105] However, only hemopexin and HO-1 are capable of fully neutralizing the noxious effect of heme, so in case heme is bound to for example albumin it remains partly bioreactive.[107] To measure these bioreactive levels of heme, we used an assay which takes advantage of the Fenton reaction of heme. When bioreactive heme comes in contact with the TMB substrate, this is oxidized resulting in a color which can be spectropho-

tometrically quantified. This assay has been used previously to measure heme levels in different patient cohorts and has been proven simple and sensitive when compared to assays using for instance toxic substances such as pyridine.[110,112,114,117] The measured plasma heme levels in our study reflect most likely the circulating levels of bioreactive heme. These levels could be even higher when measured in the microcirculation where heme is trapped in blood clots (over 350 μM) such as also is present in TMA.[120] The observed heme levels are supported by the inverse correlation between low hemopexin levels and measured heme levels. Moreover, in 15 patients hemopexin was undetectable with a strong correlation with high heme levels. This in contrast to haptoglobin, used as marker of hemolysis, which was undetectable in almost all patients. Hereby making hemopexin a reliable marker for high heme levels. Subsequently, significantly elevated HO-1 levels were observed in STEC-HUS patients compared to the healthy adult controls. HO-1 is known to be upregulated in response to various triggers like ischemia, inflammation and heme.[107] Besides intracellular upregulation of the degrading enzyme HO-1, it is also released in the bloodstream of patients in increasing amounts. However, in comparison to the well described extracellular scavengers like hemopexin, albumin and lipoproteins (all measured in g/L), concentrations of extracellular HO-1 are considerably lower (ng/ml). Most likely, HO-1 mainly exhibits its protective function intracellularly, however, we cannot exclude that it acts as a heme scavenger in serum.

We assessed the relation between measured heme levels and clinical severity. However, if any, higher heme seems to be associated with lower disease severity (lower risk of ICU admission, dialysis and multiorgan involvement). One possible explanation for this could be that a high red blood cell count, pointing to severe dehydration, is associated with worse outcome in STEC-HUS, hence interfering with our results.[121,122] In contrast, with every unit heme increases (1 μM) the chance for erythrocyte transfusion increases with approximately 80%, although this was not statistically significant. One could explain this by the fact that heme is mainly derived from red blood cells, hence a lower erythrocyte count is associated with more decay. Hence, higher heme and more erythrocyte transfusions. Another explanation could be that high heme levels amplify hemolysis as described previously.[104,106,123] A correlation between higher heme and worse outcome could still be present, but larger cohort studies are needed to confirm this.

Various studies have assessed the effect of heme *in vitro*, however using broad range of heme levels, up till 100 μM.[109,124] In our study, we used primary HGMVECs as these are the main target cell in the pathogenesis of HUS. By using a fluorescent heme analogue, ZnMP, we showed that heme is internalized by these endothelial cells. The mechanisms by which heme is internalized remains unknown. Various hypothetical theories of heme internalization have been proposed. First of all, heme possesses a highly lipophilic nature and therefore may have the ability to intercalate into the hydrophobic phospholipid bilayer of cell membranes.[104] Although we did not observe cellular injury upon exposure

to relatively low amounts of heme, we previously demonstrated that heme can cause cellular injury and death.[104,125] A role for heme transporters is perhaps a more plausible explanation for the observed internalization and efflux of heme.

Figure 5.5 Stx2 induces transcription, but inhibits protein expression of the heme-degrading enzyme HO-1

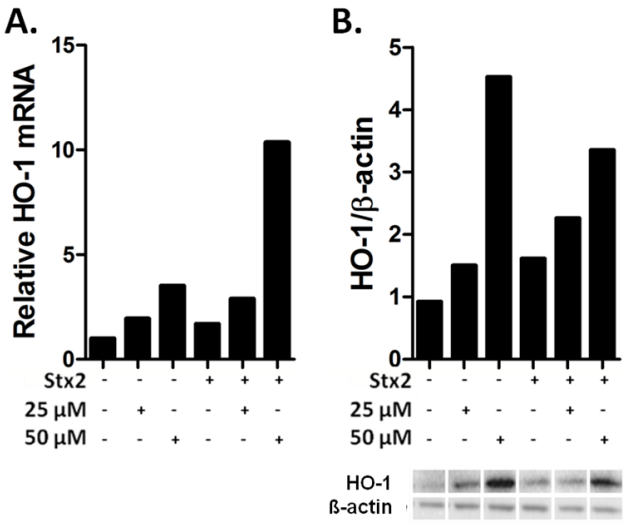

Figure 5.5 A.HGMVEC showed upregulated HO-1 mRNA levels in response to 25 or 50 µM heme, which was amplified by the co-presence of 100 pM Stx2 . B. Western blot analysis of HO-1 showed that the co-presence of Stx2 reduces HO-1 protein levels. The mRNA expression of HO-1 was plotted as relative value to the expression of GAPDH. GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, HGMVEC; glomerular microvascular endothelial cell, HO-1; heme-oxygenase 1, mRNA; messenger ribonucleic acid, Stx2; Shiga toxin 2

To assess the effect of disease specific heme levels we choose a concentration of 25 and 50 µM, taking into consideration our measurements of heme in our STEC-HUS cohort (levels up till 25 µM) and values measured by Muller-Eberhard et al. (levels up till 50 µM).[112] When exposing HGMVECs to these heme levels, we observed increased ROS production together with induction of a pro-thrombotic and pro-inflammatory state. These results are in line with previous published studies.[102] The generation of free radicals by the Fenton reaction is to date still considered the major form of ROS generation by heme and an important mechanism of heme-induced cytotoxicity.[104] Moreover, we demonstrated that heme induced nuclear translocation of NFκB, which is associated with tissue factor expression on the surface of the endothelial cells, facilitating vascular obstruction.

As previously proposed by Bitzan et al. Stx2 could inhibit HO-1 levels, when tested on human renal carcinoma-derived tubular epithelial cells.[109] Since STEC-HUS is mainly a glomerular disease we assessed HO-1 expression in HGMVECss with co-stimulation of Stx2. The presence of Stx2 caused an upregulation of HO-1 on mRNA level, however protein expression was inhibited. Most likely this could be explained by the known effect of Stx2 to inhibit protein synthesis via the ribosome. In line with these findings, HO-1 protein expression was altered by Stx2. Via a negative feedback mechanism this could explain the increased HO-1 at the mRNA level. Since HO-1 is important for degrading heme, inhibition of HO-1 activity through Stx2 would even further enhance heme-induced cytotoxicity in STEC-HUS patients. Furthermore, due to a polymorphism at the promoter region of HO-1, there is a wide variety between individuals in their ability to upregulate HO-1 expression. Individuals with longer repeats have reduced HO-1 activity and, as a consequence, are more susceptible to acute kidney injury.[126] These could partly explain the individual susceptibility to develop TMA.[102,125] For this purpose, it may be beneficial to screen individuals for HO-1 polymorphisms.[126]

Measurement of heme levels is considered quite difficult. By using the TMB assay our measured heme levels could be influenced by additional factors like free hemoglobin, also present during extensive hemolysis. However, free hemoglobin levels did not correlate with TMB results (data not shown). Haptoglobin is the scavenger of free hemoglobin, however in contrast to the strong correlation between hemopexin and heme, no correlation was found between haptoglobin and the presumed heme levels, indicating that the heme levels obtained with TMB seem specific for bioreactive heme. Hence, these observations could also be explained by interactions between heme and Toll-like receptor (TLR) 4.[127,128] As TLR4 is highly expressed on endothelial cells, and as ligation to TLR4 does generally not result in receptor-mediated endocytosis but rather in activation of pro-inflammatory signaling pathways, it is plausible that the internalization of heme is not required for the observed (pro-inflammatory) events in endothelial cells. As shown by Belcher et al. heme activates TLR-4 signaling leading to vaso-occlusion due to degranulation of Weibel-Palade bodies and expression of vascular adhesion molecules in models of sickle cell disease.[127]

Although STEC-HUS is merely treated symptomatically up till now, our findings suggest several potential future therapeutic candidates. One strategy could be to supplement the depleted scavengers like haptoglobin, to capture hemoglobin and prevents its decay into heme, or more naturally hemopexin to neutralize heme.[106,129] Various studies have already been conducted to study the effect of hemopexin infusions with promising results.[129] As the production of ROS is implicated in pro-thrombotic (TF expression) and (pro-inflammatory) NFκB-dependent signaling, it would be important to investigate whether ROS scavengers are able to prevent the TF expression or/and the nuclear translocation of NFκB in heme-treated endothelial cells. Furthermore, in a murine model

of STEC-HUS, the administration of anti-oxidants was shown to counteract important pathogenic events (e.g. platelet activation, renal damage) and ameliorate disease pathology.[130] Thus, ROS scavengers may protect against the detrimental effects of heme in HUS. Furthermore, by providing a higher amount of heme and hemoglobin scavengers, hemopexin and haptoglobin, one could prevent increased levels of bioreactive heme and consequently endothelial cell injury mediated by heme.[105,131] Various animal studies have been performed to look at the effect of therapeutic hemopexin administration with promising results.[105,132] In contrast to hemopexin, haptoglobin is already present as orphan drug. Hypothetically, by administrating haptoglobin, free hemoglobin would be bound and therefore could not be oxidized and release heme, hence no heme would be present.[106] Although up till some extent damage is already caused at time of admission, most patients still have active disease with ongoing TMA at presentation.

In conclusion, heme might be a contributing and driving factor in the pathogenesis of STEC-HUS and could potentially amplify the cascade leading to TMA. In this study we have provided evidence that elevated heme levels are present in STEC-HUS patients. Moreover, the observed heme levels have a strong inverse correlation with hemopexin levels, making hemopexin a reliable biomarker for toxic levels of heme present *in vivo*. Moreover, these disease relevant elevated heme levels promoted oxidative, pro-inflammatory and thrombotic stress *in vitro*. Importantly, via Stx2 inhibited HO-1 induction exacerbates these events by decreased protection against heme-induced insults and may promote development towards TMA.

Acknowledgements

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Supplemental data: Methods

Hemopexin measurement

Hemopexin was measured in enzyme-linked immunosorbent assay (ELISA) setting. A microtiter plate (Greiner Bio-One™) was coated with 500 ng/mL anti-human hemopexin capture antibody (Bioporto) at 4°C overnight. Subsequently, standard dilutions of hemopexin from human plasma (0-50 ng/mL, Sigma-Aldrich) as wells as diluted samples were added to the plate. Next 100ng/ mL of the detection antibody biotinylated anti-human hemopexin (Bioporto) was added to the plate followed by 1:1000 dilution of streptavidin-HRP (RPN1231V GE healthcare). Subsequently, TMB Substrate (Sigma-Aldrich) was added and the absorbance was measured at wavelength of 450 nanometer (nm) with Victor 3 V multilabel plate reader (Perkin elmer).

Heme-oxygenase 1 measurement

Plasma HO-1 levels were determined by a sandwich ELISA using the human HO-1 matched antibody pair kit (Abcam) according to the manufacturer's instructions.

Flow cytometric apoptosis assay

Confluent monolayers of HGMVECs on gelatin (fluka) coated 48-well tissue culture plate (Corning ® Costar ®) were stimulated with 4-nitroquinoline 1-oxide (4NQO; Sigma-Aldrich) or 25 and 50 µM hemin chloride porcine (designated as heme, Sigma-aldrich) prepared from 10 mM stock solution for 24 and 48 hours. Subsequently, cells were detached with 0.25 % Trypsin-EDTA (Gibco™) and collected into 1.5 mL eppendorf tubes(Eppendorf). The cells were taken up in Annexin V-buffer and stained with FITC-labeled Annexin V and propidium iodide (Sigma-Aldrich) according to the manufacturer's protocol (BioVision). All samples were analyzed on a FC500 flow cytometer with CXP software (Beckman Coulter). The flow cytometry data were analyzed with Kaluza Flow Analysis Software 1.3 (Beckman Coulter).

Reactive oxygen species (ROS) measurement

The production of intracellular ROS was measured using the fluorescence probe reagent CM-H_2DCFDA (Thermo Scientific) as described previously by Wilmer et al.[133] Briefly, confluent monolayers of HGMVECs on gelatin coated 96-wells tissue culture plate were stimulated with 25 and 50 µM heme (prepared from 10 mM stock solution) or 50 µM hydrogen peroxide (H_2O_2; Merck). ROS formation was measured by fluorometry (excitation/emission 464/530 nm) after 1 hours at 37°C with the Victor 3 V Multilabel Plate reader.

Quantitative polymerase chain reaction (qPCR) and western blot

HGMVECs were seeded on gelatin coated 6-wells tissue culture plate (Corning ® Costar ®) and grown to confluent monolayers. HGMVECs require inflammatory mediator tumor necrosis factor α (TNFα) to upregulate the Gb3 receptor on their cell surface. [111,134] Therefore, in the conditions where the effect of Stx2 in the presence or absence of heme was assessed, HGMVECs were preincubated with 10 ng/mL TNFα (Sigma-Aldrich) for 24 hours at 37 °C with 5 % CO_2. Subsequently, 100 pM Stx2 (Phoenix lab) was added to HGMVECs and incubated for 8 hours at 37 °C with 5 % CO_2. Next, 25 and 50 μM heme was added and incubated for additional 16 hours at 37 °C with 5 % CO_2.

The total RNA was extracted using TRIzol reagent (Ambion life technologies) and RNA isolation kit according to the manufacturer's instructions (NucleoSpin® RNA II from Macherey-Nagel). From the total RNA, 200 ng served as a template for cDNA synthesis in a reaction using a mix consisting of milliQ, reverse transcriptase buffer 5x (RT buffer 5x; Invitrogen), random primers (Promega), oligo dT (promega), dNTP's (self made), dithiothreitol (DTT; Invitrogen), recombinant RNasin® ribonuclease inhibitor (Invitrogen), Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Invitrogen). For qPCR analyses, a SYBR® Green master mix including primers (respectively forward and reverse primers for GAPDH were 5'-TCC AAA ATC AAG TGG GGC GA-3' and 5'-GGA CTG TGG TCA TGA GTC CT-3' and HMOX1 5'-AGA CAC CCT AAT GTG GCA GC-3' and 5'-CTG AGC CAG GAA CAG AGT GG-3) was made. qPCR was performed using the Bio-Rad CFX96™ Real-Time PCR Detection System. Experiments were performed in duplicate and the values were normalized to GAPDH using delta-delta Ct method.

Protein was isolated with standard RIPA buffer (0.15M NaCl (Sigma-Aldrich), 0.012M Sodium Deoxycholate, 0.1% nonidet P-40 (NP40), 0.1% SDS (Sigma-Aldrich), 0.05M Tris (Sigma-Aldrich) pH 7.5 and protease inhibitor cocktail (Sigma-Aldrich)). The concentration of extracted protein was determined with BCA protein assay according to the manufacturer's protocol (Thermofisher Scientific). For HO-1 protein expression, 1 ug/mL protein was boiled in Laemmli buffer (Biorad). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out using 10 % resolving gels and 4 % stacking gels. Subsequently, protein were transferred to polyvinylidene difluoride (PVDF; Merck) membranes. Blots were blocked with PBS-5 % skim milk (Merck) for an hour and incubated with primary antibodies, rabbit anti-heme-oxygenase-1 polyclonal antibody (Enzolifesciences) or anti- β-actin (Novus biological) for 1 hour at RT. Proteins were detected with HRP-conjugated goat-anti rabbit IgG antibody (Dako). Blots were developed using SuperSignal West Pico Chemiluminescent substrate according to the manufacturer's instructions (Thermofisher scientific). The visualization of the bands were done with Biorad image lab software (Biorad).

Statistical analysis

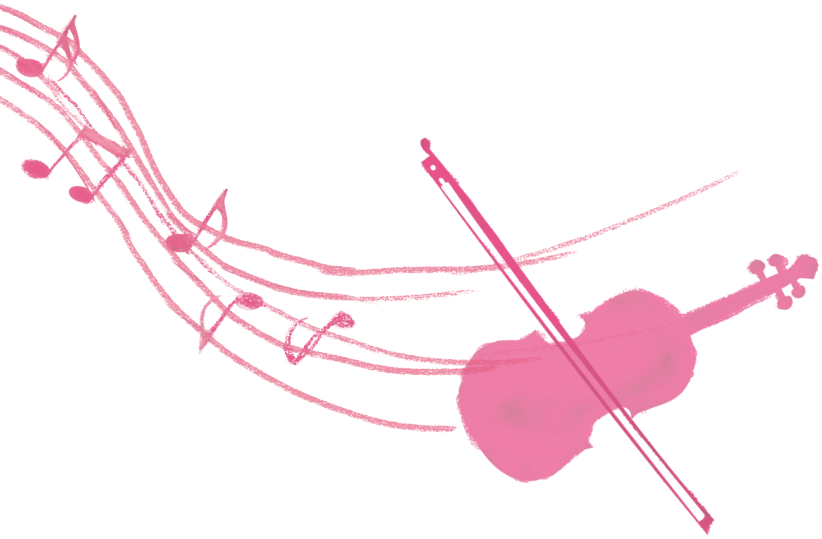
Values are expressed as valid percentages for categorical variables and as median and 25 - 75 interquartile range (IQR) for continuous variables. Log transformation was applied when data were not normally distributed to reduce skewed data. Regression analyses, with coefficient of determination R^2, was performed to determine degree of correlation between to variables. Binary logistic regression analyses was preformed to look at the risk (odds ratio: OR) of heme levels in relation to clinical parameters. Chi-square test was performed to compare categorical data and Mann-Whitney U test was used to compare continuous data. Heme levels were categorized with quartile ranges. In case a value was above the 75 IQR, the level was classified as high heme level. P values less than 0.05 were considered statistically significant and 95 confidence intervals were reported. All graphs were performed using GraphPad Prism software version 5. For statistical analyses, SPSS software (version 22.0) was used.

Part II

STRATEGIES TOWARDS PERSONALIZED TREATMENT OF aHUS

“The falling leaves drift by my window. The falling leaves of red and gold.”

Autumn Leaves - Eva Cassidy



Chapter 6

Eculizumab dosing regimen in atypical HUS: possibilities for individualized treatment

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Abstract

Recent studies indicate that eculizumab is often given in excess to aHUS patients. Individualization of treatment is thus highly requested, however, data on pharmacokinetics and pharmacodynamics of eculizumab remain limited. We analyzed 11 patients during induction (weekly), maintenance (2-weekly) and tapering (every 3-8 weeks) phases of treatment.

The trough eculizumab levels increased with each additional dose during the induction phase (depending on body weight). During maintenance, high eculizumab concentrations of up to 772 μg/ml were observed. The levels decreased with each following dose during tapering (3- and 4-week intervals), however three patients maintained target eculizumab levels over long time periods (30-48 weeks). At intervals of 6-8 weeks target eculizumab levels were no longer attained. Serum samples with eculizumab concentrations ≥50 μg/ml showed adequate complement blockade.

Our data provide essential insight for optimization of eculizumab dosing schemes and lessening of therapy burden for the patients and cost of the treatment.

Introduction

Hemolytic uremic syndrome (HUS) is a severe form of thrombotic microangiopathy (TMA) characterized by hemolytic anemia, thrombocytopenia and acute renal failure. The majority of cases are caused by an infection with Shiga toxin producing *Escherichia coli* (STEC). However, 5-10% of HUS occurs without a preceding STEC infection and is attributed to complement dysregulation.[1,10,43,54,58,59] Currently, alternative complement pathway aberrations are identified in 50-60% of atypical HUS (aHUS) patients. Genetic variants affecting *complement factor H* (*CFH*), *complement factor I (CFI), membrane co-factor protein (CD46), C3, complement factor B* and *thrombomodulin* as well as autoantibodies against CFH (anti-CFH) are associated with aHUS pathogenesis.[135-147] More recently, genetic rearrangements resulting in CFH/CFH-related hybrid proteins have also been described.[49,148,149]

Eculizumab is a humanized monoclonal complement inhibitor that binds to C5 and prevents its cleavage and activation into C5a and C5b, blocking formation of the terminal complement complex C5b-9. Eculizumab is currently approved by the Food and Drug Administration and European Medicines Agency (EMA) for the treatment aHUS and has proven to be highly efficient in the treatment of this disease.[8,58,150-153] The current international guidelines recommend life-long treatment with eculizumab. However, the resulting life-long complement blockade may increase the risk of meningococcal infection, and eculizumab treatment is associated with extreme costs.[154,155] There are no studies that would support the necessity of a life-long treatment in all aHUS patients. Before eculizumab has become available, plasma therapy was a main-stream approach for the treatment of aHUS. The guidelines for plasma therapy in aHUS advised individualized regimen and withdrawal in patients that have reached remission.[156,157] Therefore, many nephrologists use a restrictive approach for eculizumab treatment in aHUS and discontinue therapy when patients reach remission.[158-165] A subset of patients that discontinued eculizumab in these reports experienced relapse, and the therapy had to be reinitiated. This underscores the need for close monitoring of these patients for signs of relapse.

The recommended concentrations of eculizumab for efficient complement blockade are 50-100 μg/ml, however in the past our and other studies demonstrated that eculizumab levels in patients, that receive the drug every two weeks exceed this reference by up to ten fold.[158,166-168] Thus, optimization of dosing regimen in eculizumab therapy is urgently needed, however, pharmacokinetic and pharmacodynamic data to guide such personalized treatment schemes remain limited.

In this study, we followed patients who were treated with standard and extended infusion intervals, and analyzed eculizumab concentrations and complement blockade in these patients.

Methods

Patient cohort

The 11 patients, described in this study were diagnosed with aHUS and received treatment at the Radboud university medical center, Nijmegen, The Netherlands. Eculizumab was administered in the dosages that are recommended by the current EMA guidelines.[169] The current treatment schedule is split into two phases: the initial phase (weekly infusions for up to 4 weeks for patients ≥40 kg body weight), followed by the maintenance phase (life-long infusions every two weeks). For pediatric patients (<18 years old, <40 kg), the dosage regimen is adjusted to body weight. The other patients (pediatric and adult) receive the drug according to the schedule for adults.

In this report, in patients who have reached remission (normalized hematological parameters and improved/stable renal function), the therapy was reevaluated and intervals between infusions were gradually extended (while keeping maintenance infusion doses unchanged) or therapy was discontinued (**Table 6.1**). In two patients, eculizumab treatment was discontinued without extension of dosage intervals beyond two weeks due to an allergic reaction (patient 8 (P8)) and severe pneumonia (P11). In nine patients ≥40 kg, the eculizumab dose was 1200 mg in the maintenance/tapering phase and for the two children with body weight of 15 kg (P2) and 11 kg (P9), the dose was 300 mg.

During tapering/discontinuation of therapy, all patients and/or their caregivers were strictly instructed to contact their physician in case of any signs of infections, generalized malaise, fever, hematuria, edema, oliguria, paleness or other signs indicating recurrence of aHUS. In case of signs of aHUS recurrence (which did not take place during the described period), eculizumab would be restarted immediately.

Collection of blood samples

Blood samples from healthy adult controls and patients just before each eculizumab infusion were collected and placed immediately on ice (ethylenediamineteraacetic acid (EDTA) blood) or allowed to clot for 30-40 minutes (min) at room temperature (whole blood). EDTA plasma and serum samples were collected by centrifugation (10 min, 2000 x g, 4°C), aliquoted and stored at -80°C.

Assay for detection of serum eculizumab concentration

Eculizumab detection was performed using in-house enzyme-linked immunosorbent assay (ELISA). To capture eculizumab, 96-well plates (Greiner Microlon® 600 High Binding, Sigma-Aldrich, Zwijndrecht, the Netherlands) were coated with 0.5 µg/well of purified C5 (Calbiochem®, San Diego, CA, USA) diluted in carbonate buffer (pH=9.6) overnight at 4˚C. The wells were washed with phosphate buffered saline (PBS) supplemented with 0.05% Tween-20 (PBST) (Sigma-Aldrich) after this and following steps. The plates were

blocked with SuperBlock (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA, USA) 30 min at room temperature (RT) and then 45 min at RT with PBS, supplemented with 1% bovine serum albumin (PBS/BSA). Serum samples were diluted in PBS/BSA and dose response standards were prepared by adding eculizumab (Alexion Pharmaceuticals, Cheshire, CT, USA) to PBS/BSA. Samples and standards were added to the wells and incubated for 2 hours (h) at RT. Eculizumab detection was performed using incubation with Peroxidase AffiniPure Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted in PBST for 2 h at RT, o-phenylenediamine dihydrochloride tablets (Dako, Hevelee, Belgium) were added as a substrate. The reaction was stopped with 2M H_2SO_4, and samples were measured in a 96-well microtiter plate reader at 490 nm. Dilution linearity of the test was analyzed by measuring serum containing 383 µg/ml eculizumab and had coefficient of variation (CV) of 10.5% over the range of a standard curve. The detection limit of an assay was 8 µg/ml for undiluted samples. The assay showed variation coefficients of 2.9% (intra-assay, 352 µg/ml) and 5.2% (inter-assay, 328 µg/ml). Recovery was determined in 20 control samples spiked with 50 µg/ml (68 ± 31 %), 75 µg/ml (66 ± 25%) and 150 µg/ml (74 ± 17%).

C5 assay

C5 concentrations were measured in available sera or EDTA plasma, that were collected from 10 aHUS patients in acute phase, before the start of eculizumab therapy and from nine healthy volunteers. The C5 levels were measured using Human Complement C5 ELISA Kit (Abcam, Cambridge, UK), according to manufacturer's instructions.

Complement assays

The concentrations of fluid phase terminal C5b-9 complement complex (sC5b-9) in seven available EDTA plasma samples were quantified using ELISA, as previously described in detail by Bergseth et al.[170] International complement standard two was used for quantification of sC5b-9 in complement activation units per ml (CAU/ml).

Complement activity was accessed in serum samples by determining the activity of classical complement route (CH50) using an ELISA method adapted from.[171] Briefly, polyclonal goat (affinity purified) anti-human IgM (Cappel, Bioconnect Life Sciences, Huissen, The Netherlands) was coated in 96-wells plates (Nunc Maxisorb plates, Nunc, Roskilde, Denmark) at a concentration of 0.2 µg/well in carbonate buffer (pH 9.6), overnight at 4˚C. The plates were washed with Tris buffered saline (TBS) containing 0.05% Tween-20 (TBST) after each step. Residual binding sites were blocked by incubation with 10 mM TBS containing 1% gelatin (BD Difco™, Franklin Lakes, New Jersey, USA) for 1 h at RT. After blocking, 0.25 µg/well IgM (Fitzgerald Industries International, Acton, MA, USA) diluted in TBST was added and incubated for 1 h at RT. Next, standards (human pooled serum) and patient serum samples diluted in cold buffer containing 10 mM Tris,

84 mM NaCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.1% gelatin (pH 7.5), were added per well and incubated for 1 h at 37˚C. Complement binding was detected using a monoclonal mouse-anti-human C5b-9 antibody (Dako) diluted in TBST and incubated for 1 h at RT. After washing, alkaline phosphatase labeled goat anti-mouse IgG (Dako) in TBST was added for 1 h at RT. Alkaline phosphatase activity was revealed following incubation with 4-nitrophenyl phosphate disodium salt hexahydrate as a substrate (Sigma-Aldrich). The reaction was stopped with 2 M NaOH, and samples were measured in a 96-well microtiter plate reader at 450 nm. The reference value of CH50 of 67-149% was calculated with the results of 50 healthy donors.

Statistical analyses

GraphPad Prism 5 for Windows (GraphPad Software Inc.) was used to plot eculizumab levels against treatment intervals, and sC5b-9 complex concentrations and CH50% against eculizumab levels. If a patient did not reach the minimal target level of 50 μg/ml eculizumab ≥ 1 time in a treatment interval, he/she was considered not to have attained the target level for that interval. Following, the percentages of patients not reaching the eculizumab target level were plotted against treatment interval. To evaluate changes in eculizumab concentrations by the number of infusions within a treatment interval, we used IBM Statistics 22.0 for Windows (IBM SPSS Inc.). For each treatment interval, we plotted eculizumab levels against infusion number and estimated the increase or decrease in eculizumab level per infusion with 95% confidence intervals (CI), using linear mixed models with infusion numbers as fixed and patients as random effects. Body weight (in kg) and age (in years) were included in these models as potential confounders.

Results

Patient cohort

In total, five pediatric and six adult patients diagnosed with aHUS treated with eculizumab were included in this study (**Table 6.1**). In nine of these patients, pathogenic variations in the complement genes were found. One aHUS patient (P3) had anti-CFH and in one other patient (P9) no genetic abnormalities or anti-CFH were detected. In nine patients, the intervals between eculizumab infusions were extended for up to eight weeks after stable remission was achieved. In one of these patients (P1), eculizumab treatment was discontinued completely. Serum samples of this patient were continuously collected through week 26 after the last eculizumab dose (**Table 6.1**), no disease recurrence was observed during the study period. In two patients, eculizumab treatment was discontinued without extension of dosage intervals beyond two weeks due to an allergic reaction (P8) and severe pneumonia (P11).

Serum eculizumab concentrations in aHUS cohort

The eculizumab trough concentrations (lowest drug concentration in blood between two consecutive infusions) that were measured in 11 patients are depicted in **Figure 6.1A**. The detected values were 36-459 µg/ml (n=27) and 40-772 µg/ml (n=90) during the induction and maintenance phases, respectively. During tapering, concentrations of 61-367 µg/ml (n=38), 11-256 µg/ml (n=38) and 13-161 µg/ml (n=6) were measured at 3- , 4- and 5- week infusion intervals. At intervals of 6-8 weeks (n=10), the concentrations were 0-41 µg/ml. For infusion intervals of 1-5 weeks, attainment of the minimal target level of 50 µg/ml was found in 75-91% of patients (**Figure 6.1B**).

Serum eculizumab concentrations in individual patients during infusions with standard and extended intervals

In the induction phase, the eculizumab levels gradually increased with each following 1-week interval by on average 73.1 (95% CI: 56.2-90.0) µg/mL, adjusted for body weight (**Table 6.2**). Two adult patients (P10 and P11) did not attain the target eculizumab level after the first infusion, but did so after the second dose (**Figure 6.2A**). **Figure 6.3** shows that patients P10 and P11 had the lowest trough eculizumab concentrations one week after the first eculizumab infusion and the highest sC5b-9 complex levels measured before the first infusion. The serum C5 levels in patients in acute phase before start of therapy were 52-136 µg/ml, while values obtained from nine healthy controls were 80-107 µg/ml. Serum eculizumab concentrations did not change with each following dose at 2-week intervals (**Figure 6.2B, Table 6.2**), whereas the concentrations decreased on average by -11.8 (95% CI: -7.1- -16.6) µg/ml and -3.6 (95% CI: -1.1- -6.2) µg/ml with each following dose in the tapering phase of the 3- and 4-week intervals, respectively (**Figure 6.2C and 6.2D, Table 6.2**).

Table 6.1 Characteristics of aHUS patients

Patient	aHUS pathogenic change	Gender (F/M)	Age at start of the observation (years)	Body weight (kg)	Studied dose intervals (weeks)[a]	Total observation period (months)
P1	*CFH:* c.1778T>A, p.Leu593Stop	F	28	61	1-6, 8, 14, 20, 26	15.5
P2	*CFH*: c.3572C>T (p.Ser1191Leu); CFH: c.3590T>C (p.Val1197Ala)	M	2	15	2-4	24
P3	Anti-CFH	M	9	50	1-4	28.5
P4	CFH/CFH1 hybrid protein	M	11	45	1-4	30.5
P5	*CFH:* c.2572T>A (p.Trp858Arg)	F	22	114	2-7	12
P6	*CFH:* c.2120delC	F	45	71	1-6	7
P7	*C3*: c.481C>T (p.Arg161Trp)	F	62	56	1-3, 8	8
P8[b]	*C3*: c.481C>T (p.Arg161Trp)	F	11	46	1, 2	2.5
P9	No changes found	M	1.3	11	1-5	11.5
P10	*CFH:* c.1520-1G>A (splice site)	F	44	53	1-7	23.5
P11[b]	*CFI:* c.685T>C (p.Cys229Arg)	F	21	85	1, 2	2

[a] *All patients with body weight ≥ 40 kilo were treated with four weekly infusions of 900 mg at start of therapy, after that, starting from week five the patients were treated with 1200 mg. For P2 and P9 pediatric dosage was applied (600 mg infusion at first week, 300 mg at week 2 and further). Samples were taken after the indicated intervals since last eculizumab infusion.*

[b] *Eculizumab treatment in these patients was discontinued during the study due to allergic reaction (P8) and severe pneumonia (P11).*

However, only three patients had a follow-up with five or more infusions at 3- and 4-week intervals. At 3-week intervals, the eculizumab levels remained relatively high in P4 and within/slightly above the target range of 50-100 μg/ml throughout the observed periods of 10 and 11 intervals (30 and 33 weeks) in P2 and P3, respectively. At 4-week intervals, the eculizumab levels remained within the recommended range throughout 12 intervals (48 weeks) of therapy for P4 only.

Body weight had a small effect on eculizumab level in the induction period only -3.9 (95% CI: -1.8 - -6.0) μg/ml per kg, while age did not have an effect in any of the intervals. The number of available data points at 5-week intervals did not allow statistical analysis. However, four of the five patients analyzed still attained the eculizumab target concentration after the first 5-week interval and P10 also attained the target range after the second 5-week interval (**Figure 6.2E**).

Figure 6.1 Serum eculizumab concentrations in aHUS cohort

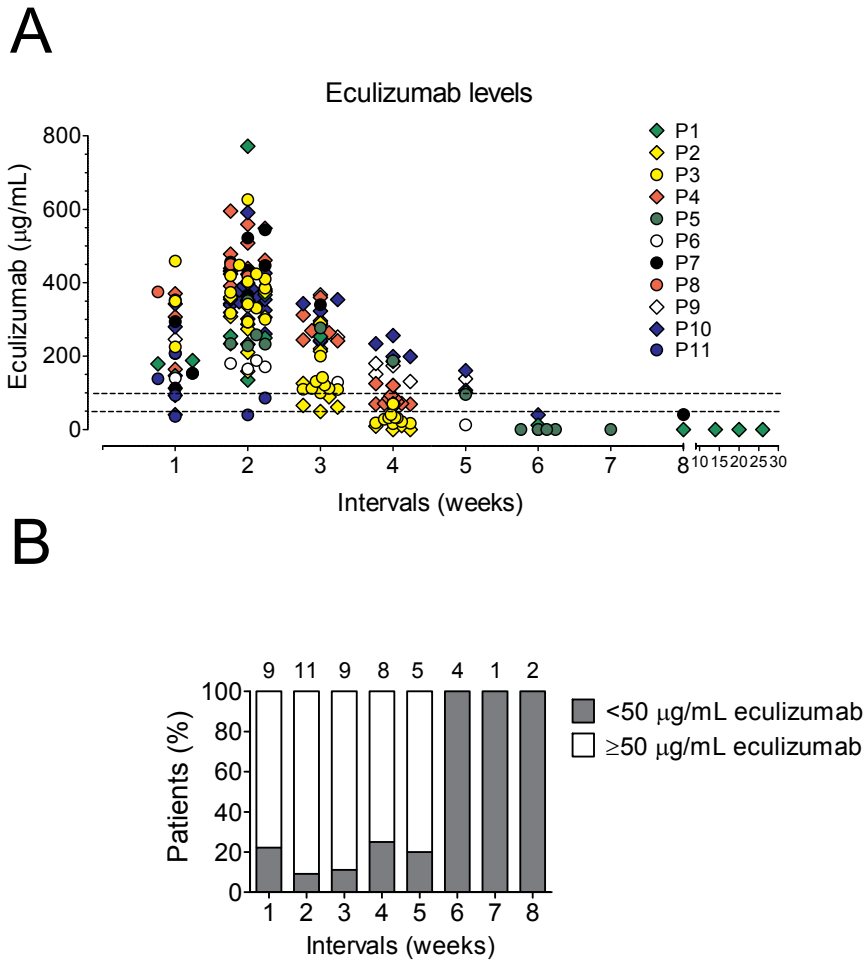

Figure 6.1 A. Eculizumab trough levels in 11 patients in the induction (weekly), maintenance (2-weekly) and tapering (every 3-8 weeks) phases of therapy (Table 6.1). From one patient (P1), samples were also collected after discontinuation of treatment (at 14, 20, 26 weeks). The dashed lines indicate the recommended range of eculizumab levels of 50-100 µg/ml. B. Percentages of patients that attained the trough eculizumab level of 50 µg/ml in all measured samples in an interval are indicated in white, whereas percentages of patients with a value < 50 µg/ml in at least one sample are indicated in gray. Total numbers of patients analyzed for each interval are shown above the columns.

Figure 6.2 Eculizumab concentrations in individual patients during infusions with standard and extended intervals

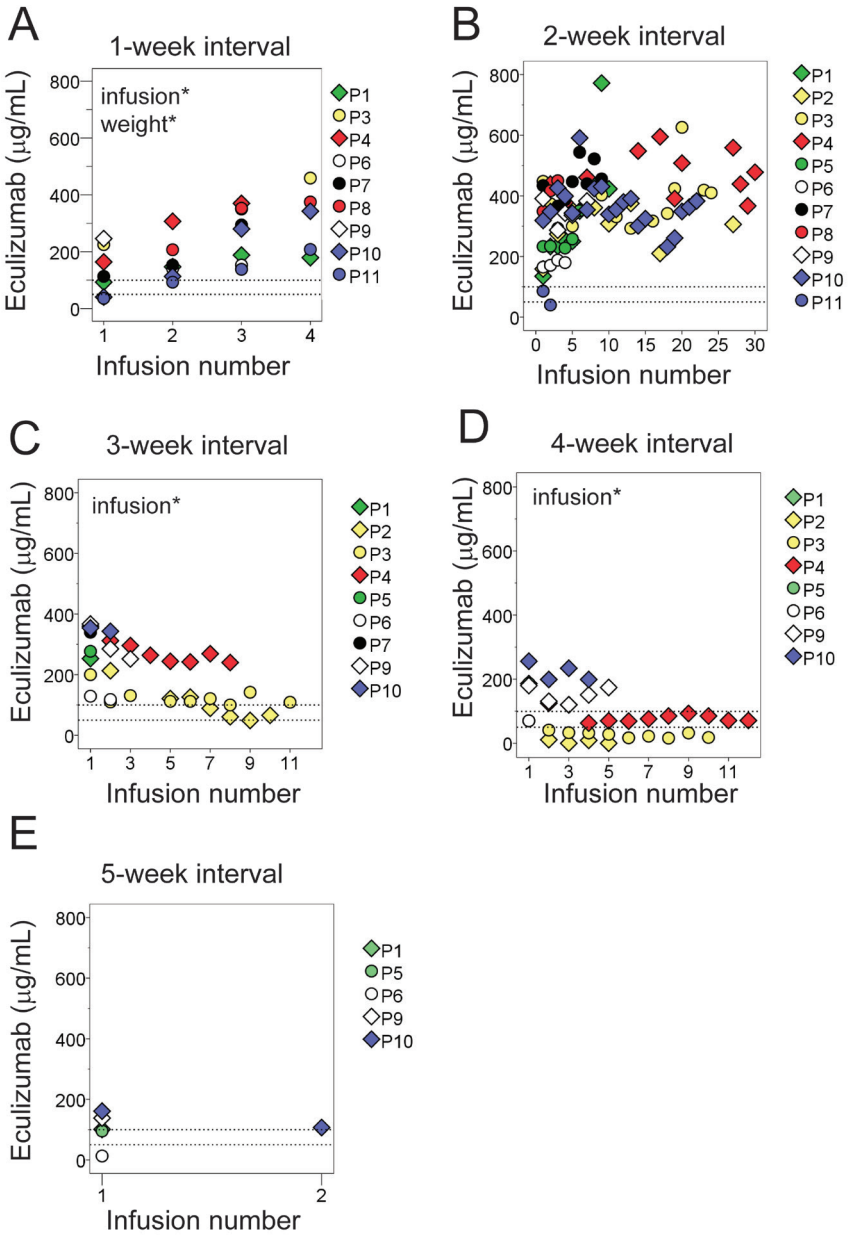

Figure 6.2 Eculizumab trough levels in 11 patients before next eculizumab infusion in the induction (1- week intervals, A. maintenance (2-week intervals, B. and tapering (3-, 4- and 5-week intervals, C, D and E. phases of therapy. The dashed lines indicate the recommended range of eculizumab levels of 50-100 µg/ml. Observed effects (*) of each following infusion or weight of a patient (kg) on eculizumab concentration as presented in Table 6.2 are indicated.

Complement inhibition under eculizumab treatment

All samples that were measured in our study and reached the eculizumab target level of 50 µg/ml had a CH50 value ≤ 12% (detection limit 10%) (**Figure 6.4**).

Table 6.2 Estimated effects of number of infusions on eculizumab concentration in serum per treatment interval

	Number of patients in treatment interval[a]	Range of samples per patient	Estimated mean eculizumab level (µg/ml)		95% CI
1-week intervals					
At start of interval	7		131.1	53.6	208.6
Increase/decrease per infusion	9	1 - 4	73.1[b]	56.2	90.0
2-week intervals					
At start of interval	10		271.9	176.9	366.9
Increase/decrease per infusion	11	2 - 20	2.3	-0.7	5.3
3-week intervals					
At start of interval	8		285.0	212.4	357.6
Increase/decrease per infusion	9	1 - 9	-11.8	-16.6	-7.1
4-week intervals					
At start of interval	6		158.2	80.8	235.6
Increase/decrease per infusion	8	1 - 11	-3.6	-6.2	-1.1

[a] *At the start of each interval, data were not collected from all patients*

[b] *Adjusted for patient's body weight (kg); weight and age did not influence the other estimates.*

Figure 6.3 sC5b-9 complex concentration at baseline and trough eculizumab levels after the first infusion

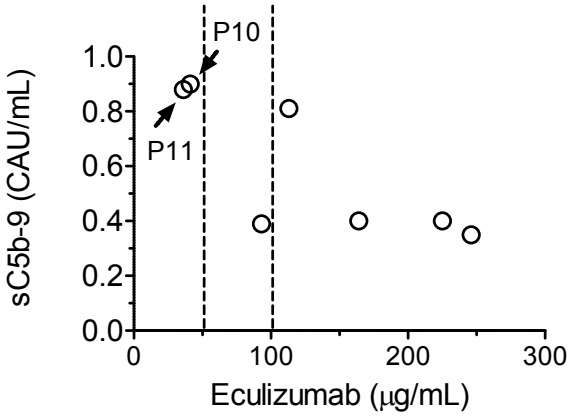

Figure 6.3 Seven patients (P1, P3, P4, P7, P9, P10, P11) of whom EDTA plasma was available were included. The dashed lines indicate the recommended range of eculizumab levels of 50-100 μg/ml. Patients that did not attain the recommended eculizumab level after first infusion are indicated (P10 and P11).

Figure 6.4 Complement inhibition during eculizumab treatment in aHUS patients

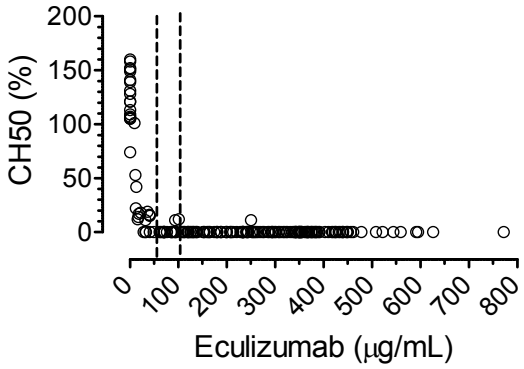

Figure 6.4 Complement activity (CH50%) data of the samples included in the study are presented as percentage of activity of normal human pooled serum. The dashed lines indicate the recommended range of eculizumab levels of 50-100 μg/ml.

Discussion

In this report we, to our knowledge, for the first time performed a systematic analysis of eculizumab levels in patients, receiving the drug with various (standard and extended) infusion intervals. In the induction phase (1-week intervals) we have observed a weight-dependent increase in eculizumab concentration with each following infusion, which is consistent with the half-life of eculizumab in aHUS patients reported as 7.8-19.5 days.[167] In two patients with high initial sC5b-9 levels, the values were below the target range one week after the first infusion. Previous report investigating efficacy of eculizumab treatment in patients with TMA after hematopoietic stem cell transplantation indicated that patients with higher levels of sC5b-9 complex before treatment have faster initial eculizumab clearance.[172] Interestingly, recent study indicated that eculizumab is able to bind to sC5b-9 *in vitro*, which suggests that part of the drug may be scavenged into a complex with sC5b-9 at first infusion.[168] Elevated sC5b-9 is a known biomarker in aHUS and thus should be taken into consideration in design of future personalized eculizumab protocols.[173,174]

In the maintenance phase (2-week intervals) the levels of eculizumab had reached extremely high values of up to 772 μg/ml. Considerable excess of eculizumab in patients was also previously reported by our and other studies.[158,166-168] Interestingly, one of the patients (P11) who had a severe pneumonia did not attain minimal target value of eculizumab and complete complement inhibition at the second infusion at 2-week interval. The reason of this is not clear, however, since levels of sC5b-9 have been shown to influence eculizumab pharmacokinetics, inflammation and increased rate of complement activation may have caused lower eculizumab concentration in this patient.

Although most patients attain eculizumab levels far above the recommended range at 2-week intervals, P11 illustrates that in some patients with ongoing active disease standard eculizumab dosing may be not sufficient for complement blockade and this should be taken into consideration when applying extended eculizumab regimen.

Interestingly, P11 developed severe pneumonia while having sub-therapeutic eculizumab levels. Although it was not a meningococcal infection, for which the terminal complement pathway is especially important, this case emphasizes the need to evaluate how much of residual complement activity is necessary to decrease risk of infection and how much of this activity may be safely allowed without risk of an aHUS relapse.

No significant correlation between eculizumab levels and number of received infusions was observed in the maintenance phase. Previously in another study, weight of the patient was correlated to the median trough concentration in a small group of aHUS patients (n=7), which was not reproduced in our study.[167] Nevertheless, with the exception for P11, all other patients attained target eculizumab levels at all 2-week infusion intervals that were analyzed.

During tapering of the treatment with extended intervals of 3- and 4- weeks, statistically significant decrease in eculizumab concentration was observed with each infusion. This indicates, that complement blockade may remain adequate at the first infusions of the extended intervals, but diminish overtime. However, at 3-week interval, in two patients (P2, P3) eculizumab recommended range of 50-100 µg/ml was attained and complement was efficiently blocked throughout the observed period of 10 and 11 intervals (30 and 33 weeks), respectively. At 4-week interval for one other patient (P4), the eculizumab levels remained within the recommended range and complement was completely blocked throughout 12 intervals (48 weeks) of therapy. Interestingly, of the three pediatric patients, who were able to sustain optimal eculizumab concentration in extended intervals, two (P3, P4) were treated with a standard adult dosage (1200 mg) and one (P2) with standard pediatric dosage (300 mg) per infusion based on their body weight (**Table 6.1**). These three cases illustrate how monitoring of eculizumab concentrations and complement inhibition allows to establish optimal infusion intervals which can be maintained over a long period of time for patients on adult and pediatric regimen.

Furthermore, our data indicate that at 5-week intervals attainment of target eculizumab range is possible at least for one or two infusions.

Overall, quite high inter-patient variability has been observed. In our manuscript we analyzed influence of body weight and age on the eculizumab levels in individual patients. Moreover, levels of the sC5b-9 complex may play a role in eculizumab clearance after the first eculizumab infusion and may contribute to variability. To facilitate individualized treatment options, inter-patient variability should be investigated further in the future. Our findings suggest that C5 concentrations vary considerably among the patients, more than among the controls. Our previous data using C5 deficient serum indicated that even a 1-2% of unblocked C5 in serum may already lead to measurable complement activity.[158] Thus, when fine-tuning the eculizumab therapy based on patient's needs, C5 concentration may be important to take into account.

In our previous work, we measured eculizumab-C5 complexes in patients, which in the situation of substantial eculizumab excess, as was observed in that study, provided a good indication of C5 levels. The data indicated some fluctuation of eculizumab-C5 complexes during the course of therapy.[158] Relevance of these findings for personalized treatment should be investigated further.

In this study only dosing intervals have been varied. However, in the future also options for the adjustment of the administered eculizumab dosage should be investigated. To this end, proportionality across the recommended dosing range of 50-100 µg/ml should be addressed at various diseases stages. Current knowledge on pharmacokinetics and pharmacodynamics indicates that eculizumab is bound to C5 and sC5b-9 *in vitro*.[168] *In vivo* deposition on other ligands, including surface of blood cells, endothelium, etc. is also

possible. It may interfere with drug's complement inhibiting activity and thus should be taken into account when lowering the treatment dose.

In this study three samples with 93-250 μg/ml of eculizumab and residual 11-12% activity in CH50 assay were observed (**Figure 6.4**). These findings are in line with previous work, where the authors also observed several samples with eculizumab >99 μg/ml (which they use as a target reference) and CH50 >10 %.[172] Interestingly, recent data point out that eculizumab inhibition of complement may not always be complete and even high drug levels would not always completely block complement in an *in vitro* assay.[175] Clinical importance of such residual complement activity in aHUS should be further investigated.

Thus, in this study we performed a thorough analysis of data of patients who were treated with eculizumab and where standard and extended intervals between infusions were applied. Our data indicate that eculizumab accumulates in the body during 1-week intervals (depending on the body weight and possibly initial sC5b-9 concentration) remains stable at 2-week intervals, and diminishes during 3- and 4- week intervals. Importantly, extended 3- and 4- week intervals may be optimal for complete complement inhibition in a considerable subset of pediatric and adult patients, as shown in this study. Our data indicate that even at 5-week intervals at least some of the patients were able to sustain optimal eculizumab concentration and complement inhibition, at least for a short time. As our study included a relatively limited number of patients, further optimization of treatment intervals in aHUS should be evaluated in a larger patient cohort.

The results of this study have laid the base to formulate the Dutch guideline for the restrictive treatment regimen of aHUS patients that is currently being introduced in the Netherlands. In the future, it will help to design correct individualized dosing schemes for patients in acute aHUS that require continuous complement blockade, as well as for patients in remission for whom therapy is being discontinued by using gradual extension of intervals. That will help to save costs and make this efficient medication more accessible to the patient.

Acknowledgement

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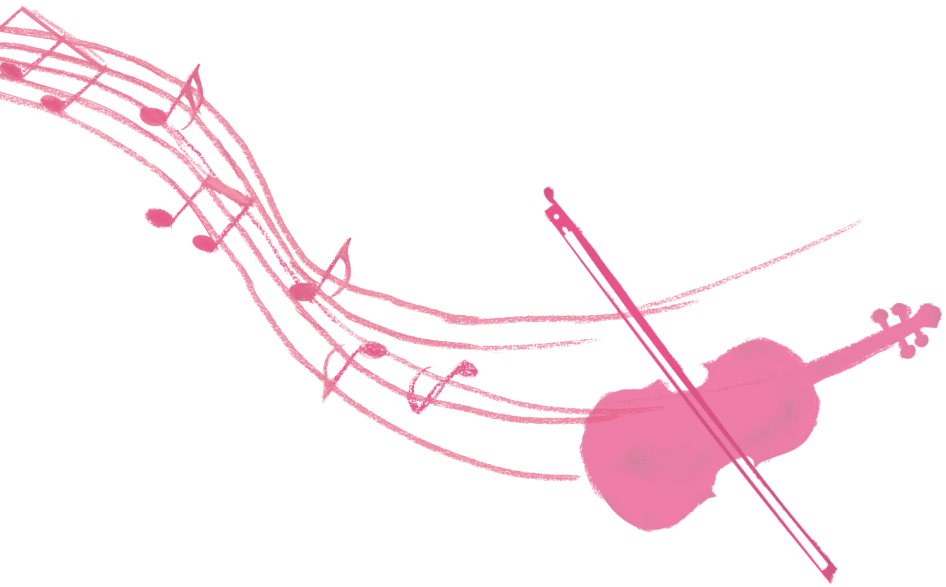
“Twee zusjes zitten voor het raam. Eén leest. De ander luistert.
Haar handje schuifelt door het haar.
Er is nog even samenhang maar dan ontgaat haar het verhaal.
De duim blijft steken tussen mond en kin.
De oudste leidt met zachte dwang haar zusje weer het sprookje in.”

Neeltje Maria Min



Chapter 7

Pharmacology, pharmacokinetics and pharmacodynamics of eculizumab

and possibilities for an individualized approach to eculizumab

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Submitted

Abstract

Eculizumab is the first drug approved for the treatment of complement mediated diseases. Current dosage schedules result in large inter-individual drug concentrations. This review will give insight into the pharmacokinetic and dynamic properties of eculizumab, both for reported on- (paroxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome, generalized myasthenia gravis) and off-label indications (hematopoietic stem cell transplantation associated thrombotic microangiopathy). Furthermore, we will discuss the potential of therapeutic drug monitoring to individualize treatment and reduce costs.

Introduction

With orphan drug status in 2003 and approval in 2007, eculizumab (Soliris) was the first drug targeting the complement system, specifically complement component C5.[176-178] Since the approval of eculizumab, many trials have been conducted and various drugs targeting different proteins of the complement system are in the pipelines.[9] Up till now, marketing authorization for eculizumab has been obtained for the treatment of paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS) and refractory generalized myasthenia gravis (gMG).[8,176,179] Noteworthy is the off-label use of eculizumab in broad spectrum of other diseases (**Table 7.1**).[180]

Like other orphan drugs, costs as high as €500,000 per patient per year are associated with eculizumab therapy.[181] Consequently, eculizumab is considered one of the world's most expensive drugs. Secondly, the burden for the patients is considerable, since eculizumab has to be administrated intravenously, every fortnight, potentially lifelong. Thirdly, there are large inter-individual variations in pharmacokinetics.[167,182] And lastly, treatment is not without risks. The most prominent risk is the susceptibility to infection with *Neisseria meningitidis* (meningococcus).[183] Furthermore, evidence is accumulating regarding the potential (long-term) adverse effects of eculizumab like hepatotoxicity.[99,184]

Taking this into consideration, the infinitesimal data regarding dose adaptations guided by either measuring drug concentration or efficacy markers (complement system) is striking. In this review we give insight into the PK and PD of eculizumab both for reported on- and off-label use. Furthermore, pharmacokinetic targets within different diseases are discussed to optimize dosing. To conclude, the possibility, and above all necessity, of dose individualization will be discussed together with the tools to achieve tailored patient care.

The complement system

The complement system is an important part of innate immunity and consists of three different pathways, all converging at C3, the central complement component (**Figure 7.1**). The classical pathway (CP) and lectin pathway (LP) are, respectively, triggered by antibodies (such as the case in gMG) and mannose containing sugars on pathogens. The alternative pathway (AP) is unique since spontaneous auto-activation is always present and can be further triggered by bacterial components such as lipopolysaccharide and bacterial toxins. When activated, an amplification loop is generated.[228] Activation of each pathway leads to the formation of the C3 convertase (C3bBb) which can cleave C3, leading to chemotaxis and opsonization with respectively C3a and C3b. Generation of large amounts of C3b results in the formation C5 convertase (C3bBbC3b). On its turn, C5 convertase can cleave C5, hereby producing the second anaphylatoxin C5a, and C5b which can bind complement proteins C6, C7, C8 and C9 to form the end product of the complement system, the membrane attack complex or C5b-C9 (C5b-C9), which causes cell lysis (**Figure 7.1**).

Table 7.1 Reported use of eculizumab in light of pharmacokinetic and pharmacodynamic data

Disease	Pharmacokinetic data available[a]	Pharmacodynamic data available[b]	References
Atypical hemolytic uremic syndrome	Yes	Yes	8,158,165-168,182,185-194
Paroxysmal nocturnal hemoglobinuria	Yes	Yes	176,177,195-208
Refractory generalized Myastenia Gravis	Yes	Yes	179,209,210
Shiga toxin producing E. Coli hemolytic uremic syndrome	No	No	211,212
Multifocal motor neuropathy	Yes	Yes	213
Antibody mediated kidney rejection	No	No	214,215
C3 glomerulopathy (including dense deposit disease)	Yes	No	216,217
Age related macular degeneration	Yes	No	218,219
AQP4 IgG positive Neuromyelitis optica	Yes		220
Systemic lupus erythematosus	Yes	Yes	221
HSCT – TMA	Yes	Yes	172,222,223
Guillain Barré	No	No	224
Psoriasis	Yes	No	178
Rheumatoide arthritis	Yes	Yes	178,207,225
Dermatomyositis	Yes	No	226
Idiopathic membranous glomerulopathy	Yes	Yes	178,207
Demyelinating neuropathy with CD59 p.Cys89Tyr mutation	No	No	227

HSCT-TMA; Hematopoietic stem cell transplantation associated thrombotic microangiopathy

[a] Data on serum eculizumab (trough) levels was considered sufficient for pharmacokinetic data

[b] Data regarding serum eculizumab levels in relation to complement blockade

Normally, the complement system is tightly controlled by regulatory proteins present both in fluid phase and on the cell surface. The most important soluble factor is considered complement factor H, followed by complement factor I. In addition to these blood derived regulators, the human body has various regulators bound to the membrane of host cells. Examples of important bound regulators on cells are respectively decay acceleration factor (DAF;CD55) and CD59 on among others erythrocytes, platelets and endothelial cells en membrane cofactor protein (MCP; CD46) on among others platelets and endothelial cells.[228,229]

Figure 7.1 The complement system

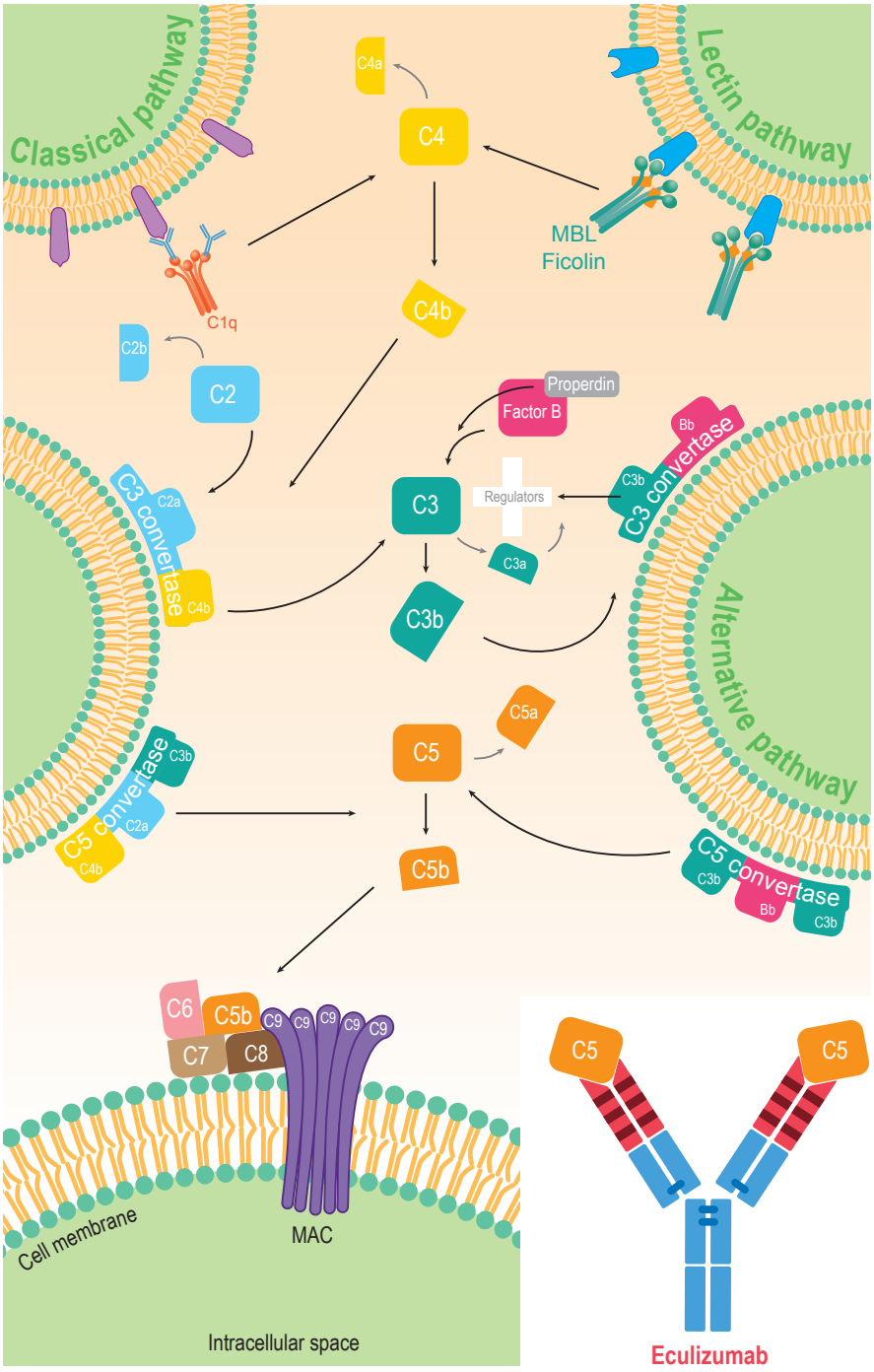

Figure 7.1 The complement system consists of three pathways which all converge at C3. The classical pathway is depicted in the left upper quadrant, the lectin pathway is depicted in right upper quadrant and the alternative pathway is depicted in the middle on the right. Each pathway is activated by a trigger such as antibodies which will be bound to C1q, mannose containing sugar which will be bound to mannose binding lectin (MBL) or ficolin, or spontaneous hydrolysis of C3 to C3a and C3b and hereby the potential to activate or enhance complement activation via alternative pathway. By formation of C3 convertases (C2aC4a or C3bBb) and subsequently C5 convertases (C2aC4bC3b or C3bBbC3b) the lytic pore and end product of the complement system is formed (C5b-C9) causing lysis of the cells. The complement system is tightly controlled by various complement regulators such as factor H and factor I. Eculizumab is a chimeric monoclonal antibody which consists of both human and murine regions (depicted as dark and red bars in Fab arms). Eculizumab is able to bind 1 or 2 C5 molecules, hereby preventing the cleavage of C5 into C5a and C5b, hence blocking formation of C5b-C9. Fb; factor b, MAC; membrane attack complex, MBL; mannose binding lectin

Pathogenesis of complement mediated diseases

Together with our growing knowledge of the complement system and its role in different diseases, evidence has emerged for complement blockage with drugs. Eculizumab was approved by the European medicines agency (EMA) and food and drug administration (FDA) as standard treatment for PNH, aHUS and gMG in respectively 2007, 2011 and 2017.[8,169,176,197] Eculizumab administration is reported in two disease situations: primarily in diseases resulting from complement dysregulation (e.g. PNH and aHUS) and subsequently in diseases with extensive complement activation with inflammation (e.g. gMG).[152,172,211,216-218,222,223,230-234] For the purpose of this review, the most prominent diseases with reported eculizumab therapy will be described, in light of sufficient PK and PD data available.

Paroxysmal nocturnal hemoglobinuria

PNH is a rare form of acquired hemolytic anemia with an estimated incidence of respectively 1-2 cases per million. PNH is caused by a non-malignant clonal expansion of hematopoietic stem cells containing a somatic mutation in a gene located on the X-chromosome called phosphatidylinositol glycan complementation class A.[177] As a result, affected stem cells are deficient of glycosyl phosphatidylinositol anchored proteins. In patients with PNH, clonal expansion results in the production of cells, like erythrocytes, granulocytes and platelets, that lack the expression of glycosyl phosphatidylinositol anchored complement regulatory proteins CD55 and CD59. Due to the absence of complement regulators CD55 and CD59, erythrocytes are susceptible for complement activation on the cell surface, in particular causing hemolysis and thrombosis [229]

PNH usually affects adult patients with an average onset at the age of 30 years, and is associated with increased mortality and morbidity.[199,235] PNH is characterized by chronic hemolysis due to complement activation. As a consequence, free hemoglobin is released which depletes nitric oxide and impairs vasodilatation. Clinical manifestations include abdominal pain, dysphagia, erectile dysfunction, pulmonary hypertension and chronic kidney disease. The most feared complication of PNH is thrombosis. Before the era of eculizumab, thrombosis accounted for up to 65% of the deaths with thrombosis incidence of 29-44%.[235]

Atypical hemolytic uremic syndrome

Atypical HUS is a rare and severe form of thrombotic microangiopathy (TMA) resulting in mechanical hemolytic anemia, thrombocytopenia and acute kidney injury.[1] Atypical HUS results from an uncontrolled overactivation of AP due to pathogenic mutations of or acquired auto-antibodies directed against, complement regulatory proteins. Overactivation of the AP causes excessive generation of respectively C3 and C5 convertases, leading to excessive deposition of C5b-C9 on endothelial cells. This causes endothelial activation and injury, resulting in platelets aggregation, formation of thrombi and scattering of erythrocytes when squeezed through these narrowed microvessels in mainly the glomeruli of the kidney.

Atypical HUS has an estimated incidence of 1-2 cases per million and affects all ages with a slightly higher incidence in childhood when compared to adults.[1,6] Although TMA affects predominantly the renal vasculature, extra-renal involvement of central nervous system (10-15%), heart (3-10%), pancreas, liver, intestines, lungs, and skin is reported.[1,6] Before the implementation of eculizumab, mortality was as high as 25% in the acute phase of the disease and over 50% of the patients reached end state renal disease after their first presentation.

Refractory generalized myasthenia gravis

Generalized MG is a rare and acquired form of neuromuscular auto-immune disorder. It occurs in the presence of antibodies, in up to 80% directed against the acetylcholine receptor at the postsynaptic membrane of the neuromuscular junction. Antibodies composed of IgG1 and IgG3 isotypes are known to activate CP, ultimately leading to C5b-C9 deposition on the postsynaptic membrane and consequently disruption of the acetylcholine receptors. Due to chemotaxis and cell lysis associated with complement activation, the postsynaptic membrane becomes less sensitive to the released acetylcholine, hence nerve impulses will be inhibited.[209,236-238]

Generalized MG has an estimated incidence up to 10 per million. Patients suffer from progressive muscle weakness and fatigue, starting usually with ocular muscle weakness. However, when generalized, it affects bulbar, axial, limb and respiratory muscles as well. Due to respiratory failure patients are frequently admitted to the intensive care unit and in case of delayed intervention, eventually this can lead to death.[209,236]

Hematopoietic stem cell transplantation associated thrombotic microangiopathy

Secondary TMA is frequently seen in patients after hematopoietic stem cell transplantation (HSCT).[172] Mimicking aHUS, the complement system seems to play a crucial role in developing severe HSCT-TMA.[172,222,239] Especially in patients presenting with severe HSCT-TMA, the mortality rate and severity of chronic sequelae are high. Severe TMA affects 20-30% of the HSCT recipients and is characterized by proteinuria and elevated levels of sC5b-C9 in addition to TMA.

Pharmacokinetic properties of eculizumab

Eculizumab (Soliris, Alexion Pharmaceuticals, Inc.) is a humanized chimeric monoclonal antibody consisting of a human framework build of IgG2 and IgG4 composed with variable regions of murine origin (**Figure 7.1**). The eculizumab dose depends on the indication and weight of the patient. Pharmacokinetics of eculizumab in healthy subjects have not been studied.[240] At the end of the previous century, phase I studies were performed in rheumatoid arthritis (RA, C97-001-01) and systemic lupus erythematosus (SLE, C97-002-01) patients. Phase II multiple dose studies were conducted in patients with RA (C01-004), idiopathic membranous glomerulopathy (IMG, C99-004) and PNH (C02-001) Pilot studies have been performed in patients with dermatomyositis (C99-007) and psoriasis (C99-007) as well.[208,214,220,221,225,226,241]

Eculizumab is administrated as an intravenous infusion in 25-45 minutes, with a maximum of two hours in patients above 12 years of age and four hours in children less than 12 years of age (see **Table 7.2** for different dosage regimens per disease).[240] After intravenous administration, eculizumab is primarily distributed in blood plasma. Limited distribution in cerebrospinal fluid has been described, with concentrations 5000-fold lower than in plasma.[220] Distribution to other tissues has not been described in human studies. In vivo animal studies and in vitro studies with normal human tissues showed intracellular distribution to a wide variety of cells, consistent with expected C5 localization.

The specific metabolism of eculizumab has not been described. As described for other monoclonal antibodies, they are catabolized via the mononuclear phagocyte system consisting of monocytes, macrophages, Kupffer cells and histiocytes. By either endocytotic digestion or binding to the Fcγ-receptors on phagocytic cells, monoclonal antibodies will be degradated by lysosomes to peptides and amino acids. Eculizumab contains no known active metabolites.[240,242,243] It has an average half-life of approximately 11.3 days (standard deviation of ±3.4).[178] Due to the molecular size, eculizumab is not excreted in urine, except in patients with heavy proteinuria where eculizumab concentrations as high as 56 μg/ml have been detected. [158,168]

Table 7.2 Dosage scheme in different patient populations

Patient group	Induction	Maintenance
PNH	**600mg**, every week, for 4 weeks	**900mg** in fifth week, every 14 days thereafter
aHUS – gMG	**900mg**, every week, for 4 weeks	**1200mg** in fifth week, every 14 days thereafter
Pediatric population PNH & aHUS <40 kg		
30 to < 40 kg	**600mg,** every week, for 2 weeks	**900mg** in third week, every 14 days thereafter
20 to < 30 kg	**600mg**, every week, for 2 weeks	**600mg** in third week, every 14 days thereafter
10 to < 20 kg	**300mg** once	**300mg** in second week, every 14 days thereafter
5 to < 10 kg	**300mg** once	**300 mg** in second week, every 21 days
HSCT-TMA	**900mg,** second dose when*	**1200 every 2 weeks**, when steady CH50 suppression is achieved and TMA parameters together with sC5b-C9 levels normalize
Pediatric population <40 kg		
30 to < 40 kg	**600mg** second dose when*	**900mg every 2 weeks**, when steady CH50 suppression is achieved and TMA parameters together with sC5b-C9 levels normalize
20 to < 30 kg	**600mg** second dose when*	**600mg every 2 weeks**, when steady CH50 suppression is achieved and TMA parameters together with sC5b-C9 levels normalize
10 to < 20 kg	**600mg** second dose when*	**300mg every 2 weeks**, when steady CH50 suppression is achieved and TMA parameters together with sC5b-C9 levels normalize
5 to < 10 kg	**300mg** second dose when*	**300mg every 2 weeks**, when steady CH50 suppression is achieved and TMA parameters together with sC5b-C9 levels normalize

aHUS; atypical hemolytic uremic syndrome, CH50; classical pathway inhibition, gMG; generalized myasthenia gravis, HSCT; Hematopoietic stem cell transplantation, PNH; paroxysmal nocturnal hemoglobinuria, sC5b-C9; soluble C5b-C9, TMA; thrombotic microangiopathy

** Within 72h when sC5b-C9 is > 244ng/mL, when CH50 was no longer suppressed or after seven days. In case of remaining complement activity (CH50>10% and elevated sC5b-C9, dose should be increased by 300mg/dose to maximum of 1200mg/dose.*

Eculizumab pharmacokinetics.

The eculizumab concentration in serum depends on various factors. The adequate dosage is based upon weight of the patient and the underlying disease.

To identify the optimal dose of eculizumab necessary to block complement, six single dose studies were performed in patients with RA (C97-001-01) and SLE (C97-002-01).[178] Patients were infused with a single dose, ranging from 0.1 mg/kg up till 8mg/kg of eculizumab in 30 minutes (**Table 7.3** and **Table 7.4**). As reported in the scientific discussion of the EMA, these single dose studies in RA patients yielded a mean clearance (CL) of 0.26 ml/kg/hr with a central volume of distribution (Vd1) of 15 ml/kg and peripheral volume of distribution (Vd2) of 20 ml/kg.[208] The estimated Vd at steady state was calculated at 35 ml/kg. With this Vd, the estimated half-life was 93 hours (3.9 days). The area under the curve (AUC) was calculated at 24467.6 µg.hr/ml. One highly interesting observation was the presence of a second peak after two days post dose, however no further information regarding this observation was published. Two-fold increase of the eculizumab dosage from 4 to 8mg/kg in respectively RA and SLE yielded an increase of 60% and 15% in mean maximum concentration (C_{max}) (**Table 7.3**). The AUC increased by 70% and 103% in RA and SLE respectively (**Table 7.3**). Both distribution and half-life are dose dependent (**Table 7.3**).[178,208]

Multiple-dose studies were conducted in three patient groups with RA (C01-004), IMG (C99-004) and PNH (C02-001). With the use of a two-compartment model, PK parameters were calculated (see **Table 7.4**). At therapeutic doses, eculizumab shows linear pharmacokinetics, indicating saturation of the drug target.[208]

The population pharmacokinetics of eculizumab in PNH patients have previously been described using a one-compartment model (**Table 7.4**). Clearance was estimated to be 0.3 ml/kg/hr with Vd of 110.3 ml/kg which was slightly larger than estimated serum volume of patients.[178] Elimination rate (K) of 0.0028 1/h which resulted in a half life of approximately 271.7 hours (11.3 days). In PNH, eculizumab trough concentration (C_{trough}) measured at week 26 were 97 ± 60 µg/ml. C_{trough} levels remained stable during maintenance phase (see **Table 7.2** for dosage regimen).[178,194] During follow-up of patients in both pivotal trials (extension study, E05-001), C_{trough} concentrations below 35 µg/ml were repeatedly observed in 10% of the patients. This was correlated with rapid eculizumab CL above 0.4 ml/kg/hr or a shorter half-life < 130 hours. In half of the patients the breakthrough hemolysis was successfully treated with decreasing the eculizumab dosing interval. Overall, 10% of the patients needed frequent changes in dosing interval to sustain remission.[203]

In the group of aHUS patients, pharmacokinetics were best described with a one-compartment model. In total, 57 patients from C08-002A/B, C08-003 A/B and C09-001r were included in the analysis (see **Table 7.3** and **Table 7.4**). Clearance was estimated at 0.2 ml/kg/hr and Vd was 87.7 ml/kg. C_{trough} concentrations measured at week 26 in aHUS were 242 ± 101 µg/ml with an estimated half life of 291h (12.1 days).[194] Pharmacokinetic data of both prospective trials (C08-002 A/B and C08-003 A/B) showed C_{trough} levels of respec-

tively 93 and 113 µg/ml in adults and 104 and 109 µg/ml in adolescent patients. The AUC during maintenance phase in both trials were 77,693 µg/hr/ml and 104,228 µg/hr/ml in adults and 104,228 µg/hr/ml and 99,956 µg/h/ml in adolescents respectively. Observed C_{max} were up to 431 µg/ml.[194]

Pharmacokinetic properties of eculizumab in gMG were best described using a two-compartment model (see **Table 7.4**).[179,237,244] Patients from two studied were included, with resp. 14 participants of double-blind placebo-controlled randomized cross-over trial (C08-001) and 126 participants of a double-blind placebo-controlled randomized trial (ECU-MG-301). Clearance was estimated at 0.09 ml/kg/hr with Vd1 of 27.6 ml/kg and Vd2 of 30 ml/kg (median weight of 80kg in eculizumab arm). Eculizumab C_{trough} concentrations were approximately twofold different at steady state comparing both studies. Following a maintenance dose of 1200mg, C_{max} and C_{trough} levels reported at week 26 were 738 ± 288 µg/ml and 341 ± 172 µg/ml. Interindividual variability was high, CL up to 42% and Vd up till 24%.[179,244]

Finally, a study assessed PK properties in patients with HSCT-TMA. Eculizumab clearance ranged from 0.23 to 3.39 mL/kg/hr during induction phase. Important to note is the high number of erythrocyte and platelets transfusions due to severe and persistent gastro-intestinal bleeding explaining the high clearance. At the fifth week CL was decreased to mean CL (±SD) of 0.35 ml/kg/hr (± 17.7). Pharmacokinetic modeling with one compartment model of eculizumab therapy in HSCT recipients showed high variability of eculizumab clearance of 1.4 ml/kg/hr (relative standard error of 9%), especially within the first weeks of treatment.[172]

Factors influencing eculizumab concentrations

Several covariates have been identified to impact pharmacokinetics of eculizumab. Next to age and weight, these are C5 concentrations, C5b-C9 concentration, human anti-human antibodies (HAHA), plasma exchange therapy, and pregnancy. The impact of these covariates will be discussed in the next paragraphs.

Effect of age and weight

The most important covariate between different populations is the difference in weight. Since weight influences the clearance and volume of distribution of eculizumab the dosage is weight based.[167] Eculizumab pharmacokinetics in children have been investigated in two open label studies in both PNH (n=7) and aHUS (n=22) patients.[166,204] In the open label phase I/II study (M07-005) conducted in seven PNH patients ranging from 11-17 years, patients received eculizumab according the standard protocol (600mg weekly for 4 weeks, followed by 900mg at week 5 and every 14 days thereafter). Eculizumab concentrations reached a plateau after 4 weeks. After 12 weeks median (range) C_{trough} concentrations were 192.5 (124.2-321.1) µg/ml with median C_{max} of 425.4 (220.5-556.1) µg/ml.[204] No data regarding CL and Vd were described.

Based on population PK analysis of the three pivotal trials of aHUS (C08-002, C08-003 and C09-001r), (±SD) per weight category (>40kg, 30-40kg, 20-30kg, 10-20kg, 5-10kg) was estimated at respectively 15.1 (± 6.5), 7.6, 6 (± 1.46), 5.49 (± 0.24) and 3.6 (± 1.3) ml/kg/hr, resulting from an allometric relationship between weight and clearance.[194] Furthermore, in the prospective open-label non-randomized single arm C10-003, 22 pediatric aHUS patients above 1 month and 4.9 kg, were included.[166] Median weight was 20 kg (range 7 – 95 kg). Patients received eculizumab in different dosage with interval of 14-21 days based on weight as described in summary of products.[193] Clearance and Vd were 10.4 ml/hr and 5230 ml. Overall, patients had a C_{max} of 515.4 µg/ml (range 264.7-1094.4) and C_{trough} of 256.7 µg/ml (50.2-531.1), which is higher than in the adult population. The elimination half-life was 290 hr (12 days). The parameters AUC, C_{max} and C_{trough} were different between different age groups. Overall, patients had a median AUC (range) of 141,741.4 µg.ml/hr (43,652.9 – 261,814.4) at steady state. Power function of body weight was estimated at 0.715 (95% CI: 0.59-0.84). Eculizumab appeared safe and effective in pediatric patients.[166]

Soluble C5b-9 levels

Recently, three articles were published which suggested that soluble C5b-9 (sC5b-C9) levels may impact eculizumab exposure.[168,172,182] Since eculizumab is not only capable of binding C5 but also sC5b-C9 with lower affinity, it physiologically plausible that increased levels of sC5b-C9 at initiation of therapy correlate with lower eculizumab concentrations. Jodele et al. even showed that pre-treatment measured sC5b-C9 levels correlated with eculizumab clearance. [172]

Intravenous administration of immunoglobulins

Monoclonal antibodies can be cleared via non-specific endocytosis in various phagocytes, however are protected intracellular from degradation by the neonatal Fc receptor. This receptor is mainly present in endothelial cells and proximal renal epithelial cells. In case of saturation of this neonatal Fc receptor, due to, for example, a high load of immunoglobulins, increased elimination of monoclonal antibodies can occur.[242] A study performed in patients with multifocal motor neuropathy, who were treated with eculizumab according to PNH protocol, and concomitant intravenous immunoglobulins (IVIg), showed significantly lower eculizumab C_{trough}. In patients who received IVIg therapy, eculizumab concentrations were measured with a median (interquartile range; IQR) of 79 (55-108) µg/ml versus 120 (96-147) µg/ml in patients who did not receive IVIg.[213] No statistical significance was observed in hemolytic complement activity, although more patients with IVIg had detectable total complement activity above 20%.[213]

Human anti-human antibodies

As with any humanized antibody therapy the risk of developing (neutralizing) human anti-human antibodies (HAHA) is present. Analysis of a cohort of 75 PNH patients with median of 7.5 years of eculizumab therapy revealed no HAHAs.[245] In the TRIUMPH trial one patient developed HAHAs, however it seemed not to effect eculizumab therapy.[176] One aHUS patient, 30 months old, was reported with HAHAs. The patient had inadequate C_{trough} (17.1 µg/ml) ongoing hemolytic activity, together with nonresponsive TMA after the start of treatment.[166]

Plasma infusion and exchange

As expected, plasma exchange and plasma infusion have a clear influence on clearance of eculizumab and subsequently lead to a marked reduction of the half-life (see **Table 7.3**). Clearance was measured in aHUS patients with plasma exchange and increases from 14.6 ml/hr to 3660 ml/hr with an estimated half-life of 1.26 hours. Therefore, it is recommended to supplement an extra dosage (600mg, unless patient received only 300mg in maintenance phase, then only 300mg suppletion) of eculizumab within 60 minutes after plasma exchange. In case of infusion of fresh frozen plasma, it is recommended to give 300mg in addition to normal dosage, 60 minutes prior to infusion. [240,244]

Effect of pregnancy

No formal pharmacokinetic studies have been performed for pregnant women receiving eculizumab. Different articles and case reports describe the use of eculizumab in pregnant women with either PNH or aHUS.[200,246-251] Kelly et al. reported therapeutic concentrations of eculizumab measured in two out of three PNH patients, without detectable eculizumab in cord blood and breast milk.[200] Servais et al. described five pregnancies in respectively three aHUS patients receiving eculizumab. Similar as reported by Kelly et al. no eculizumab was detected in cord and neonatal blood. However, eculizumab dosage had to be increased in two patients, as high as 1800mg every fortnight, to maintain complement blockage. Both patients showed signs of partial blockage already in the first trimester [250] In contrast to the previous discussed studies, in a cohort of 61 pregnant PNH patients, in 35% of the cord blood samples eculizumab concentrations were detectable, ranging from 11.8 to 21.2 µg/mL. This in contrast to the breast milk samples where eculizumab was not detectable.[251] Moreover, pregnancy was associated with lower and even inadequate C_{trough} levels of eculizumab. Of the 61 patients evaluated, half of the patients needed increased dosage of eculizumab.[251]

Table 7.3 Non-compartmental analysis

Population	Number of participants	Eculizumab dosage	Clearance (ml/hr) ± SD	Central volume of distribution (ml) ± SD
RA (C97-001)	6	4 mg/kg	16.2 ± 7.2	7500 ± 4700
RA (C97-001)	6	8 mg/kg	20.3 ± 7.2	5000 ± 3600
SLE (C97-002)	3	4 mg/kg	19.3 ± 5.2	4200 ± 1500
SLE (C97-002)	3	8 mg/kg	19.1 ± 8.1	3900 ± 1800
aHUS patients with PT (C08-003, C09-001r)	48	See table 7.2	3660	unknown
Pediatric aHUS patients (C10-003)	22	See table 7.2	10.4	5230

aHUS; atypical hemolytic uremic syndrome, AUC; area uder the curve, Cmax; maximum concentration, Ctrough; trough concentration NA; not applicable, PNH; paroxysmal nocturnal hemoglobinuria,

Table 7.4 Population pharmacokinetic analyses

Population	Nr. of participants	Clearance (ml/hr/kg ± SD)	Central volume of distribution (ml/kg ± SD)
RA (C97-001)	10	0.262	15.04
RA (C97-001)	121	0.3 ± 0.12	12.7 ± 8.54
SLE (C97-002)	6	0.3 ± 0.11	18.7 ± 4.8
RA (C01-004)	111	0.230	12.5
IMG (C99-004)	71	0.41 ± 0.14	64.9 ± 31.5
PNH (C02-001)	11	0.25 ± 0.069	32.5 ± 16.5
PNH (C04-001)	40	0.311 ± 0.13	110.3 ± 17.9
gMG (ECU-MG-301, C08-001)	75	7.37 ml/kg [6.62-8.2 95%CI]	2210 ml [1940-2530 95%IC]
aHUS (C08-002, C08-003, C09-001r)	57	0.208	87.7
HSCT-TMA	18	1.4 (RSE of 9%)	81.7 (RSE of 21%)

aHUS; atypical hemolytic uremic syndrome, gMG; generalized myasthenia gravis, HSCT-TMA; Hematopoietic stem cell transplantation associated thrombotic microangiopathy, IMG; idiopathic membranous glomerulopathy,

Peripheral volume of distribution (ml) ± SD	AUC∞ (µg.hr/ml) ± SD	Cmax (µg/ml) ± SD	Ctrough (µg/ml) ± SD	Half-life (hr) ± SD	Reference
NA	22,200± 13,700	111 ± 53	unknown	281 ± 298	[178]
NA	37,800 ± 5900	182 ± 19	unknown	197 ± 198	[178]
NA	15,600 ± 5000	139 ± 25	unknown	162 ± 88	[178]
NA	31,600 ± 9100	160 ± 10	unknown	141 ± 6	[178]
unknown	unknown	unknown	unknown	1.26	[208]
NA	Median 141,741.4 (range 43,652.9 – 261,814.4)	515.4 (range 264.7-1094.4)	256.7 (50.2-531.1)	290	[166]

PT; plasmatherapy, RA; rheumatoid arthritis, SD; standard deviation, SLE; systemic lupus erythematosus

Peripheral volume of distribution (ml/kg ± SD)	Intercompartment clearance (ml/hr/kg)	Elimination constant (1/hr)	Half-life (hr) ± SD	Effect weight on Vd	Reference
20	unknown	unknown	92.9	NA	[208]
42.3 ± 9.7	0.54 ± 0.16	unknown	unknown	NA	[207]
20.6 ± 10.84	0.59 ± 0.56	unknown	unknown	NA	[207]
44.4	unknown	unknown	131.33	NA	[208]
149.5 ± 44.92	0.21 ± 0.17	unknown	unknown	NA	[207]
26.5 ± 7.37	0.21 ± 0.06	unknown	unknown	NA	[207]
NA	NA	0.0028 ± 0.0008	271.7 ± 81.6	NA	[207]
2400 ml [2040 – 2820 95% IC]	1820 ml/hr [73.3- 45.4 95% CI]	unknown	unknown	0.634 [0.494 - 0.774 95% CI]	[179]
NA	NA	unknown	291	NA	[193]
NA	NA	unknown	unknown	NA	[172]

NA; not applicable, PNH; paroxysmal nocturnal hemoglobinuria, RA; rheumatoid arthritis, RSE; relative standard error, SD; standard deviation, SLE; systemic lupus erythematosus

Pharmacodynamic properties of eculizumab

Formal dose and concentration response studies were not performed in any of the patient groups reported below. Data of various clinical trials were used to assess efficacy and exposure-response of eculizumab. Only the data published regarding PD in relation to PK will be described here.

Monitoring of eculizumab therapy

In all trials conducted by the pharmaceutical company, hemolytic activity was used to determine PD properties of eculizumab.[8,166] Hemolytic activity reflects total complement activity (reported as CH50) by testing the capacity of patient serum to lyse sheep or chicken erythrocytes coated with antibodies. In case of a functional complement system, the CP will be activated, subsequently leading to C5b-C9 deposition on the erythrocytes thus causing hemolysis. CH50 levels correlated with eculizumab serum trough levels and a completely blocked complement (no hemolytic activity measured) was the aim of treatment.

It is important to realize that besides the classical hemolytic assay based on lysis of erythrocytes, various other assays have been introduced to measure CH50 and AP50. A well-known and characterized assay is the so called "Wieslab" test. In this ELISA CH50 and AP50 can also be detected, however instead of hemolysis the readout is C5b-C9 formation, detected using a C9 neoantigen. However,the Wieslab ELISA is less sensitive[252]

Exposure-effect relationship of eculizumab

The exposure-effect relationship in the phase I and II trials in respectively RA, SLE and IMG patients was mainly evaluated using the serum hemolytic activity to assess complement inhibition.[178] The results of the single dose studies in RA (C97-001) and SLE (C97-002) showed a concentration-dependent inhibition of hemolytic activity with inverse relationship between eculizumab concentration and C5 complement blockade. Hemolytic activity was completely blocked within 15 minutes, in almost all patients who received a 2.0 mg/kg dose, but hemolytic activity reappeared within two days. By increasing the dose to 4 or 8mg/kg complement activity was completely suppressed for respectively 7-14 and 11-21 days. Complete blockade of complement hemolytic activity was measured at eculizumab concentrations as low as 29- 55 μg/ml and 11- 35 μg/ml in respectively RA and SLE patients.[178]

Maximum PD effect was modeled using an Emax model with the above described data from RA patients pooled with data of IMG, and PNH patients with an half maximal effective concentration (EC50) of 43 μg/ml (95% CI: 39.04-47.78 μg/ml). Since eculizumab concentrations are reported in both bound to C5 and unbound proportion of eculizumab, this could indicate an overestimation of the required eculizumab concentration for com-

plement blockade.[207] The EMA scientific report with pooled data from all single and multiple dose studies (performed in various patient populations) suggested that an eculizumab serum concentration of 35 µg/ml is sufficient to completely block complement activation. Hence, the dosing schedule of 600mg in the initiation phase followed by 900mg every fortnight was selected as most optimal dosing regimen to suppress complement inhibition in almost all PNH patients (**Table 7.2**).[178,208]

In aHUS patients, higher eculizumab concentrations of 50-100 µg/ml for complete complement blockade are recommended. This higher target exposure was based on meta-analysis of data of 177 PNH patients. In this cohort, some patients (up to 10%) had remaining complement activity.[197,203] It was expected that this residual activity could result in clinical manifestations in the aHUS patient group. The main expected clinical manifestations reported was the potential rapid loss of kidney function in case of insufficient blockage with ongoing active TMA.[193] In the FDA approval package an analysis is shown which described the necessity of minimal eculizumab concentration of 50 µg/ml to achieve more than 90% decrease in free C5.[194]

Paroxysmal nocturnal hemoglobinuria

Different trials, C02-001 (n=11), TRIUMPH (n=43) and SHEPHERD (n=97), and the extension trial comprising all previously reported patients (E05-001) reported efficacy of eculizumab in PNH.[176,177,198] To assess pharmacodynamic properties, serum hemolytic activity was evaluated. Unfortunately, both the TRIUMPH as SHERPERD trial mention the use of the serum hemolytic assay, however no values were reported.[176,197] Only in the extension trial, eculizumab concentrations in relation to hemolytic activity were described. In total 43 patients of the TRIUMPH trial and 97 patients of the SHEPHERD trial were included. In total, 36% of the patients had eculizumab concentrations below 35 µg/ml after the first infusion of eculizumab.[203] Of these 36%, 74% exhibited hemolytic activity (>20%). During follow up, C_{trough} concentrations below 35 µg/ml were repeatedly observed in 14 (10%) patients. In half of them complement activity was blocked with decreasing the interval between the administrations. In total, 21 patients of the whole population experienced a median of 22 decreases in dosing interval.[203]

Reiss et al. described the results of a phase I/II study in seven pediatric PNH patients, all above 11 years of age. Eculizumab concentrations above 124 µg/ml correlated with complete complement blockade. The C_{max} and C_{trough} concentration together with AUC of eculizumab was associated with change in LDH.[204]

Latour et al. assessed efficacy of eculizumab in 22 PNH patients.[253] The association between CH50 and LDH was assessed with linear mixed model with intercept and slope for therapy duration. The association between CH50 and eculizumab was analyzed using generalized linear models with logistic link function. They reported suboptimal eculizumab concentrations (< 35µg/ml) in 5% of the samples. C_{trough} varied from 18-643 µg/ml

and half-life was highly variable ranging from 4-21 days. Furthermore, CH50 measured in these patients (median follow up of 13 months with a minimum of 6 months treatment) showed a CH50>10% in 49% of the patients prior to the next eculizumab administration. Complete blockade of complement (CH50 <10%) was associated with lower LDH levels (longitudinal Tobit regression model). An increase of CH50 by 1.4% per 100 U/l (95% CI 0.03-2.7) increase of LDH was observed. All patients with eculizumab serum levels above 150 µg/ml never experienced breakthrough hemolysis. Inter-patient variation was broad with $T_{1/2}$ ranging from 4-21 days.[253]

Atypical hemolytic uremic syndrome

To analyze efficacy of eculizumab in aHUS, free C5 is used as read out. In both prospective studies (C08-003 and C08-003) a median reduction of 50 and 62% respectively was observed in C5 activity and eculizumab C_{trough} levels above 50 correlated with decrease of free C5 by >90%.[194] CH50 levels were reduced or undetectable after seven days of therapy.[194] After 24 weeks, they report complete inhibition of complement activity in all participants. However, no specific values are reported regarding complement activation and eculizumab concentrations.[8,185] The prospective study in pediatric aHUS patients (C10-003) did not report complement inhibition data, only that all patients showed signs indicative of complement inhibition after 24 hours.[166]

Gatault et al. described a concentration-effect relation in seven aHUS and two PNH patients treated with eculizumab. With regression technique (A=20.6 x exp (-0.083 x C) the relation between hemolytic activity and pharmacokinetics was described. No lysis was detected when C_{trough} levels were above 20 µg/ml, using a CH50 SPAPLUS kit which measures liposome lysis. However, they state this assay is less sensitive, hence not recommended. They also used and ELISA kit to measure C9, to detect low to moderate depression of complement activity which corresponds with CH50. Complement activity was detected with this assay when C_{trough} levels were below 59 µg/ml.[167]

Other populations.

No exposure effect studies have been conducted in HSCT-TMA patients. Like HSCT-TMA patients, pharmacodynamic data are sporadically reported for gMG. EMA assessment report describes the population pharmacokinetic/dynamic analysis with free C5 as target engagement and hemolytic activity for proof of pharmacology.[179]

Covariates for eculizumab pharmacodynamics

In some patient with PNH or aHUS population a suboptimal (defined as persistent disease activity) response to eculizumab is described despite adequate eculizumab concentrations and suppression of CH50. Although CH50 is suppressed, sC5b-C9 levels can remain elevated, indicating ongoing complement activation. One possible mechanism for this is residual C5 activity despite adequate eculizumab levels, especially during strong complement activation. Careful characterization and monitoring of these patients is necessary to understand this mechanism.[175]

Nishimura et al. described 11 PNH patients (3.2% of the PNH population receiving eculizumab) with a poor response to eculizumab.[254] Despite adequate C_{max} and C_{trough} concentrations patients had ongoing hemolysis. All had a single missense C5 heterozygous sequence variant c.2654GàA which corresponds to the polymorphism p.Arg885His. This genetic variant prohibits the binding of eculizumab to C5, and subsequently C5 capacity to form the lytic C5b-C9 remains present. Further screening revealed the same prevalence of this variant among healthy controls in Japan.[254] Separately, an Asian PNH patient with poor response to eculizumab had a c.2653CàT mutation in C5 which predicts p.Arg885Cys. Both mutation were not found in a cohort of 220 Chinese PNH patients and 259 healthy controls.[255] Schatz-Jakobsen et al. investigated the structural changes in C5 as a consequence of these mutations. Due to the replacement of arginine by histidine or cysteine, the arginine binding pocket is too small, hence eculizumab cannot bind to C5.[256]

Incremental value of therapeutic drug monitoring of eculizumab therapy

We have reviewed the basic pharmacology of eculizumab in all diseases for which this drug is currently licensed. Next we described the pharmacokinetic and pharmacokinetic-dynamic relations as well as covariates that impact both pharmacokinetics and dynamics. We conclude, that there is high diversity between the different patient groups. As a consequence, both set C_{trough} targets as the pursued effects are different per indication. However, not only between but also within patient groups large variations are noted. We advocate for an individualized approach, to provide the best tailored care.

We hypothesize that by performing Therapeutic Drug Monitoring (TDM) one could adjust the dose and/or dosing intervals and hereby maximize treatment response and reduce treatment costs. As an illustration, we performed a simulation study of the effect of TDM of eculizumab in PNH patients. We simulated a population of 1000 virtual patients based on the population pharmacokinetics and pharmacodynamics of eculizumab in PNH patients.[207] We simulated the pharmacokinetics of eculizumab at steady state (after eight weeks of standard treatment including induction phase) and applied a protocol for TDM based on the measurement of the steady state C_{trough}, with the following algorithm: the absolute dose at each administration (900 mg) remained the same, but the dosing interval was adjusted, based on the measured C_{trough} levels. When C_{trough} levels were below the target of 30 μg/ml, we decreased the dosing interval from two weeks to one week. When the C_{trough} was between 30-90 μg/ml the usual two week interval was maintained. At higher exposure, the dosing intervals were extended: a C_{trough} of 90-120 resulted in a three week dosing interval, a C_{trough} between 120-210 μg/ml resulted in a four week interval and a C_{trough} above 210 μg/ml resulted in interval of five weeks. The results of the simulation are presented in **Figure 7.2**, where the predicted C_{trough} and associated inhibition of C5 activity are shown before and after the TDM intervention. Without TDM, large inter-individual variation in predicted exposure can be observed, with median (range) C_{trough} level of 76 (4 to 362) μg/ml when using standard dosage regimen. By using TDM, median C_{trough} levels decrease to 58 (30-131) μg/ml (**Figure 7.2A**). Furthermore, simultaneously with decreasing the range of C_{trough} levels, a decrease in the variation of C5 inhibition could be observed with more patients reaching target attainment (**Figure 7.2B**). Moreover, with our TDM regimen an overall cost reduction of 11% in 1000 simulated patients was achieved by diminishing eculizumab administrations. We calculated the costs of eculizumab administrations alone per patient for one year of treatment and compared this with standard treatment (patients who are treated one year according to treatment scheme described in **Table 7.2**). Every patient received eight weeks of standard therapy after which TDM was applied with adjustment of the interval between eculizumab administrations. Obviously, costs largely depend on the cumulative

dose per patient, ranging from €211,618 per patient per year with five weekly interval to €726,524 per patient per year with one weekly interval.

In conclusion, we would like to stress the potential of TDM for the use of eculizumab in various diseases. To fully determine efficacy of therapy, we advise to monitor both eculizumab serum levels as well as complement blockade by CH50. One could argue that eculizumab levels alone would be sufficient since there seems to be a clear correlation between C_{trough} and complement blockade. In our opinion, CH50 is especially important in the case of clinical deterioration despite adequate exposure.

Figure 7.2 Effect of therapeutic drug monitoring in PNH

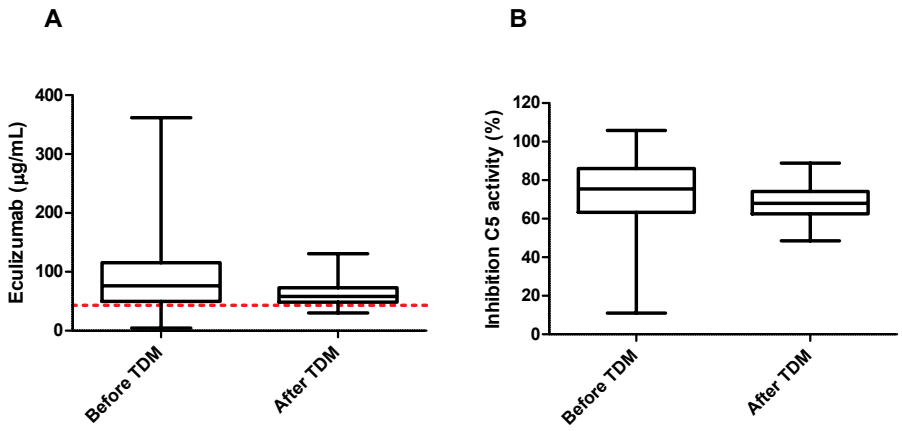

Figure 7.2. A. Large variations are observed in C_{trough} levels when simulated in 1000 PNH patients according compartiment model as described by pharmaceutical company. A substantial amount of patients does not reach the target of 35 µg/ml (depicted as red dottet bar) and in contrast some patients even reach C_{trough} levels up till 362 µg/ml. B. By applying therapeutic drug monitoring the distribution can be largely diminished, with almost all patients reaching target attainment and adequate inhibition of C5 activity as measured by a serum complement hemolytic activity.

Discussion

Eculizumab is known as one of the most expensive orphan drugs worldwide. Despite multiple studies in various patient populations, little is known regarding the potential for TDM in patients receiving eculizumab. In this review we extensively discussed PK and PD parameters in various patient populations (PNH, aHUS, gMG and HSCT-TMA) treated with eculizumab. Furthermore, strategies to target optimal C_{trough} levels via TDM with preserving adequate complement blockade are discussed.

Although CH50 is used as PD outcome in most of the articles, one should realize the high diversity in the assays used. Taken together with the uncertain concentration of both bound and unbound proportion of eculizumab measured in the assay, we would advocate to set up a close collaboration with national reference laboratory in each country. Furthermore, the potential influence of high soluble C5b-C9 levels on eculizumab levels should be further evaluated.

Eculizumab was first approved for PNH patients, with a set C_{trough} target level of 35 μg/ml based on multiple-dose studies performed in PNH patients. However, long-term studies revealed breakthrough hemolysis during maintenance therapy and half of all patients had insufficient blockage of complement prior to the next infusion.[253] Different etiologies could be the cause of this ongoing hemolysis. The most prominent ones are considered C3 opsonisation of erythrocytes, making them more vulnerable to hemolysis, and insufficient C5 blockage by either inadequate C_{trough} levels or in case of strong complement activation.[175,257] Multiple studies have described ex vivo detection of hemolytic activity, indicating ongoing complement activation, despite eculizumab treatment with adequate C_{trough} levels. Besides discordant results between different assays used in the clinics, remaining hemolytic activity could persist in light of strong complement activation.[175]

On the other hand, the determined C_{trough} target level of 50-100 μg/ml in aHUS patients is based rather on assumptions than on PK modeling. Taking into account the breakthrough hemolysis in PNH patients with a C_{trough} of 35 μg/ml, and the risks of ongoing TMA in aHUS patients, the C_{trough} target was set at 50-100 μg/ml. Especially in patients with aHUS, the duration of eculizumab treatment is a highly debated topic. Important to note is the trend towards withdrawal of eculizumab in patients with aHUS and only reinitiate treatment at recurrence of disease.[5,192,258] Of note, most studies focused on complete complement blockade to prevent ongoing disease activity. However, it is unknown if complete blockage is necessary to prevent disease progression in acute and/or remission phase. Ardissino et al. recently described eculizumab treatment in aHUS patients with impaired instead of fully blocked complement activity.[259] With CH50<30% instead of completely suppressed no recurrences were observed.

Only recently, eculizumab was approved for the treatment of gMG. Striking is the lack of data correlating complement inhibition to clinical deterioration in patients with gMG. Furthermore, no relation between eculizumab concentration and primary efficacy endpoint was seen. Hence, monitoring complement inhibition while on eculizumab therapy needs to be evaluated. Future (extension) studies should provide more data regarding eculizumab treatment in gMG. Since IVIgs are used as therapy in gMG one should realize that eculizumab C_{trough} levels could be influenced hereby. During trials patients had a washout period before receiving eculizumab.

In conclusion, to minimize unnecessary use of the highly expensive orphan drug eculizumab, and to assess the optimal treatment scheme, TDM may be performed using eculizumab C_{trough} levels. Furthermore, disease activity should be monitored to look for possibilities to taper treatment. Treatment algorithms should be developed to explore the possibility of individual dosing regimens based on set C_{trough} levels together with CH50.

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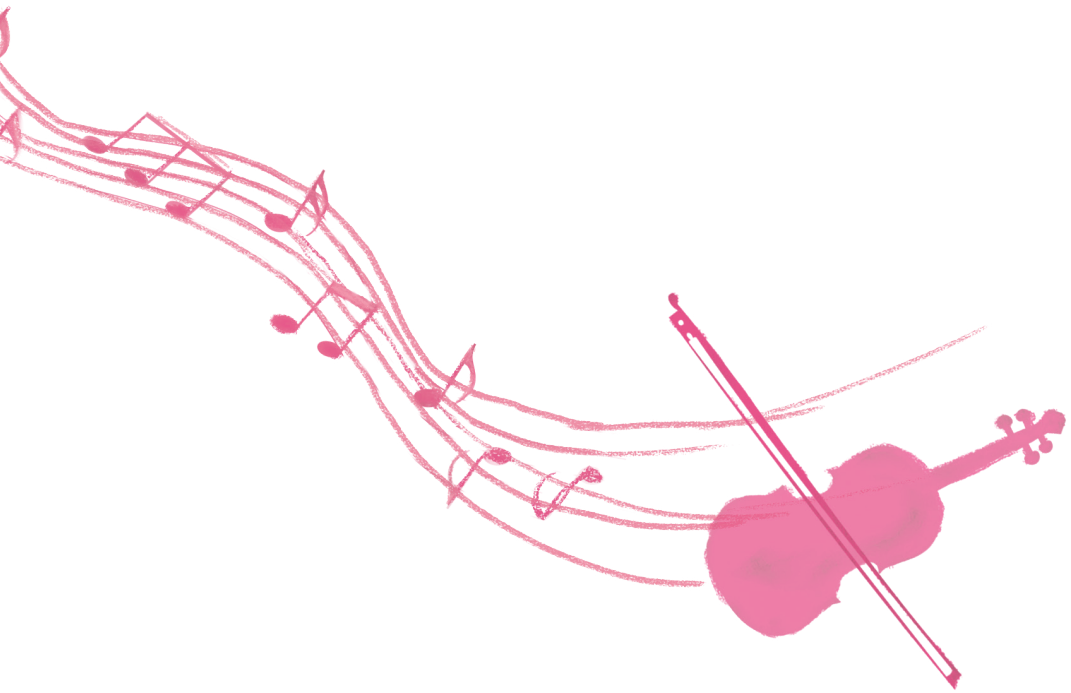
"Cooking is about emotion, it is about culture, it is about love, it is about memory"

Massimo Bottura – Chef of Osteria Francescana, Modena, Italy



Chapter 8

Safety and effectiveness of restrictive eculizumab treatment in atypical hemolytic uremic syndrome

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Abstract

Background

Atypical hemolytic uremic syndrome (aHUS) is a rare, but severe form of thrombotic microangiopathy, as a consequence of complement dysregulation. Atypical HUS has a poor outcome with high mortality and over 50% of patients developing end stage renal disease. Since the end of 2012, these outcomes have greatly improved with the introduction of eculizumab. Currently, the duration of treatment is debated. Most guidelines advise lifelong treatment. However, there is no hard evidence to support this advice. Historically, a substantial number of aHUS patients were weaned of plasma therapy, often without disease recurrence. Moreover, the long-term consequences of eculizumab treatment are unknown. In this retrospective study we describe 20 patients who received a restrictive treatment regimen.

Methods

All aHUS patients who presented in the Radboudumc, Nijmegen, the Netherlands, between 2012-2016, and who received eculizumab are described. Clinical, diagnostic and follow up data were gathered and reviewed.

Results

Twenty patients (14 adults, 6 children) with aHUS have received eculizumab. Eculizumab was tapered in all and stopped in 17 patients. Atypical HUS recurrence occurred in five patients. Due to close monitoring, recurrence was detected early and eculizumab was restarted. No clinical sequela such as proteinuria or progressive kidney dysfunction were detected subsequently. In total, eculizumab has been discontinued in 13 patients without aHUS recurrence, of which five are event free for over a year now. With this strategy approximately €11.4 million has been saved.

Conclusions

A restrictive eculizumab regimen in aHUS appears safe and effective. Prospective studies should further evaluate the most optimal treatment strategy.

Introduction

Atypical hemolytic uremic syndrome (aHUS), is a severe form of thrombotic microangiopathy (TMA). Atypical HUS is considered the consequence of complement dysregulation caused by mutations in complement genes or acquired auto-antibodies against complement regulatory proteins such as complement factor H (CFH).[260] Atypical HUS can occur at any age and, before the era of eculizumab, outcome was very poor with up to 10% mortality in the acute phase and up to 50% of patients developing end stage renal disease (ESRD).[43,261]

In 2012, the new orphan drug, eculizumab, was approved for the treatment of aHUS. Initiation of eculizumab treatment in the acute phase of aHUS resulted in significant recovery of renal function and hematological remission of TMA occurred in almost all patients.[8,166] According the Summary of Product Characteristics (SPC) of eculizumab published by the European Medicines Agency (EMA), treatment with eculizumab consists of weekly infusions in the initial phase (up to four weeks) followed by eculizumab infusions every 14-21 days depending on the body weight of the patient, potentially their entire life.[240]

Currently, the duration of treatment is debated. There is no strong evidence in favor of a lifelong treatment. Historically, plasmatherapy (PT) was the only available treatment option, but failed in a number of aHUS patients, leaving them dependent on dialysis. In contrast, in some patients who developed complete remission, PT could be tapered and stopped. Thus, only a small proportion of aHUS patients remained on chronic PT.[1,262] It is noteworthy that there are no studies that provide strong evidence in favor of lifelong treatment with eculizumab. This together with the high expenses of eculizumab has stimulated a debate regarding duration of treatment. Eculizumab is not only one of world's most expensive drugs, the burden of lifelong treatment is substantial.[8] Patients have to return to the hospital every fortnight. Also, the long-term consequences of eculizumab treatment are unknown. The most prominent risk is the development of a severe meningococcal infection. Other adverse outcome include: the possibility of developing (neutralizing) human antihuman antibodies (HAHAs) [166,203], or immune-mediated drug reactions. Other side effects are not yet fully understood. For example, only recently a study was published about the potential hepatotoxicity of eculizumab.[99]

Even though the outcome of patients with aHUS has improved greatly with the implementation of eculizumab, the costs of treatment are striking. The costs effectiveness of eculizumab, considering the price per quality adjusted life year, is a matter of international debate. For example, one important requisite for the approved funding of eculizumab by the National Institute for Health and Care Excellence in the United Kingdom is the exploration of dose adjustment or discontinuation of eculizumab treatment.[263]

These findings underline the need for studies that evaluate optimal dosage and duration of eculizumab therapy. A growing number of case reports have emerged that suggest that eculizumab therapy can be reduced or discontinued in patients with aHUS.[158,160-162,165,190,192,264,265]

Eculizumab therapy became available in the Netherlands in November 2012. In view of the limited evidence for lifelong therapy, the high expenses and the yet unknown side-effects of long-term eculizumab therapy, lifelong treatment of aHUS patients was considered unnecessary and undesirable. Eculizumab was tapered or stopped, as early as three months after initiation of treatment, in patients who had reached stable remission. In this retrospective study we describe 20 patients who received eculizumab according our local practice between 2012 and 2016.

Materials and Methods

In this descriptive study, we provide information on all pediatric and adult patients with aHUS who were treated with eculizumab, from November 2012 until October 2016 at the Radboud university medical center (Radboudumc), Nijmegen, the Netherlands. Medical ethical committee approval was waived, according Dutch law, since the study was observational and retrospective. Patients were followed regularly by one of the treating physicians (NvdK is the treating physician for the pediatric patients and JW for adults patients). Patients were coded, and clinical and laboratory data were analyzed anonymously.

TMA was defined as: signs of hemoglobin level below the lower limit of normal for the specific age together with signs indicative of hemolysis, thrombocytopenia < 150 x 10^9/l, and acute renal failure. To exclude the presence of Shiga toxin producing *Escherichia coli* causing HUS (STEC-HUS) at presentation, fecal diagnostics and serological antibody assay for O157 serotype were performed. These test were obligatory in children and performed on indication in adult patients. The ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) activity was determined to exclude thrombotic thrombocytopenic purpura (TTP). Medical history was reviewed for signs of previous (suspected) aHUS episodes. Possible triggers such as drugs or malignant hypertension causing TMA and specific aHUS triggers such as pregnancy or (viral) infections were reviewed. In cases where aHUS was suspected, the presence of auto-antibodies against CFH were determined in serum. Simultaneously, DNA was collected for genetic screening of mutations in complement genes. Initially, patients were screened for mutations in: *complement factor H (CFH), complement factor B (CFB), complement factor I (CFI), complement component C3,* and *membrane cofactor protein (MCP/C46).* In case no explanatory mutation was found, patients were further screened for mutations in: *factor H related proteins 1-5 (CFHR1-5), diacylglycerol kinase-*ε *(DGK*ε*), thrombomodulin (THBD) and plasminogen (PLG).* Multiplex ligation-dependent probe amplification (MLPA) was used to screen for possible rearrangements in *CFH/CFHR* region. Hypertension was defined as the use of antihypertensives, blood pressure repeatedly measured above 140/90 millimeter of mercury (mmHg) in adult patients or blood pressure above the 95th percentile for sex, height and age, in pediatric patients.

Eculizumab treatment

Eculizumab was started in adult patients with a (suspected) diagnosis of aHUS in the native kidneys, who did not respond to PT, or remained PT dependent. In children and in patients with recurrent aHUS after kidney transplantation, eculizumab therapy was started immediately without PT. The dose of eculizumab in the initial and maintenance phase was dependent on the body weight of the patient.[240] All patients received antibiotic prophylaxis and vaccination as appropriate. Clinical remission was defined as normalized

platelet count, normalized hemoglobin level without signs of hemolysis, and improved and stable kidney function.

When clinical remission was reached, eculizumab treatment was evaluated. In adult patients who received eculizumab, and who had: improved and stable kidney function, well controlled blood pressure and no evidence of ongoing TMA after three months, eculizumab was withdrawn. In patients with aHUS after kidney transplantation, or in patients who needed retreatment because of a relapse after eculizumab withdrawal: treatment interval was first gradually extended when clinical remission was reached. In pediatric patients the interval was extended, and patients were observed with eculizumab at extended intervals with suppressed titers of total hemolytic complement activity (CH50). However, in case of stable disease (no signs of TMA), eculizumab was gradually further extended and finally stopped. When the treatment interval was eight weeks and no signs of TMA were present, eculizumab was stopped.

In case of therapy discontinuation, patients were closely monitored for any signs of disease recurrence. Patients were seen frequently within the first year of discontinuation for blood and urine workup, initially in the first four months with an interval of two to four weeks, and every two months thereafter. Blood was drawn to look for signs of hemolytic anemia (values determined: hemoglobin, haptoglobin, lactate dehydrogenase), thrombocytopenia and renal impairment. Furthermore, urine was collected to screen for proteinuria and hematuria.

Patients were advised to measure their blood pressure at home, and in case of increased blood pressure to contact their physician. All patients and their caregivers were strictly instructed to pay close attention to any signs of disease recurrence and were advised to contact their physician in case of any signs of infections, generalized malaise, fever, hematuria, edema, oliguria, paleness or other signs indicating recurrence of aHUS. In case of aHUS recurrence, eculizumab was restarted immediately.

All kidney transplant recipients were treated according the protocol as described by Verhave et al.[266] To prevent endothelial damage and hereby lowering the risk of aHUS recurrence, among others, patients were strictly monitored to prevent high calcineurin inhibitor levels, had aggressive blood pressure control, preferably with ACE inhibition, and were prescribed statins. In patients with aHUS in their native kidneys, we also adhered to tight blood pressure control. However, patients with well controlled blood pressure and low cholesterol levels did not necessarily receive ACE inhibitors or statins.

Statistical analysis

Clinical variables were expressed for the total number of patients of whom data were available. Values were expressed as absolute numbers and percentages given in parenthesis for categorical variables and as mean and standard deviation (SD) or median and 25 - 75 interquartile range (IQR) for continuous variables, as appropriate.

Results

Patient characteristics

Between 2012 and 2016 a total of twenty patients, both adult (n=14) and pediatric (n=6) patients, received eculizumab as treatment for aHUS, in the Radboudumc, Nijmegen, the Netherlands (**Table 8.1**).

The median (IQR) age at first presentation with aHUS was 24 (7 - 40) years and 13 (65%) patients were female. Five patients had a history of kidney transplantation. In 11 (55%) patients a pathogenic mutation in complement genes or auto-antibodies against CFH were found and 7 (35%) patients had a mutation of unknown significance (**Table 8.1**). Of the 20 patients who received eculizumab treatment between 2012-2016, four patients (patient 1, 3,16, 17) already received treatment for aHUS when eculizumab became available in the Netherlands in November 2012. Patient 3, 16 and 17 were PT dependent before they were switched to eculizumab and patient 1, previously PT dependent, continued eculizumab therapy after the clinical trial conducted by Alexion Pharmaceuticals, Inc ended. (**Table 8.1**). Since November 2012, 16 patients presented with a new TMA episode of which seven patients initially received PT. However due to either PT dependency (n=2) or PT resistance (n=5), eculizumab therapy was initiated. Nine patients received eculizumab without PT.

All pediatric patients reached remission with initiation of eculizumab. Of the 14 adult patients, all but one showed signs of aHUS remission after initiation of eculizumab. Patient 7, with a history of chronic renal failure e causa ignota for over ten years, did not show a renal response after eculizumab initiation. At presentation this patient had a high blood pressure, signs of TMA and further decline of renal function. Renal biopsy showed signs of mainly chronic TMA. Eculizumab was started immediately, but did not result in improvement of renal function, most likely due to the chronic condition. Therefore eculizumab was stopped.

In the 19 responding patients the median (IQR) time until recovery of creatinine, and thrombocytes was respectively 39 (19 - 74) days and 5 (2 - 6) days (**Table 8.2**).

Withdrawal of eculizumab therapy

Adults:

Thirteen of the fourteen adult patients have responded to eculizumab therapy. Therapy has been discontinued in all but one patient (**Figure 8.1**). Patient 6, who received a kidney transplant, has been treated with eculizumab for many years now. Treatment with eculizumab at extended intervals resulted in low grade TMA for which therapy recently was intensified. Of the twelve patients where therapy was discontinued, three experienced TMA recurrence at respectively two weeks, three months and twelve months after eculizumab withdrawal. Immediate initiation of eculizumab was effective and no clinical

sequelae such as more severe proteinuria or decline of kidney function were observed (**Table 8.3**). In patient 3 eculizumab was again withdrawn after a period of receiving eculizumab with an extended interval. The median (IQR) duration of initial therapy was 3.8 (2.8 - 5.8) months. The median (IQR) time of follow up after start of eculizumab therapy was 27.4 (7.8 - 42) months.

Children:
In total, eculizumab therapy was given to six pediatric patients between 1 - 11 years of age. Until now, eculizumab treatment has been discontinued in four patients. In the remaining two patients the dosing interval was prolonged (**Figure 8.1**). Patient 19 experienced a relapse at twelve months after eculizumab withdrawal, which was treated successfully for a period of three months with eculizumab (**Table 8.3**). After reaching remission eculizumab was again withdrawn. The pediatric patients had a median (IQR) time of follow up after start of eculizumab therapy of 31.3 (18.5 – 42.3) months.

Relapses
In total, five patients experienced a recurrence of aHUS (**Table 8.3**). Due to close monitoring and rapid reinitiation of eculizumab therapy, no chronic sequelae were detected in these patients (**Table 8.3**). After clinical remission was reached, eculizumab was again withdrawn in two patients (**Figure 8.1**). The rate of relapses differed between the patient groups with various genetic variants: respectively four out of the eight patients with a *CFH* mutation and one out of the four patients with *C3* mutation experienced a relapse, In patients with identified genetic variants in respectively, *CFB* (n=1), CFI (n=1), *CFH-CHFR1* (n=3), patients with auto-antibodies against CFH (n=1) or patients were no pathogenic mutation was detected (n=2), no aHUS recurrence was detected after therapy adjustment.

Figure 8.1 Follow up aHUS patients

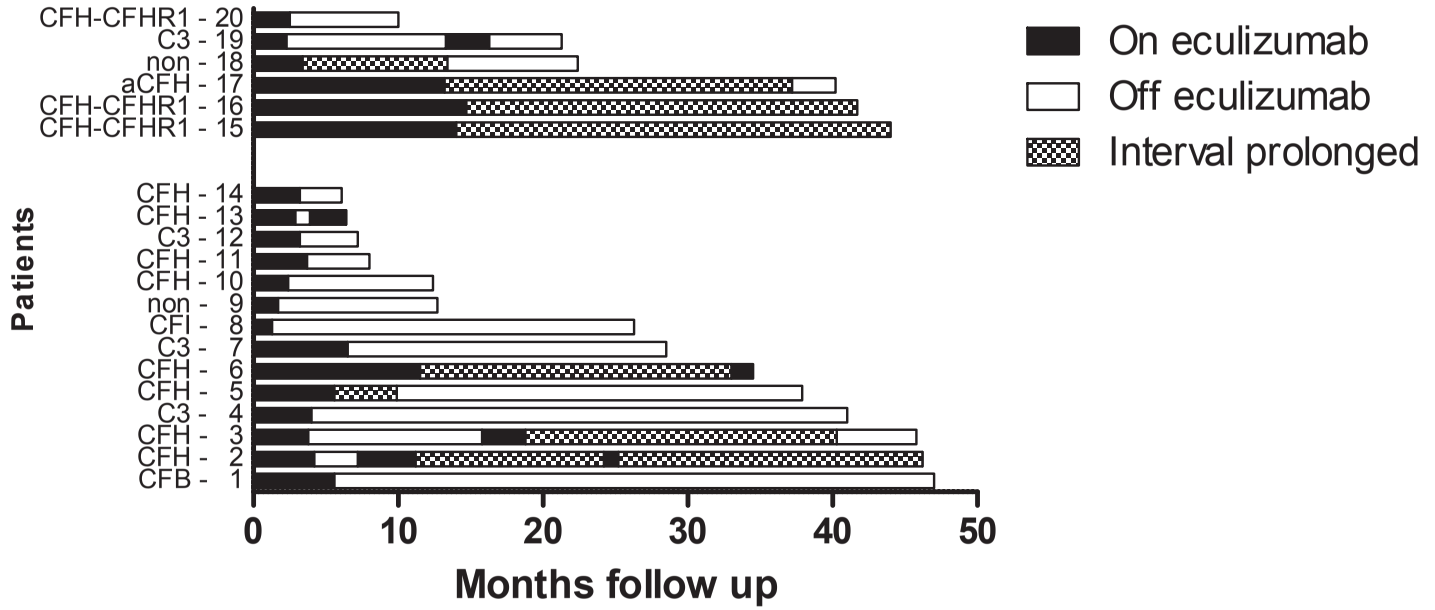

Figure 8.1 Clinical course during follow up in all twenty aHUS patients who received eculizumab. The numbers 1-14 and 15-20 represent respectively adult and pediatric patients. The identified genetic variant is described for each individual patient. The white bars indicate eculizumab discontinuation, the black bars indicate the time with eculizumab treatment as described in Summary of Product Characteristics. In some patients the intervals between the eculizumab infusions were prolonged as indicated with the dotted bars. aCFH; auto-antibodies directed against complement factor H, C3; complement component C3, CFB; complement factor B, CFH; complement factor H, CFHR; complement factor H related protein, CFI; complement factor I

Table 8.1 Patient Characteristics

Patient number	Gender	Previous TMA episodes	History of kidney transplantation	Age first onset (years)	Age eculizumab initiation (years)	Identified genetic variant
1	F	Yes, multiple aHUS episodes, PT dependent and received eculizumab in Alexion trial C08-002A	No	3	19	CFB: c.967A>G (p.(Lys323Glu))
2	F	No	No	21	21	*CFH*:c.2572T>A (p.(Trp858Arg))
3	F	Yes, First episode of TMA in October 2012, for which PT, however patient was PT dependent	No	43	43	*CFH:* c.2120delC (p.(Pro707fs))
4	F	No	No	31	31	*C3*: c.481C>T (p.(Arg161Trp))
5	F	No	No	27	27	*CFH:* c.1778T>A (p.(Leu593*))
6	F	Yes, pre-eclampsia after which ESRD for which peritoneal dialysis	Two kidney transplantations	31	44	*CFH:* c.1520-1G>A (p.(?))
7	F	No	No	62	62	*C3*: c.481C>T (p.(Arg161Trp))
8	F	No	No	21	21	*CFI*: c.685T>C (p.(Cys229Arg))

	Mutation classification	Initial PT	Total duration eculizumab treatment (months)	Relapse after eculizumab withdrawal?	Remarks
	Pathogenic *Gain of function mutation*[143]	No	5.6	No	When Alexion trial ended patient received eculizumab 5.6 months before therapy was discontinued.
	Variant of unknown significance	Yes, no response	43.2	Yes, after 3 and twelve months	Two relapses : 1.Three months after therapy discontinuation, occurred in parallel with decreasing the beta blocker and an increase of blood pressure. 2. At eculizumab interval of 8 weeks, triggered by gastro-enteritis
	Pathogenic *Frameshift*	Yes, plasma dependent	28.3	Yes, after 12 months	Relapse most likely triggered by viral infection
	Pathogenic *Gain of function mutation*[143]	Yes, no response	4	No	
	Pathogenic *Nonsense mutatie*	Yes, plasma dependent	9.6	No	Interval between eculizumab administrations was gradually extended before eculizumab discontinuation due to variable serum creatinine values
	Variant of unknown significance *Splice site mutation*	No	34.5	Yes, at moment of extended interval	Decline in kidney function for which kidney biopsy was performed. Signs of TMA activity for which eculizumab treatment was intensified.
	Pathogenic *Gain of function mutation* [143]	Yes, no response	6.8	No	Eculizumab was stopped after extended period due to unresponsiveness
	Variant of unknown significance	Yes, no response	1.3	No	After six eculizumab infusions patient was admitted to the ICU with a severe pneumonia. Due to this severe infection and the lack of ongoing TMA, eculizumab was discontinued

Table 8.1 Continued

Patient number	Gender	Previous TMA episodes	History of kidney transplantation	Age first onset (years)	Age eculizumab initiation (years)	Identified genetic variant
9	F	Unknown	Two kidney transplantations	42	42	No pathogenic mutation found in: *CFH, CFI, CFB, C3, MCP, CFH/CFHR region*
10	F	Yes, hypertension and eclampsia	One kidney transplantation	51	51	*CFH:* c.2669G>T (p.(Ser890Ile))
11	F	Yes, malignant hypertension	One kidney transplantation	21	28	*CFH*:c.1548T>A (p.Asn516Lys)
12	M	No	No	33	33	*C3:* c.481C>T (p.(Arg161Trp))
13	F	Yes, multiple aHUS episodes	Two kidney transplantation	31	41	*CFH:* c.2034g>t (p.Trp678Cys)

	Mutation classification	Initial PT	Total duration eculizumab treatment (months)	Relapse after eculizumab withdrawal?	Remarks
	NA	No	1.7	No	TMA developed as complication of her first pregnancy. Caesarian was performed. Due to incomplete recovery of hemolysis and renal function eculizumab was started. After 1.7 months it was considered safe to withdrawn eculizumab due to elimination of the initial trigger and no signs of active TMA.
	Variant of unknown significance[141]	No	2.4	No	
	Variant of unknown significance[267]	No	4.7	No	
	Pathogenic *Gain of function mutation*[143]	Yes ,no respons	3.2	No	
	Variant of unknown significance	No	5.4	Yes, after 2 weeks	Patient had a complicated disease course with changes in immunosuppressive therapy. First, calcineurin inhibitor nephrotoxicity was considered likely, and patient was switched to belatacept with calcineurin inhibitor withdrawal. Two weeks after the last eculizumab administration patient presented with fever,an increase of serum creatinine, mildy decreased thrombocytes and high LDH values. Although kidney biopsy showed no signs of active TMA, eculizumab was restarted. However, after eculizumab initiation serum creatinine did not decline, questioning the presence of TMA.

Table 8.1 Continued

Patient number	Gender	Previous TMA episodes	History of kidney transplantation	Age first onset (years)	Age eculizumab initiation (years)	Identified genetic variant
14	M	No	No	49	49	*CFH*:c.2850C>T (p.(Gln950His))
15	M	Yes, multiple aHUS episodes, treated with PT and eculizumab in Alexion trial C10-003	No	5 months	2	de novo rearrangement *CFH- CFHR1*[a]
16	M	Yes, multiple aHUS episodes, patient was PT dependent	No	4 months	11	*Hybrid CFH-CFHR1 gene*[b]
17	M	Yes, multiple aHUS episodes, patient was PT dependent	No	6	9	Auto-antibodies against CFH
18	M	No	No	16 months	16 months	No pathogenic mutation found in *CFH, CFI, CFB, C3, MCP, PLG, THBD, DGKε, CFHR1-5, CFH/CFHR region*

	Mutation classification	Initial PT	Total duration eculizumab treatment (months)	Relapse after eculizumab withdrawal?	Remarks
	Variant of unknown significance	Yes, PT dependent	3.2	No	
	Pathogenic[148]	No	44	NA	Experienced relapse three months after eculizumab withdrawal due to end of Alexion trial. Eculizumab was restarted.
	Pathogenic[148]	Yes, PT dependent	41.7	NA	
	Pathogenic[47]	Yes, PT dependent	37.2	No	The auto-antibody CFH remained high, for which rituximab and mycophenolate mofetil was administered. The titer was successfully reduced after which eculizumab was withdrawn according recently published guidelines[4]
	NA	No	13.4	No	Patient received eculizumab for over a year with an extended interval up to four weeks, after which therapy since patient showed no signs of TMA.

Table 8.1 Continued

Patient number	Gender	Previous TMA episodes	History of kidney transplantation	Age first onset (years)	Age eculizumab initiation (years)	Identified genetic variant
19	F	Yes, multiple aHUS episodes treated with PT	No	9	11	*C3:* c.481C>T (p.(Arg161Trp))
20	M	No	No	10	10	*Hybrid CFH-CFHR1 gene*[b]

aHUS, atypical hemolytic uremic syndrome; C3, complement component C3; CFB, complement factor B; CFH, complement factor H; CFHR, complement factor H related protein; CFI, complement factor I; CMV, cytomegalovirus; DGKε; diacylglycerol kinase-ε; ESRD, end stage renal disease; ICU, intensive care unit; F, female; LDH, lactate dehydrogenase; M, male; MCP, membrane cofactor protein; NA, not applicable; PCR, polymerase chain reaction;

Mutation classification	Initial PT	Total duration eculizumab treatment (months)	Relapse after eculizumab withdrawal?	Remarks
Pathogenic *Gain of function mutation*[143]	No	5.3	Yes, after 12 months	At start maintenance phase (1200mg) patient showed signs indicative of an anaphylactic reaction after eculizumab infusion. Temperature increased up to 38.7 Celsius, patient experienced shivers, and erythematous spots were seen at the face and shoulders. The reaction was observed during four consecutive infusions and patient responded good at anti-histamine treatment. Twelve months after therapy discontinuation, patient experienced a relapse, wherefore patient was treated again with eculizumab for 3 months
Pathogenic[148]	No	2.5	No	

PLG, plasminogen; PT, plasma therapy; THBD, thrombomodulin; TMA, thrombotic microangiopathy
[a] identified genetic variants in CFH: c.3572C>T (p.(Ser1191Leu)) and c.3590T>C (p.(Val1197Ala)). [b] Caused by deletion CFH exon 23, CFHR3 and CFHR1 exon 1-5

Table 8.2 Laboratory values at start of treatment and after recovery

Patient number	**Values at start of eculizumab**		
(genetic variant in)	**Screat (µmol/l)**	**Hemoglobin (mmol/l)**	**Trombocytes (x10⁹/l)**
1 (*CFB*) *	159	6.9	277
2 (*CFH*) [a]	648	5.5	125
3 (*CFH*) [b]	270	6.9	129
4 (*C3*) [a]	605	5.2	78
5 (*CFH*) [b]	164	5.2	138
6 (*CFH*)	275	4.9	150
7 (*C3*)	291	6.1	135
8 (*CFI*) [a]	817	5.1	130
9 (-)	224	5.9	177
10 (*CFH*) [a]	376	5.0	84
11 (*CFH*) [a]	1063	4.5	46
12 (C3)	341	5.1	45
13 (*CFH*)	163	6.8	165
14 (*CFH*) [b]	250	6.2	181
15 (*CFH-CFHR1*)	95	3.4	109
16 (*CFH-CFHR1*) [b]	81	7.0	180
17 (αCFH) [b]	46	7.4	288
18 (-) [a]	200	5.2	514
19 (*C3*)	122	4.8	12
20 (*CFH-CFHR1*) [a]	552	6.0	76
Total, median (IQR)	**260 (160-508)**	**5.5 (5-6.8)**	**135 (84-180)**

Table 8.2. Values indicating TMA at start of eculizumab are depicted. Serum creatinine value at recovery and days until recovery of creatinine are given. For a detailed description of the genetic variants per patient, see Table 8.1. αCFH; auto-antibodies directed against complement factor H, C3; complement component C3, CFB; complement factor B, CFH; complement factor H, CFHR; complement factor H related protein, CFI; complement factor I,

Table 8.3 Follow up of patients with aHUS recurrence after eculizumab withdrawal

Patient (genetic variant in)	**2** (*CFH*)		**3** (*CFH*)	
Value	Screat (µmol/l)	PCR (g/10mmol)	Screat (µmol/l)	PCR (g/10mmol)
Initial presentation	1860	2.5	628	7.5
Recovery	143	0.24	122	<0.10
First Relapse	1022	7.7	305	1.7
Recovery first relapse	146	<0.10	118	<0.10
Second Relapse	330	0.8	NA	NA
Recovery second relapse	141	<0.10	NA	NA
Last follow up	138	<0.10	100	<0.10

Table 8.3 Five patients with recurrence of aHUS after eculizumab discontinuation are presented in this table with serum creatinine (Screat) and protein-to-creatinine ratio (PCR) as value for the proteinuria during this period.

Values at start of eculizumab		Lowest values at recovery	
LDH (U/l)	**Haptoglobin (g/l)**	**Screat (µmol/l)**	**Days until recovery creatinine**
151	0.31	132	NA
360	<0.10	159	83
420	<0.10	124	11
1368	-	154	99
231	0.82	160	NA
331	<0.10	160	15
260	<0.10	NA	NA
338	0.89	146	65
395	0.42	149	20
1329	<0.10	147	50
1701	<0.10	203	132
1905	<0.10	88	54
426	<0.10	111	21
230	0.45	166	39
982	<0.10	29	48
244	0.48	84	NA
186	0.28	47	NA
632	<0.10	39	19
-	0.43	56	15
2490	<0.10	45	32
377 (241-1069)	**0.01 (0.01-0.43)**	**139 (77-159)**	**39 (19-65)**

LDH; lactate dehydrogenase, NA; not applicable, Screat; creatinine

**patient received already eculizumab as participant in a clinical trial conducted by Alexion Pharmaceuticals, Inc.*

[a] patient was on dialysis at start of eculizumab

b patient was PT dependent at start of eculizumab

6 (*CFH*)		**13** (*CFH*)		**19** (*C3*)	
Screat (µmol/l)	PCR (g/10mmol)	Screat (µmol/l)	PCR (g/10mmol)	Screat (µmol/l)	PCR (g/10mmol)
275	0.6	175	0.3	121	14.4
160	<0.10	98	0.2	56	<0.01
225	<0.10	128	0.2	136	8.8
150	<0.10	116*	<0.10	55	<0.01
NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA
150	<0.10	120	<0.10	52	<0.01

C3; complement component C3, CFH; complement factor H

**The disease course of this patients is described in Table 8.1.*

Discussion

Although eculizumab is recommended as lifelong therapy for patients with aHUS, this study shows that a short treatment period with eculizumab is feasible and more importantly: safe and effective. Of the twenty patients, only two patients currently receive eculizumab, according SPC advise, continuously at two weeks interval. In total, eculizumab has been discontinued without aHUS recurrence, in thirteen patients of which five are event free for over a year now.

Our local restrictive treatment is effective, with 19 out of the 20 patients showing TMA remission and improvement of kidney function. The duration of treatment in our center deviates from the current international guideline and is based on the following principles: first, there is no proof that lifelong eculizumab therapy is needed and/or associated with improved survival. Moreover, in former times PT could be withdrawn in a substantial amount of aHUS patients without disease recurrence. Additionally, most relapses occurred during the first year after disease onset. This risk decreased from over 80% to approximately 25% in almost all aHUS patients, after the first year.[261] Secondly, treatment with eculizumab is not without risk, with as most prominent one the risk of developing meningococcal meningitis. Finally, the risk of aHUS recurrence is depending on risk factors such as age of onset, genetic background of the patient, disease history (prior recurrence) and the presence of triggers, such as the use of calcineurin inhibitors after kidney transplantation, or the presence of rejection.[1,8,44,57,267]

Based on these principles we have pursued eculizumab withdrawal in all aHUS patients. In adults with a primary aHUS episode in their native kidney, therapy withdrawal was considered after three months of therapy. In patients considered of high risk for relapse, such as: adults who received kidney transplantation, or patients with disease recurrence after eculizumab withdrawal, treatment was gradually extended. Extending the dose interval of eculizumab dosage may seem irrational. However, we recently showed that the standard treatment regimen often leads to eculizumab serum levels that largely exceed the target level.[158]

In pediatric patients it can be difficult to differentiate between complaints possibly related to the use of eculizumab, such as infections due to their immune compromised status or possible side effects of the drug itself. The optimal treatment schedule of eculizumab in pediatric aHUS patients has yet to be established. Because children, especially young children, tend to experience more infections which are an important trigger for aHUS recurrence, in most of our pediatric patients eculizumab treatment was first tapered before discontinuation.[44,57]

Other studies have reported the successful discontinuation of eculizumab in patients with aHUS. [160,161,165,190,192,265] The largest cohort was recently described by Fakhouri et al.[165] This study included 108 patients who were treated with eculizumab for aHUS. Eculizumab was withdrawn in 38 patients (35%) after a median duration of treatment of 17.5 months.

Follow up after eculizumab withdrawal amounted 22 months. During follow up 12 patients (32%) experienced a relapse. Retreatment with eculizumab was successful. Although this and other studies thus provide evidence that eculizumab treatment can be stopped in some patients, there are some limitations. Most patients were treated with eculizumab for a long period of time. Most importantly, withdrawal of eculizumab was only attempted in a limited amount of patients, which may have introduced selection bias.

Our study adds important new information. We have tried to withdrawn eculizumab in all patients as early as three months after start of therapy. Clearly, outcome in our patients was favorable with approximately 30% of patients experiencing a relapse which could be reversed with re-introduction of eculizumab. Obviously, we cannot exclude that patients with a relapse after eculizumab withdrawal are at risk for more severe chronic kidney disease later in life. Therefore, it is important to be able to predict early relapses. Based on theoretical considerations, we have been reluctant to withdrawn eculizumab in young children or in patients who had received a kidney transplantation. In these patients we have extended the treatment interval to allow evaluation of TMA signs in a period of less intensive complement blockade.

Literature data suggest that knowledge of the genetic defect may also add in predicting relapses. Indeed, in the study of Fakhouri et al.[165] and Ardissino et al.[160,192] most relapsing patients had a mutation in *CFH*. Likewise, four of five patients with a relapse in our study had a mutation in *CFH*. Still, in our view predictive accuracy is too low: four patients in our cohort with a mutation in *CFH* did not experience a relapse after eculizumab discontinuation. Well designed, prospective, multicenter, international studies may allow the identification of high risk mutations which could guide individual therapy.

A restrictive treatment regimen is important in reducing possible long-term side effects and lowering the burden of lifelong eculizumab treatment. Furthermore, another important issue in our society comprises healthcare expenses. Eculizumab is known as one of world's most expensive drugs at this moment and treatment of one patient can cost up to half a million euro's.[263] A restrictive treatment strategy will reduce costs considerably. Recently, a cost-effectiveness analysis concerning treatment of aHUS in kidney transplant recipients was published.[268] Van den Brand et al. showed that induction therapy with eculizumab followed by a lifelong treatment was inferior to eculizumab upon recurrence. This restrictive treatment strategy appeared cost-effective. In addition, our data provides important information to allow cost-effectiveness analyses of treatment of patients with aHUS in the native kidney.

Our treatment strategy allowed early eculizumab withdrawal and hereby reduced drug costs in our patients. The total costs of treatment with eculizumab in these twenty patients were approximately €7.3 million. This comprises a cost reduction of 62% when compared to the treatment regimen according the SPC of EMA, which would have cost around €18.7 million. Obviously, we have not performed a formal cost-effectiveness analysis.

One important limitations of our observational study is that eculizumab withdrawal was not protocolized but rather based on clinical information and the assessment of the treating physician. Furthermore, it remains important to consider the importance of early recognition of symptoms indicating aHUS recurrence, which is sometimes difficult in young children. Future studies should help to identify a reliable predictor of disease recurrence. The most reliable markers may reflect complement activation in blood and on the cell surface as previously suggested.[173,269]

Prospective studies are needed to determine optimal duration of treatment with eculizumab and evaluate prediction markers. Recently, a national, prospective study called CUREiHUS (NTR5988) has started in the Netherlands. This study will include all aHUS patients and will monitor and evaluate the restrictive treatment regimen according the new Dutch guideline. With the CUREiHUS study we hope to show that a restrictive treatment regimen is feasible. Moreover, we want to investigate the potential efficacy of this new treatment regimen from a societal perspective with the design of a cost-effectiveness analysis. In conclusion, discontinuation of eculizumab treatment in patients with aHUS is safe and effective, with close monitoring for signs of disease recurrence. This restrictive eculizumab regimen reduces costs considerably.

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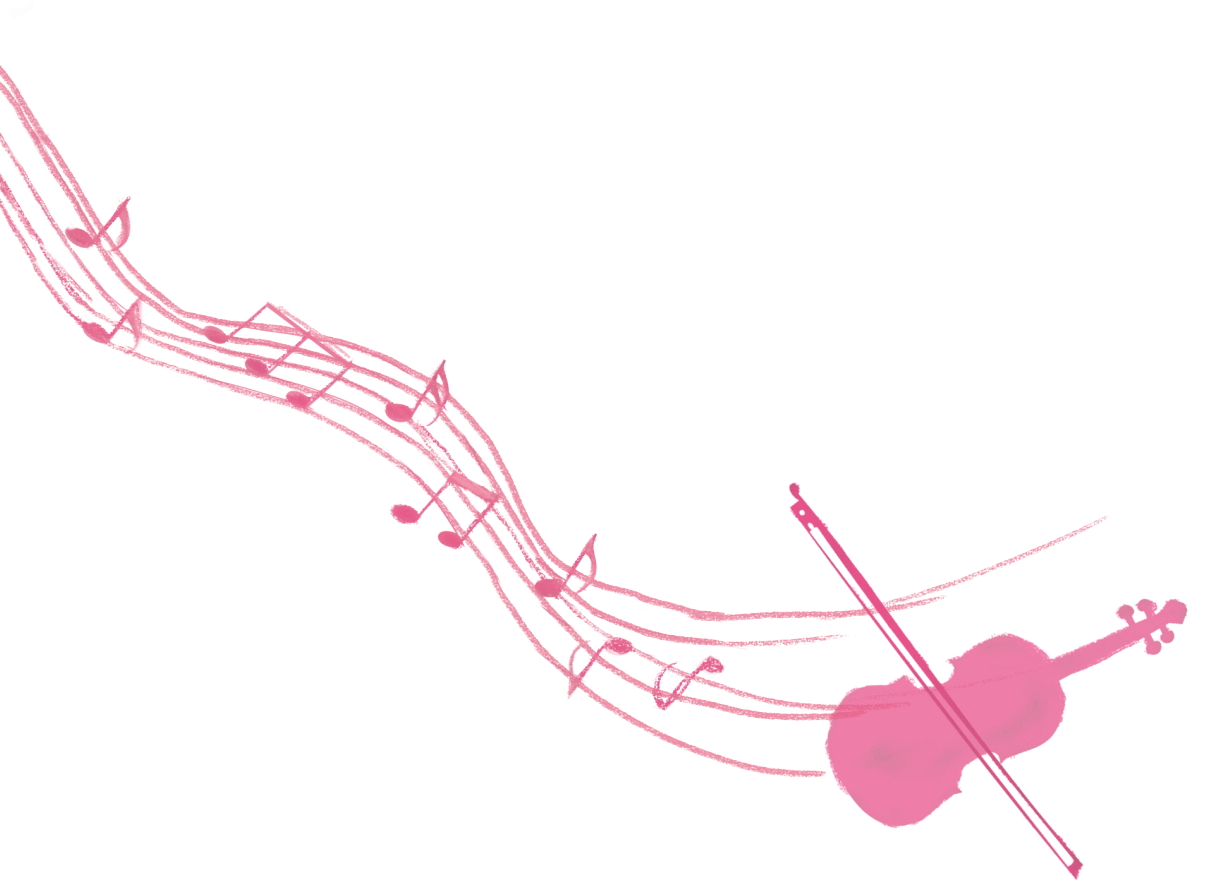
"Shoot for the moon. If you miss you'll land among the stars."

Oscar Wilde



Chapter 9

Eculizumab in atypical hemolytic uremic syndrome: strategies toward restrictive use

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Abstract

With the introduction of the complement C5-inhibitor eculizumab, a new era was entered for patients with atypical hemolytic uremic syndrome (aHUS). Eculizumab therapy very effectively reversed thrombotic micro-angiopathy and reduced mortality and morbidity. Initial guidelines suggested lifelong treatment and recommended prophylactic use of eculizumab in aHUS patients receiving a kidney transplant. However, there is little evidence to support lifelong therapy or prophylactic treatment in kidney transplant recipients. Worldwide there is an ongoing debate regarding the optimal dose and duration of treatment, particularly in view of the high costs and potential side effects of eculizumab. An increasing but still limited number of case reports and small cohort studies suggest that a restrictive treatment regimen is feasible. We review the current literature and focus on the safety and efficacy of restrictive use of eculizumab. Our current treatment protocol is based on restrictive use of eculizumab. Prospective monitoring will provide more definite proof of the feasibility of such restrictive treatment.

Introduction

Over the last decade, the knowledge of the pathogenesis of atypical hemolytic uremic syndrome (aHUS) has increased substantially.[1,5,57] Atypical HUS is a rare and severe form of thrombotic microangiopathy (TMA) which predominantly affects the renal vasculature. The disease course is characterized by relapses and both onset and recurrence are often triggered by a gastro-intestinal or pulmonary infection or pregnancy.[1,57] Atypical HUS is caused by dysregulation of the alternative complement pathway, resulting in excessive production of the terminal complement complex C5b-9, and subsequently endothelial cell injury. In up to 60% of the aHUS patients pathogenic mutations in complement (regulatory) genes, such as *complement factor H* (*CFH*), *membrane cofactor protein* (*MCP*), and *C3*, can be detected. Additionally, about 10% of the patients, predominantly adolescents, have auto-antibodies directed against CFH.[1] Atypical HUS is associated with increased mortality, although the reported mortality varies in the different cohort studies. Mortality, largely dependent on the underlying pathogenic mutation, seems to be higher in children than in adults, and could be estimated at 2-4% in adults and 8-14% in children at 3-5 years of follow up.[53,54] In the absence of effective treatment approximately one third of the pediatric patients and half of adult patients with aHUS who did not died in the acute phase required, often permanent, dialysis treatment.[53,54] The risk of recurrent disease after kidney transplantation was estimated to be 50 to 80%, with a overall five year graft survival of 36 ± 7% in patients with a recurrence compared to 70 ± 8% in patients without a recurrence.[10,58,267]

Plasmatherapy (PT), either plasma exchange (PE) or in some cases plasma infusions, became the cornerstone of treatment in aHUS in the early nineties, despite the lack of randomized clinical trials and most information being based on retrospective studies with variable treatment protocols.. In 2009, the European Pediatric Study Group for HUS was the first who published a guideline to promote standardized high volume and early initiated PT in aHUS patients.[156] The results of an audit analysis of this guideline were published in 2014 by Johnson et al. who evaluated 71 pediatric patients treated with PT.[262] Only 59 (83%) patients received some form of PT, and of these 59 patients 13 patients received early high volume PT as recommended by the guideline. Median (range) time until hematological remission in all patients was 11.5 (0-119) days and 83% of the patients reached hematological remission within 33 days. After 33 days, renal function had not fully recovered in most patients: 17% of the patients were still dialysis dependent, 46% suffered from persistent renal impairment and 11% had either residual proteinuria and/or hypertension.[262] Of note, PT is not without risks: in most studies various adverse events were noted, more in the pediatric population (up to 80%, especially young patients with low body weight had an increased risk) than in adults (26%), mostly catheter infections (50%) and thrombosis (19%).[57,58,262,270]

By unraveling the role of complement in aHUS, novel therapeutic options emerged.[8] With the introduction of eculizumab in 2011, a new era was entered for treatment of aHUS patients. Eculizumab is a humanized, chimeric monoclonal antibody directed against complement component C5. By blocking the cleavage of C5 into C5a and C5b and subsequently the assembly of C5b-C9, endothelial injury is prevented.[8] Based on two prospective open label phase two pivotal trials (including adults and adolescents) and a retrospective analysis of aHUS cohort (including pediatric, adolescent and adult patients) who received eculizumab outside the trials, eculizumab gained marketing approval and was adopted as first line therapy in patients with aHUS.[8,57,185] In these trials eculizumab was administered following a standard treatment scheme, which is currently recommended by the European Medicines Agency (EMA) and Food and Drug Administration (FDA) (**Table 9.1**). In the prospective studies TMA event free status and complete TMA response was observed in respectively >92% and 50-85% of the aHUS patients who were respectively resistant to PT or PT dependent.[8] PT could be discontinued in all patients. Overall eculizumab treatment was well tolerated. Efficacy of eculizumab in the pediatric population was confirmed in a prospective open label phase 2 trial conducted in 22 children below 18 years of age.[166] Complete TMA response was achieved after a median of 8.6 (1-22) weeks.[166] In the pivotal trials eculizumab treatment was continued for at least two years.[8,185] No advice was given how to proceed when a patient was stable and in remission. Of note, TMA event free status and complete TMA response should not be interpreted as complete remission or full renal recovery (see also table 6 in the manuscript of Loirat et al. for an extensive overview of definitions and attainment of endpoints).[57]

Although these trials showed excellent results of treatment with eculizumab, the introduction of the drug initiated a worldwide debate regarding the optimal treatment strategy. Different questions were raised such as: what is the optimal duration of therapy? How can therapy be monitored? Is it safe to stop eculizumab therapy? Is there a need for prophylactic use of eculizumab in case of kidney transplantation? Guidelines, written by Kidney Disease Improving Global Outcome (KDIGO) or clinical recommendations generated by HUS international (a group of HUS experts), are inconclusive.[57,233] This review will focus on the safety, effectiveness and feasibility of restrictive eculizumab treatment.

Eculizumab therapy: a none-ending story

Although no official document or international guideline directly addresses the duration of eculizumab therapy, it is assumed (and advocated in various scientific meetings and publications) that standard therapy is eculizumab in two-weekly dosages lifelong.[57,233] Indeed approval reports of both EMA and FDA emphasize the risks of withdrawal of eculizumab.[169,194,233] In most guidelines, both treatment duration and dosage of eculizumab are debated.[57,233] There are reasonable arguments against the advised standard therapy.

Table 9.1 Eculizumab dosage regimen, standard therapy according to EMA/FDA

Weight category	Induction phase	Maintenance phase
Above 40 kg	900mg, every week, for 4 weeks	1200mg, in fifth week, every 14 days thereafter
30 to < 40 kg	600mg, every week, for 2 weeks	900mg, in third week, every 14 days thereafter
20 to < 30 kg	600mg every week, for 2 weeks	600mg, in third week, every 14 days thereafter
10 to < 20 kg	300mg once	300mg, in second week, every 14 days thereafter
5 to < 10 kg	300mg once	300mg, in second week, every 21 days thereafter

Eculizumab has to be administrated intravenously.

EMA; European medicines agency, FDA; food and drug administration

First of all, there is little evidence to support lifelong therapy in every patient with aHUS. Before introduction of eculizumab, when PT was the mainstay therapy, renal outcome of aHUS patients was poor. However, some patients responded well to PT with hematological remission and recovery of kidney function and were not in need of chronic PT. Geerdink et al. evaluated a Dutch cohort of 45 pediatric aHUS patients.[44] Of these, 12 patients (25%) were not in need of chronic PT and did not relapse after the first aHUS episode. Fremeaux-Bacchi et al. reported 214 patients (89 children and 125 adults) with aHUS, of which 146 were treated with PT and followed for 4-5 years.[54] In 42% of the children and 34% of the adults outcome was favorable, the remaining patients relapsed, reached ESRD after the first aHUS episode or died. In 2006 Caprioli et al. reported the outcome of 60 aHUS patients with a mutation in *CFH*, *MCP* or *complement factor I (CFI)*. The majority of the patients was treated with PT for a period of 2 days to 6 weeks. After long-term follow up renal function had normalized in 38% of the patients, including in 22.5% of the patients with a *CFH* mutation.[141] Jamme et al. evaluated the outcome of 156 adult aHUS patients treated with 5-20 sessions of PE. Overall outcome was poor as 14 patients died from aHUS or complications of treatment. After 1-year follow up renal function (according to Modification of Diet in Renal Disease equation (MDRD)) had recovered to an estimated glomerular filtration rate (eGFR) of ≥60ml/min/1.73m² in 19% of the patients.[271] Some authors argue in favor of lifelong therapy while referring to the

underlying genetic abnormality. However, many patients only present with disease in adulthood, and have been free of disease in childhood despite contact with triggers such as vaccinations or infections.[1]

Secondly, eculizumab treatment is not without risks. Although eculizumab is safe and well tolerated, potential (serious) adverse events need to be taken into account. The most prominent risk factor is the susceptibility to infections with encapsulated bacteria, especially meningogoccal disease. By blocking the complement system, which is part of innate immunity, patients are more prone to infections with encapsulated bacteria, especially *Neisseria meningitidis*. The risk of invasive meningococcal disease is estimated at >2000-fold increased compared to the normal population.[272,273] To minimize the risk, patients are vaccinated against serotypes A,C,W, Y and recently B and receive prophylactic antibiotics.[272] Furthermore, booster vaccinations every five years are highly recommended, since it is unknown what degree of protection is reached in patients with complement deficiencies.[272] Yet, a few cases have been described of fulminant meningogoccal disease despite extensive vaccination and adequate antibodies titers, questioning the effectiveness of the vaccines.[155,274] The protective efficacy of vaccinations is less potent in patients who are treated with immunosuppressive drugs. Hence, a higher risk of infections can be expected in patients with aHUS after kidney transplantation. Furthermore, there is a risk of developing human anti human antibodies (HAHA) which could neutralize eculizumab. Thus far, all case reports described non-neutralizing HAHA, which did not interfere with eculizumab efficacy.[166] Other (long term) chronic sequelae of eculizumab therapy are not yet fully comprehended. Only recently, emerging evidence appeared regarding the hepatotoxicity caused by eculizumab.[99,184,275] In retrospect, hepatotoxicity was already noted in 10 patients during the pivotal trials as described in the approval package of the FDA.[194] Liver enzyme abnormalities were observed 10-29 days following initiation of eculizumab. In most cases liver enzyme derangement was transient, however in some patients eculizumab was withdrawn due to suspicion of drug induced liver injury.[99,184,275]

Thirdly, pharmacokinetic and pharmacodynamic data are only sparsely described in the pivotal trials and are insufficient to endorse the current treatment scheme.[169,208] The recommended trough level of 50-100 μg/ml is based on a meta-analysis of patients with paroxysmal nocturnal hemoglobinuria (PNH) treated with eculizumab. Some PNH patients showed residual complement activity (based on a hemolytic assay to asses total complement activity; CH50) at targeted eculizumab trough levels of 35 μg/ml. Hence, trough levels of 50-100 μg/ml were advised for aHUS patients to minimize the risk of residual complement activation.[169,208,276]

And last but not least, with costs up to €500,000 per year per patient, eculizumab is unaffordable in many countries, or can only be afforded with specific restrictions. For example, The UK National Institute for Health and Care Excellence (NICE), located in the United Kingdom, stated in 2015 that eculizumab could only be reimbursed if the following

arrangements were in place: coordination of eculizumab use through an expert centre, a registry to monitor these patients, a national protocol with start and stop criteria and a research program to evaluate a restrictive treatment regimen.[263,265] These criteria are under development and have not been published.

In this review we discuss the available evidence and address the unanswered questions regarding eculizumab therapy, its prophylactic use, treatment dose and duration of treatment. Prospective studies are needed to answer the open questions. Prospective evaluation of our current treatment protocol will provide some answers and expand the evidence.

Strategies towards a restrictive use of eculizumab in patients with native kidneys

When to start eculizumab therapy?

Early initiation of treatment (<24-48 hours) is highly recommended to stop TMA activity and to prevent chronic sequelae.[57,189,262] In pediatric patients treatment with eculizumab is the preferred option, also in view of the high complication rate of PE in children.[262,270] In adults, initial therapy with PE for five days is recommended. This will allow a detailed diagnostic work-up to exclude secondary causes of TMA, such as hypertension, drugs or auto-immune diseases. This is in agreement with the pivotal study protocol, which included patients with progressive TMA after four or more sessions of PE and showed favorable outcomes. Of note, actual median (range) time to start eculizumab therapy in PE resistant patients was 0.8 months (0.2-3.7) in this study.[8,277] A similar approach may also be feasible in adolescents, who have high prevalence of CFH auto-antibodies.

If the suspected diagnosis of complement mediated aHUS is not refuted and patient did not respond to PE, eculizumab therapy should be initiated. Of note, TMA activity may disappear either spontaneously or with PE only in some patients with aHUS. In the latter cases, eculizumab therapy is not needed and PE can be gradually withdrawn as described by the European Pediatric Study Group for HUS.[156,262]

Can we reduce eculizumab dosage?

Following the summary of product characteristics, eculizumab should be administered as two- or three-weekly infusions during the maintenance phase (**Table 9.1**).[169] The pivotal trials aimed at reaching a trough level between 50-100 μg/ml considered necessary to fully block complement (CH50<10%). Therapeutic drug monitoring was not done, and all patients were treated with the advised fixed dose. It became clear that often serum eculizumab levels above target were reached, with reported trough levels up to 700 μg/ml in adults and up to 1100 μg/ml in children.[194] This has stimulated clinical investigators to adapt the treatment schedule, by either increasing the interval or decreasing the dosage to maintain trough levels between 50-100 μg/ml.[167] Volokhina et al. evaluated 11 aHUS patients in whom treatment intervals were prolonged. Eculizumab concentrations ranged from 40-772 μg/ml, 61-367 μg/ml, 11-256 μg/ml and 13-161 μg/ml after respectively 2, 3, 4 or 5 weeks interval. At intervals of 4-5 weeks, 80% of the patients had eculizumab trough levels > 50 μg/ml. All patients with trough levels above 50 μg/ml had a fully blocked complement system as measured by CH50 (<10%).[182] Willrich et al. showed complete complement blockade with eculizumab trough levels of 100 μg/ml, confirming the target levels as established in the pivotal trials.[276] Ardissino et al. reported that a median dose of 0.75 mg/kg/day (IQR 0.67-0.95) eculizumab was sufficient to block complement up to four weeks.[259] In comparison, the recommended dose of 1200 mg two-weekly for a

70 kg adult provides 1.2 mg/kg/day and a 900 mg dose in a 40 kg child provides 1.6 mg/kg/day. Gatault et al. developed a one-compartment model to predict pharmacokinetics and pharmacodynamics of eculizumab using the data of seven patients. Following their model, it would be possible to extend the interval to four weeks in patients less than 90kg and even to six weeks in patients with body weight below 70kg.[167] Of note, eculizumab trough levels are quite variable between patients with an inter-individual variation coefficient of 45%.[167,182]

The added value of therapeutic drug monitoring in patients treated with eculizumab is still unproven. Interpretation of eculizumab levels is difficult since the assays differ and all detect (to a variable degree) both bound and unbound eculizumab (**Table 9.2**). Instead of measuring eculizumab levels, the pharmacodynamic effect (total complement activity expressed as CH50) could also be used to monitor therapy. Most authors target a fully blocked complement system (CH50<10%). In contrast, a recent study evaluated efficacy of reduced dose of eculizumab, targeting CH50<30%. In a substantial amount of patients this resulted even in less effective complement blockade with CH50 between of 10-70%. All 38 patients remained in remission.[259] Indeed, various studies reported relapse free remissions in patients treated with eculizumab at extended intervals and incompletely blocked complement. [182,190,259] This suggest that complete complement blockade may not be necessary. Other markers of eculizumab activity and endothelial damage have been proposed. However, inconclusive and conflicting results have been published regarding the correlation between C3, C3d, C5, C5a, soluble C5b-C9, *ex vivo* endothelial cell assay, alternative pathway (AP50) activity, and efficacy of eculizumab therapy.[168,182,252,269] Also, although treatment with eculizumab reduced the levels of markers of endothelial damage, their value in clinical practice is not proven.[278]

Is withdrawal of eculizumab therapy possible?

Evidence to support lifelong therapy, as suggested shortly after the introduction of eculizumab, is limited.[8,233] In the past years, an increasing number of case reports and small cohort studies have provided information on eculizumab withdrawal. Nine reports have summarized the data of both children and adult patients in which therapy was either tapered and/or withdrawn (**Table 9.3**). In these studies, eculizumab was withdrawn in 171 patients after a median (range) of 6 (0.5-50) months. Median (range) follow up was 12 (0-47) months. In the individual studies relapse rate ranged from 20-67%.[160,165,192,258,265,279,280] Overall, 44 (27%) patients developed disease relapse. The median (range) time to relapse was 3 (1-29.5) months. This is in agreement with earlier reports, dating from the pre-eculizumab era, indicating that 57-82% of relapses occurred in the first year of follow up.[54] Due to close monitoring (among others screening for proteinuria and hematuria, and rigorous control of blood pressure) and rapid re-initiation of eculizumab at the time of relapse, chronic sequelae could be prevented.[160,165,192,258,265,279,280]

Table 9.2 Monitoring of eculizumab therapy and complement activity in aHUS

Parameter	Interpretation
Serum eculizumab level	Target is set at trough levels of 50-100 μg/ml to fully block complement
Eculizumab-C5 complex	In contrast to serum eculizumab levels, one could also determine eculizumab bound to C5, hence only the bound proportion of eculizumab is determined.
Total complement activity (CH50)	CH50 levels correlated nicely with eculizumab serum trough levels, and suppressed CH50 (<10%) is reached with trough levels >30-50 μg/ml.[182,193,208,252]
Alternative pathway activity (AP50)	AP50 levels can be suppressed by eculizumab, however ongoing activation has been noted despite adequate eculizumab levels.[168,252]
C3d	C3d is a breakdown product of C3, hence elevated C3d complement levels reflect activation at level of C3 is present.
C3	Can be both normal as decreased in aHUS patients during acute phase and remission
C5	Eculizumab binds to C5. Eculizumab trough levels correlate with C5 levels
C5a	C5a is released after cleavage of C5. In case of eculizumab therapy, C5 cannot be cleaved, hence less C5a is present.
Ex vivo endothelial cell assay	By determining the C5b-C9 deposition after adding patient serum on activated endothelial cells, complement blockade could be assessed with good reproducibility
Ham test	The Ham test is modified from the assay used to detect PNH. By acidifying the patient serum, AP is activated and results in erythrocyte lysis in PNH. In the modified Ham test, PNH like cells are incubated with serum of aHUS patients and depending on AP dysregulation present in the serum, will be lysed.[282]
Soluble C5b-C9 (TCC)	Soluble C5b-C9 should decrease during eculizumab therapy

AP; Alternative complement pathway,CP; Classical complement pathway, ELISA;Enzyme linked immunoabsorbent assay,

	Remarks
	Reports differ regarding the measurement of only the free proportion of eculizumab[182] versus measurement of eculizumab both free and bound to C5 [8,166] Furthermore, eculizumab can bind a maximum of 2 C5 molecules per eculizumab molecule and is able to bind both C5 as C5b incorporated in C5b-C9 complex. Hence, measurement of eculizumab can comprise free (excess) eculizumab, eculizumab bound to 1 C5, eculizumab bound to 2 C5, or in combination with C5b-C9 complexes.[281] The assay used in the trials to determine the trough levels measured both bound and free proportion.[193,194]
	It is known that eculizumab can also bind C5b-C9. Furthermore one eculizumab molecule could bind respectively 1 or 2 C5 molecules, hence the remaining capacity is unknown.[158] Clinical use is unknown
	There are different assays to measure CH50. With this test total complement activity (also known as CH50) is tested to determine the capacity of patient serum to lyse sheep or chicken erythrocytes coated with antibodies. In case of a functional complement system, the CP will be activated, consequently leading to C5b-C9 deposition on the erythrocytes and consequently cause hemolysis. With the Wieslab test, CH50 can be measured withC5b-C9 formation, detected using a C9 neoantigen, as read-out.[252] Recently, Willrich et al. reported CH50 measurement during eculizumab treatment with liposome immunoassay with stable and reliable results.[276]
	There are different assays to measure AP50. Specific assessment of alternative pathway activation is possible with a hemolytic assay based on untreated rabbit erythrocytes (AP50). Puissant-Lubrano et al. compared both the hemolytic assays as used in all trials with the Wieslab ELISA in 16 patients treated with eculizumab, and found conflicting results.[252] Residual activity of the AP was observed with the hemolytic assay, in the presence of (sufficient) eculizumab levels. In contrast to the Wieslab ELISA which showed complete blockade of the AP. Moreover, they assessed sensitivity of all assays and concluded that the Wieslab ELISA is less sensitive, hence the residual activity measured with the hemolytic assay is most accurate.[252]
	C3d levels are elevated in acute phase of aHUS and decreased in the majority of patient with eculizumab therapy[42,168]
	[269]
	C5 levels fluctuate between and within patients due to among others disease activity.[276]
	Interestingly, values of C5a do not decrease to zero, although no C5a should be present in light of sufficient eculizumab.[168] Furthermore, C5a has a very short half-life of approximately 1 minute.
	This assay has one major drawback since it is a highly specialized technique which cannot be easily performed in any laboratory.[269] Although Noris et al. advocates that persistent complement activation tested *ex vivo* (whereas CH50 remained low) is a reason to increase eculizumab dosage or decrease interval, Merril et al. showed different results using a ham test.[269,280]
	Merril et al. showed no correlation was seen between positive or negative Ham test, hence the presence of complement activation, and aHUS recurrence. Moreover, various patients remained positive with the Ham test without disease recurrence and withdrawal of eculizumab therapy.[280]
	Various studies report different results. Due to the ability of eculizumab to bind C5b-C9, it could be possible that these complexes have a lower clearance, hence are elevated during remission. [168,182,252,269,276]

PNH; paroxysmal nocturnal hemoglobinuria, TCC;terminal complement complex

Obviously, the abovementioned studies have a relative short follow up time of 12 months. Furthermore, they could be biased, since eculizumab was withdrawn in selected patients and not per protocol. Also, the duration of eculizumab therapy before withdrawal was quite variable. This selection process could have led to a more favorable outcome. However, a comparable relapse rate was noted in a study where eculizumab therapy was stopped per protocol at 3-6 months after start of therapy in adolescent and adults patients in remission.[258] In pediatric patients eculizumab could be stopped in 4 patients and tapered in the remaining two children. A recurrence developed in one out of the six pediatric patients. Still, long term follow up is needed to provide more information regarding chronic sequelae of treatment withdrawal. It is also important to study possible predictors of relapse. In this respect, information regarding the presence and type of genetic mutation could be relevant.

Due to still limited data on eculizumab withdrawal in children and adults with various pathogenic mutations, evaluating data deriving from the pre-eculizumab era is the best alternative up till now to estimate risk of relapse. Several large cohort studies have been published which looked at disease relapse in patients with different pathogenic mutations.[53,54] Patients were treated with various protocols of PE or received only conservative therapy. In respectively children and adults the risk of relapse was estimated at 43% and 35% respectively, mostly within the first year after presentation.[53,54] The risk of recurrence seemed to be higher in patients with pathogenic mutation in *CFH* (31-55%), *MCP* (18-52%) and *C3* (50%).[53,54] Long term outcome in general was more favorable, despite the frequently reported relapses, in patients with a mutation in *MCP*.[160,165,192,258,265,279,280] Before the introduction of eculizumab, relapse rates were approximately 30% in patients without a known pathogenic mutation.[54]

The risk of relapse in the nine mentioned studies is 27% after withdrawal of eculizumab. This compares favorably with the above mentioned relapse rates. Notably, since the introduction of eculizumab, to our knowledge, only one case of disease relapse after eculizumab withdrawal is reported in a patient without a proven pathogenic mutation.[265] In contrast, the risk of relapse is reported to be higher (up to 75%) in patients with a genetic variant in *CFH*.[165] In patients with a *MCP* mutation a relative high risk up till 50% is reported after eculizumab withdrawal.[165] Our own data showed a relapse rate of 50% in patients with a *CFH* mutation, which is lower than previously reported in literature.[258] Limited data are published regarding the relapse rate after discontinuation of eculizumab in children, which ranges between 16-50%.[156,262] In conclusion, the risk of relapse after eculizumab withdrawal is estimated at 30% and most relapses occurred within the first year after withdrawal. Pathogenic mutations in *CFH* and *MCP* seem to be associated with higher chance of recurrence. In contrast, withdrawal of eculizumab in patients without a proven pathogenic mutation was associated with a low risk of disease recurrence. In all reported cases of relapse,

rapid re-initiation of eculizumab treatment allowed remission of aHUS and full renal recovery. [165,258]

Future studies should provide more insight in the relation between specific pathogenic mutations and risk of relapse and chronic sequelae.

Is withdrawal of eculizumab therapy possible after relapse of aHUS?

There are very limited data on eculizumab withdrawal in patients with relapse of disease. In total, 17 patients were described who had a recurrence after eculizumab withdrawal. In three patients, with respectively pathogenic mutations in *CFH, MCP and C3*, eculizumab was again discontinued after 3 to 20 months of treatment. No relapses have been reported.[165,258] This suggests that even in a proportion of patients with disease recurrence, lifelong treatment is not necessary. However, future studies should provide more insight in the risks of multiple relapses and ability of eculizumab therapy to prevent chronic damage when given "on demand" (i.e. during active TMA episodes).

Table 9.3 Studies describing a restrictive eculizumab regimen in aHUS patients

Author & year of publication	Number of participants	Age (years)	Duration standard eculizumab therapy (months)	Number of participants with tapered therapy
[283] Cugno et al. 2014	18	Mean 21 (range 2-40)	Up till 40 months	18; interval was extended up to 4 weeks based on CH50
[160,192] Ardissino et al. 2014-2015	22	18 (1-53)	4.3 (0.5-14.4) months	0
[265] Sheerin et al. 2016	43	Unknown	6 (0.5-8.5) months	0
[165] Fakhouri et al. 2017	108	30 (2-79)	17.5 (2-50) months	0
[280] Merril et al. 2017	17	46 (19-69)	3 (0.5-18.2) months	NA
[279] Macia et al. 2017 Summary of authors' case reports	6	37 (16-39)	6 (1-14) months	1; patient received 900mg every 4 weeks
[258] Wijnsma et al. 2017	20	28 (1-62)	3.8 (1.3-14.7) months	5
[259] Ardissino et al. 2017	47	25 (0.5-60)	2.6 (0.4-24.6) months	38
[279] Macia et al. 2017 Summary of clinical series	130	26 (0-80)	6.3 (0.2-53.7) months	0

Numbers are expressed as median (range) unless otherwise specified

The different cohort studies and case reports include patients with aHUS after kidney transplantation and patients

Number of participants in whom therapy was discontinued	Follow up period after therapy adjustment (months)	Recurrence, number (%)	Time until recurrence (months)	Outcome
0	Up till 43 months	0	NA	No chronic sequelae
16	Up till 40 months	5/16 (31%)	1.2 (0.7-16.3) months	No chronic sequelae after restart of eculizumab. Serum creatinine and proteinuria returned to baseline values
12	12 months	3/12 (25%)	2.5 (1.5-9) months	Full renal recovery was seen in 1 patient. The remaining two patient were still dialysis dependent on time of withdrawal and presented with hemolysis and hyperkalaemia which resolved quickly with reintroduction of eculizumab.
38	22 (5-23) months	12/38 (31%)	7.5 (3-29) months	No chronic sequelae after restart of eculizumab
15	10.2 (1.2-46.3) months	3/15 (20%)	2 (1.8-3.3) months	Two patients received eculizumab after which kidney function was restored .One patient died during PE for recurrence after non adherence with antihypertensive drugs.
5	Unknown	4/6 (67%)	3 (2-12) months	Unknown
15	27.4 (6-47) months	5/20 (25%)	7.5 (1-12) months	No chronic sequelae after restart of eculizumab. One relapse occurred during tapering eculizumab.
9	26.9 (0.8-80.9) months	0	NA	No chronic sequelae after restart of eculizumab
61	5.6 (0-35.1) months	12/61 (20%)	3 (1-29.5) months	Limited data available. One patient progressed to ESRD despite re-initiation of therapy.

with aHUS due to auto-antibodies directed against complement component factor H.
ESRD; end stage renal disease, NA; not applicable, PE; plasma exchange

Strategies towards a restrictive use of eculizumab in kidney transplant recipients

Kidney transplantation is associated with an estimated risk of aHUS recurrence of 50% to 80%.[58,284,285] Most recurrences occur early in the post-transplant period. Recurrent aHUS usually leads to graft loss and curative PE does not improve graft survival.[267,284] The risk of recurrence depends on the genetic variant, and especially patients with a pathogenic mutation in genes that encode circulating complement components, such as *CFH, CFI, complement factor B (CFB)* or *C3,* have a high risk of recurrence (50-80%). In contrast, the recurrence rate is low (8%) in patients with a single genetic variant in *MCP*, a membrane bound factor. This is not unexpected since the endothelium of the graft expresses a functional variant.[58,284,285] In patients without a pathogenic mutation the risk of recurrence is reported to be moderate (18-28%).[53,141] A lower percentage of aHUS recurrence (20%) and graft failure was reported in children[286,287], however, presumably in most of these studies patients with STEC HUS were included.[288-291] Sellier-Leclerc et al. more accurately diagnosed aHUS and reported a higher recurrence rate of 53% among pediatric patients, and even of 80% in patients with a mutation in the gene encoding *CFH*. Graft survival at one year was 62%.[292] The recurrence risk is influenced by transplantation related factors such as pre-donation kidney injury, ischemia reperfusion injury, acute rejections, infections, the use of mammalian target of rapamycin (mTOR) inhibitors and calcineurin inhibitors (CNI).[284,285,290,293,294]

Prophylactic eculizumab therapy in kidney transplantation

Effectiveness of prophylactic eculizumab therapy

The reported high recurrence rate and absence of effective treatment urged many transplantation centers to be very restrictive with offering a kidney graft to aHUS patients with end stage renal disease (ESRD). After the introduction of eculizumab many centers started to perform kidney transplantation using eculizumab prophylaxis in various different schemes.[295] The first case series of aHUS patients treated prophylactically with eculizumab was published by Zuber et al. in 2012.[295] Since then, in total, 53 patients, including 14 pediatric patients have been reported in literature (**Supplementary Table 9.1**). As expected, the overall outcome of transplantation with prophylactic eculizumab therapy was favorable. In most patients allograft function remained well preserved. Median (range) serum creatinine values, available in 38 patients, was 88 (44-187) μmol/l at 15 months (1.5-44 months) after transplantation. Four patients had signs of TMA after transplantation, which resolved after increase of eculizumab dose.[265,296-298] One patient lost its allograft due TMA and a renal artery thrombosis, while on eculizumab therapy. No biopsy was performed.[299]

Discontinuing prophylactic eculizumab therapy

In the patients who were treated prophylactically, eculizumab was discontinued in eight, all adults, at 1 to 28 months after transplantation.[192,279,300-303] Two patients, both recipients of a living donor kidney, developed aHUS recurrence after discontinuation of eculizumab (**Supplementary Table 9.1**).[279,303] The remaining patients did well, without aHUS recurrence and serum creatinine ranged from 67 to 118 μmol/l after a follow up of 4 to 26 months (**Supplementary Table 9.1 and Table 9.4**). These data indicate that continued eculizumab treatment is not needed in all adults patients after kidney transplantation.

Kidney transplantation in aHUS patients without eculizumab prophylaxis

The reported high incidence of recurrent aHUS after kidney transplantation was based on studies that mainly included patients who received a deceased donor kidney, were treated with high dose CNI, and often experienced rejection episodes. Small studies suggested better outcomes after living donor kidney transplantation.[300,304,305] Based on these data, we hypothesized that kidney transplantation without eculizumab prophylaxis should be feasible and developed a transplantation protocol to limit endothelial cell injury of the allograft and subsequent complement activation. (**Supplementary Table 9.5**). Initial data analysis showed a low aHUS recurrence rate. [305] Meanwhile, we have transplanted 19 adult patients with a living donor kidney. Eighteen patient had a high risk of recurrence according to the KDIGO classification, in seven patients a mutation in the gene encoding *CFH* was present. After a median follow-up of 42 (4-78) months, two patients (one patient with a *CFH* mutation and one patients with a *C3* mutation) developed aHUS recurrence, respectively after two and four months after transplantation, for which eculizumab was restarted. In these 19 patients median (range) serum creatinine concentration at last follow-up is 138 (79 -185) μmol/l.

Effectiveness of rescue therapy with eculizumab

The pivotal trials included 25 patients who received eculizumab for aHUS recurrence after kidney transplantation. The median (range) interval between onset of aHUS recurrence and start of eculizumab was relatively long: 1.25 months (0.03-36.7). In 88% of the patients a TMA event-free status was reached. In twenty patients who completed the 18 months follow-up mean eGFR was 44 ml/min/1.73m² (SD 27).[306] After extended follow-up two patients lost their grafts: one never responded to eculizumab and the second reportedly experienced TMA after a reduction in eculizumab dosage leading to ESRD.[279] In addition, we identified 56 patients, including five children (5,10, 13, 15 and 17 years old), from literature who had been treated with rescue therapy (**Supplementary Table 9.2**). Patients with a known genetic mutation more often had experienced a recurrence in a previous allograft (**Supplementary Table 9.3**). In the majority of the patients (68%) the onset of recurrence was within three months after transplantation. The interval between the

onset of aHUS and initiation of eculizumab varied between 0 days and 279 days. Eculizumab was started within seven days after onset of recurrence in only 22 (39%) patients. Of note, late initiation of eculizumab is associated with less recovery of kidney function.[295] Graft function was maintained in 47 (84%) patients. The remaining patients experienced graft loss despite rescue therapy (n=9;16%), in three patients caused by aHUS of which one had received insufficient eculizumab treatment [186,300,307]and six patients lost their graft due to non-TMA related causes (rejection, infectious complications, acute tubular necrosis.[186,300,302,308-310] Graft function after aHUS recurrence was reported in 38 patients: median (range) serum creatinine was 137 μmol/L (48-486) with a median (range) follow up of 14 months (2- 82) after onset of aHUS. Serum creatinine values exceeded 200 μmol/L in five patients.[186,311-314] In all of them eculizumab therapy had been suboptimal (late initiation of therapy or too early discontinuation) and/or renal function had been compromised before the onset of aHUS. We identified 10 patients[305,315-324], with at least four patients at high risk of recurrence, [305,317,318,323] in whom eculizumab was initiated within seven days after onset of aHUS. Early treatment resulted in full recovery of renal function: median (range) serum creatinine was 113 μmol/L (53-159) before aHUS recurrence and 116 μmol/L (53-180) after treatment of recurrence.

These data seemingly contrast with those reported by Legendre et al. These authors suggested that the recovery of kidney function in transplant patients treated with eculizumab was limited.[306] In a pooled post hoc analysis, which included 26 kidney transplant patients and 74 patients with a native kidney, recovery of kidney function was less pronounced in kidney transplant patients compared to patients with a native kidney: respectively a mean eGFR after 26 weeks of 37 ml/min/1.73m^2 (SD 36), mean change from baseline 11 ml/min/1.73m^2 (SD 20) versus 61 ml/min/1.73m^2 (SD 41) and 38 ml/min/1.73m^2 (SD 36) (P=0.0092). These differences may partly be explained by the type of renal injury which differed between the groups: patients with aHUS in the native kidney had experienced a more rapid decrease of eGFR in the time interval compared to patients with post transplant aHUS which showed a more gradual renal function deterioration. In addition, the time from aHUS diagnosis to introduction of eculizumab was in both patients groups much longer than currently accepted: in transplant patients median 1.25 months (range 0.03-36.7 months) versus 0.69 months (0.03-47.4 months) in patients with native kidneys. The above data suggest that rescue therapy with eculizumab in adult patients may result in acceptable recovery of renal function, however early initiation of therapy is of paramount importance.

Discontinuing rescue therapy

In the above mentioned 56 patients eculizumab was discontinued in sixteen adult patients (29%) (**Supplementary Table 9.2**). A new recurrence occurred in 11 of 16 patients (63%).[294,300,302,305,307,309,310,314,319,325] The recurrence rate in this group is higher compared to the recurrence rate reported in patients who stopped eculizumab prophylaxis after transplantation (2/8 patients; 25%). Of note, in all patients (n=4)with mutation in the gene encoding *CFH* discontinuation of eculizumab led to a recurrence (**Supplementary Table 9.3**).[300,302,305,307] In nine of 11 patients eculizumab was restarted. In six patients renal function improved, however not to baseline values. [294,300,305,314,319,325] Graft failure occurred in the other three, but was not attributed to aHUS by the authors[300,302,310] In contrast, a remarkably lower recurrence rate was described by Macia et al. in 2017.[279] In their study 16 patients from the pivotal trials, who had been treated with eculizumab for aHUS recurrence after transplantation, discontinued therapy. During a median follow up of 24 weeks one patient (without identified genetic variant) experienced aHUS recurrence for which eculizumab was restarted resulting in improvement of kidney function.[306] The high recurrence rate calculated from the case reports may be the consequence of publication bias. Otherwise, Macia et al. may have included several patients with de novo TMA after transplantation, not caused by aHUS, but by transplantation related factors, explaining the low recurrence rate after discontinuation of eculizumab. Future studies are necessary to determine recurrence risk after discontinuation of rescue therapy and the consequences for renal outcome.

Reduction of eculizumab dosage after kidney transplantation

No systematic data are available on the safety of tapering eculizumab therapy after transplantation to reach trough levels of 50 to 100 µg/ml. However, patients have been described who were successfully treated with eculizumab at extended intervals.[152,321,326,327]In contrast, several patients developed aHUS activity after interval prolongation.[295,328] Furthermore, patients have been reported with TMA while on standard maintenance therapy.[265,296-298] In two of the latter cases eculizumab trough levels were above target range and/or complement was completely blocked.[297,298] Therefore, it has been suggested that higher eculizumab levels are necessary in patients after kidney transplantation when exposed to strong triggers of complement activation.[297] Alternatively, other causes of TMA in kidney transplant patients, such as infections, antiphospholipid antibodies, antibody mediated rejection or immunosuppressive drugs, must be considered in these situations.

Monitoring

After therapy withdrawal strict monitoring is essential, therefore we would recommend to see the patient regularly in the outpatient clinic under supervision of an physician with expertise in aHUS (**Table 9.4**). Since signs indicative of a subclinical or early phase of TMA can be subtle, and aHUS is a rare disease, it can be difficult to recognize and adjust treatment appropriately. Especially in kidney transplant recipient, aHUS can present as a smoldering disease. A allograft biopsy may discloses only subtle changes, mostly limited to swelling of vascular endothelial cells in capillaries and small arterioles.[293]

Time for a paradigm shift in the treatment of aHUS with eculizumab

Based on literature we have developed a treatment protocol of restrictive eculizumab therapy (**Figure 9.1**). Our protocol is founded on the principle that eculizumab can be withdrawn in patients with aHUS in remission after a first episode in native kidneys, routine eculizumab prophylaxis is not needed in adult patients before kidney transplantation, and that reducing the eculizumab dose is often possible in patients who need long term therapy. Of note, decisions concerning individualization are based on age, disease history, co-morbidities, renal function, and patient and physicians preferences. Many restrictive treatment strategies can be considered (**Figure 9.2**). Obviously, the best strategy is currently unknown. Prospective studies are needed to allow comparisons and to compose evidence based recommendations.

In the Netherlands national guideline was drafted based on our restrictive treatment protocol. The guideline was composed by the Dutch aHUS working group, comprising a nephrologist and pediatric nephrologist from every academic hospital in the Netherlands. The guideline was approved by the professional societies and implemented in January 2016. In the Netherlands several criteria must be met before orphan drugs can be (re)imbursement by the National Healthcare Institute: the presence of an indication committee, the definition of clear start and stop criteria, monitoring of this process and performing a cost effectiveness analysis. In accordance with this policy start of eculizumab therapy in the Netherlands must be approved by the national aHUS working group (with four members being available on a daily basis) and treatment should follow the new guideline. Furthermore, a national prospective, observational study (abbreviated as CUREiHUS, NTR5988) was designed to monitor the guideline and eculizumab therapy. The study will be closed in august 2020 Analysis of our study data will provide evidence to develop optimized treatment protocols.

Because the optimal treatment strategy in aHUS is currently under investigation and our treatment scheme was designed as a study protocol we recommend that all aHUS patients who are withdrawn of eculizumab will be included in a registry or national study to allow comparison and to aid future research. Furthermore, due to the rarity of aHUS and because treatment can be complicated, we believe monitoring of eculizumab therapy requires consultation with a aHUS referral center and access to a specialized laboratory capable of performing eculizumab and complement assays.

Table 9.4 Monitoring disease activity in aHUS patients

Characteristics aHUS	Regular workup during eculizumab therapy[a]	Regular workup after therapy withdrawal	Recurrence aHUS[b,c]
1. (acute) Kidney injury	Serum creatinine	Serum creatinine	Serum creatinine greater than upper limit of normal per age or increase of >15% compared to baseline
	Proteinuria (protein – creatinine ratio)	Proteinuria(protein – creatinine ratio) Dipstick analyses twice per week at home	Increase of >25% in proteinuria
	Bloodpressure (aim for P50)	Bloodpressure measurement twice per week at home	NA
2. Thrombocytopenia	Platelets	Platelets	Platelet count < 150,000 x 10^3 µl
3. Mechanical hemolytic anemia			*Mechanical hemolysis is defined by the presence of at least 2 or more of the following criteria:*
	Hemoglobin	Hemoglobin	Below lowest limit of normal per age
	LDH	LDH	Greater than upper limit of normal
	Haptoglobin	Haptoglobin	Undetectable
	Schizocytes	Schizocytes	Appearance of schizocytes

Table 9.4 After therapy withdrawal strict monitoring is essential. Regular workup after at least 1,2, 3, 6, 9 and 12 months is required. We would advise to monitor blood pressure at home. We aim for blood pressures around P50 for height and age (children) or <130/80 mmHg (adults). Urine dipstick analysis at home could be considered, especially in children. Moreover, comprehensible instructions to the patient (and caregivers) when and how to contact their treating physician are essential. In case of signs indicating aHUS recurrence such as high blood pressure, petechiae, fatigue, oliguria, jaundice, or a possible triggering event like infection, the patient has to seek contact immediately. Of note, recurrent aHUS after kidney transplantation can present as a smouldering disease, with a slow increase in serum creatinine without overt systemic hemolysis. A allograft biopsy may disclose only subtle changes, mostly limited to swelling of vascular endothelial cells in capillaries and small arterioles.[293]

[a] Consider to monitor liver enzymes in light of potential hepatotoxicity, especially in patients with pre-existing liver disease. [b] Recurrece of aHUS is defined by the occurrence of all 3 characteristics of aHUS; acute kidney injury, thrombocytopenia, and mechanical hemolytic anemia. [c] a kidney biopsy to detect (smoldering) TMA can be of additional value. LDH; lactate dehydrogenase NA: not applicable, P50; median percentile for height and age, TMA;thrombotic microangiopathy

Figure 9.1 Treatment algorithm

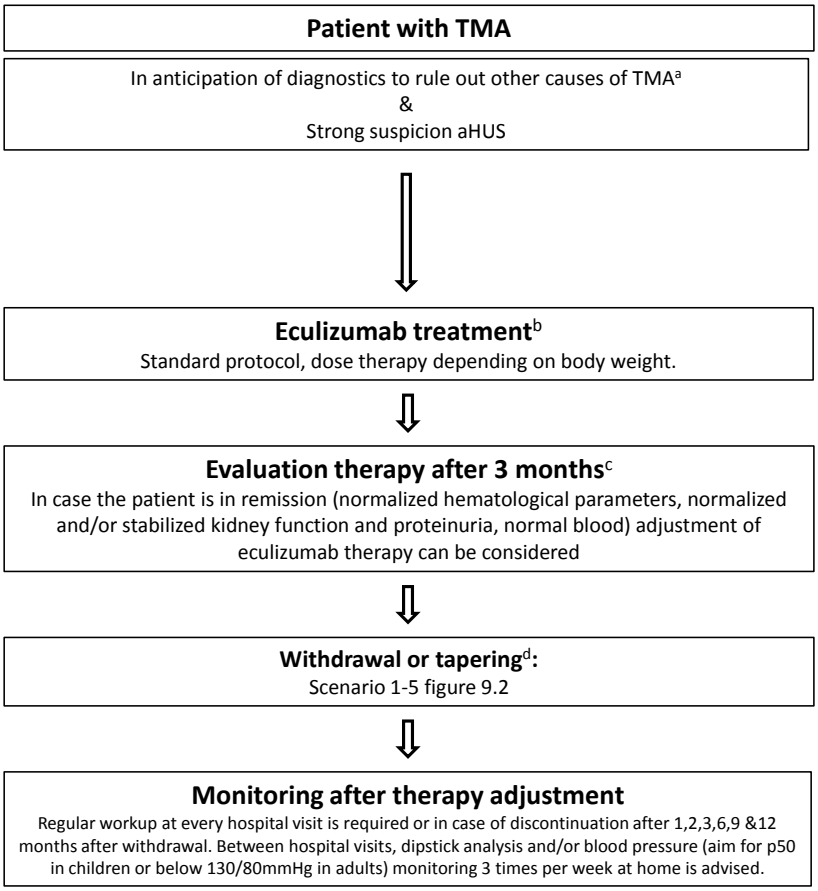

Figure 9.1 After adequate exclusion of other causes of TMA such as TTP, STEC-HUS or secondary TMA and in patients with strong suspicion of aHUS, eculizumab treatment should be started within 24 hours after presentation. When the patient is stable and in remission, withdrawal or tapering can be considered, depending on patient characteristics (see ***Figure 9.2****). After therapy adjustment, strict monitoring is essential. NB in case of antibodies against complement factor H a different treatment protocol has to be initiated as described by Loirat et al.[57]*

[a] For extensive overview of practical diagnostics approach for TMA see Fakhouri et al. 2017 Lancet.[5] [b] Treatment should preferably be started within 24 hours after presentation. In adults with first episode of aHUS in native kidney, treatment with plasma exchange for 4 days (high volume PE with 1.5 plasma volume) is advised to allow diagnosis of secondary causes of aHUS. Adolescents may be considered as adults. [277] After exclusion of secondary causes of aHUS and if the patient does not show a favorable response after four days of PE, treatment should be switched to eculizumab. Starting treatment with eculizumab within seven days after presentation in PE resistant patients was effective in the clinical trials.[189] In case the patient is PE sensitive, PE should be tapered and discontinued in the course of one month.[156,262] [c] Improvement of platelets and LDH is expected within 2-4 weeks. If no response, consider alternative diagnosis or inefficacy of eculizumab (C5 polymorphism p.Arg885His).[254] [d] See figure 2 for the different scenario's to withdrawn or taper eculizumab, depending on patient characteristics.

Other studies of eculizumab discontinuation

To our knowledge there are two other, registered, studies which evaluate safety and efficacy of eculizumab discontinuation. The first is a prospective study conducted in France (STOPECU, NCT02574403). The study protocol is well described, with criteria when and how to withdraw eculizumab. According to the protocol, eculizumab will be discontinued after six months of therapy. The primary end-point is the incidence of aHUS recurrence in follow-up period of two years. The study will end inclusion in November 2019. The second study is conducted by Alexion Pharmaceuticals (EVIDENCE studie, NCT02614898) with a planned end date of April 2020. This study is observational, without defined protocol, and will document TMA events and outcome of all included patients.

Since aHUS is a rare disease, and disease course is quite variable, we feel that a prospective study with inclusion of all treated patients and used of defined treatment algorithms is the best option to gain more insight in the pros and cons of different treatment options. We realize that withdrawal of eculizumab is not without risks. Still, lifelong therapy will expose many patients unnecessary to a drug which can cause side effects. Strict monitoring of the patients and rapid re-initiation of eculizumab therapy should limit risks. Obviously, the enormous costs of eculizumab cannot be disregarded. For a societal perspective, the health care budget is not infinite. Therefore, we must aim to develop cost-effective treatment strategies. The data provided by the CUREiHUS and STOPECU studies should help to develop cost-effective scenario's which may vary per country depending on the local willingness to pay per quality adjusted life year. We have calculated cost-effectiveness of prophylactic eculizumab therapy after kidney transplantation and showed that the costs exceeded the willingness to pay threshold in the Netherlands.[268]

Conclusions

Eculizumab therapy has changed the lives of patients with aHUS. The optimal treatment strategy is unknown. There is no evidence to support the need for lifelong therapy and untargeted treatment. Limiting the initial treatment period to three months in incident carefully evaluated patients with native kidney aHUS and withholding prophylactic therapy in patients with aHUS at transplantation is the first step toward restrictive use of eculizumab. Future studies, preferably at a multinational level, should evaluate the best strategies to prevent, predict and treat relapsing disease. This includes targeted therapy aiming at complete and incomplete complement blockade, and development of biomarkers that predict preclinical TMA activity.

Figure 9.2 Scenario's for the treatment of aHUS patients with eculizumab

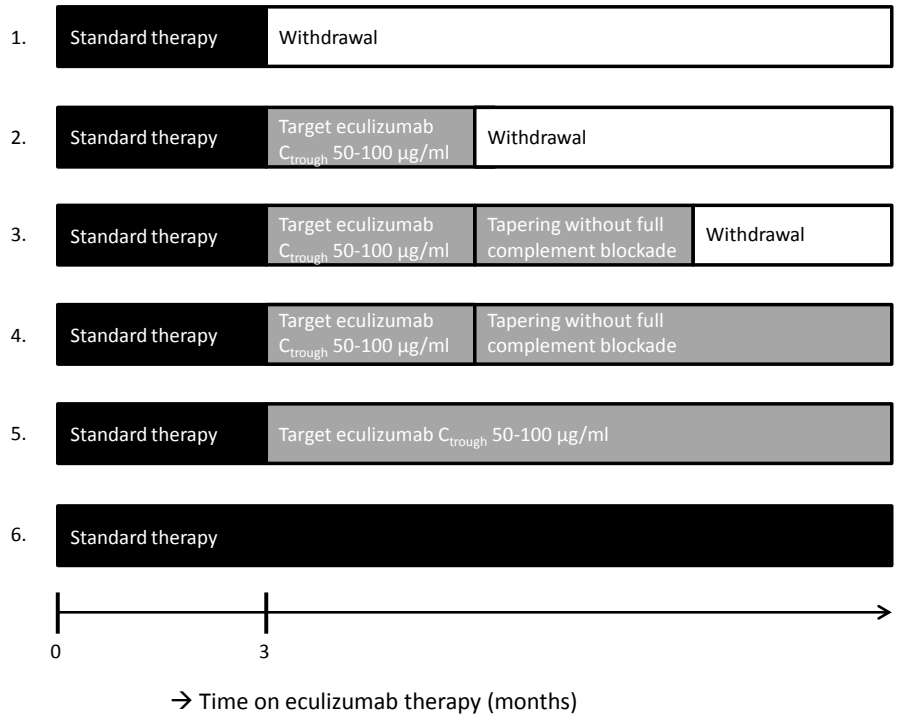

Figure 9.2 The current standard regime is reflected by scenario 6, lifelong therapy with standard dose eculizumab at biweekly intervals.

Scenario's 1-5 illustrate options of restrictive eculizumab therapy. According scenario 1 eculizumab is given at standard dose, at biweekly intervals, with withdrawal after 3 months. According to scenario 2 eculizumab dose will be adapted to target trough levels of 50-100 µg/ml with complete blockade of the complement system. After an observation period eculizumab therapy will be withdrawn. In scenario 3, drug withdrawal is preceded by a period of eculizumab therapy at reduced dose and incomplete complement blockade (CH50 < 30%). For logistical reasons dose reduction will be done by extending the dose interval. In scenario 4 and 5, treatment with eculizumab will continue for undefined period ("lifelong", waiting for more data), either aiming at incomplete (scenario 4) or complete complement blockade (scenario 5). Of note, the observations periods are not strictly defined. The choice for a scenario as well as the length of the observation period will be dependent on patient characteristics, disease history, renal function and patient or physician preferences. In patients with uncontrolled blood pressure, active (viral or bacterial) infection, reduced eGFR with evidence of continuous improvement (i.e. the nadir of serum creatinine has not been reached), or evidence of ongoing (extra-renal) TMA activity, eculizumab treatment should be continued until stable remission is reached.

*Typical examples of the patient profiles which may best fit a proposed scenario are presented below. To aid the choice for a certain scenario a score can be calculated based on patient characteristics. The sum of the points will guide the selection of a scenario (**Supplementary table 9.6**).*

Scenario 1. This is the proposed scenario used in adults patients with a first episode of aHUS in the native kidneys, who have adequately responded to treatment, with well controlled blood pressure, stable renal function and no signs of TMA.

Figure 9.2 Continued

Scenario 2. This is the proposed scenario used in adults with a first relapse of aHUS in native kidneys, occurring more than 12 months after treatment withdrawal, who have adequately responded to treatment with recovery of eGFR, well controlled blood pressure, and no signs of TMA. This scenario is also suitable for pediatric patients above 6 years of age and kidney transplant recipients with no pathogenic mutation.

Scenario 3. This is the proposed scenario used in adults with first relapse of aHUS in native kidneys, occurring within 3 – 12 months after treatment withdrawal, who have adequately responded to treatment with recovery of eGFR, well controlled blood pressure, and no signs of TMA. This scenario is also suitable for kidney transplant patients with a aHUS recurrence, successfully treated with eculizumab and pathogenic mutations in other genes than CFH.

Scenario 4. This is the proposed scenario for patients with multiple relapses and kidney transplant recipients with a relapse (and CFH mutation) in which lifelong therapy is necessary.

Scenario 5. This scenario is proposed for adult patients with relapse occurring during treatment with incomplete complement blockade, or with early (< 3 months) relapse after eculizumab withdrawal. This scenario is also used in pediatric patients below 6 years of age. They have an increased risk for frequent relapse since they are exposed to various infectious triggers during childhood. Therefore withdrawal of eculizumab is not advised, but tapering of therapy to target serum trough levels could be beneficial to limit potential side effects and prevent overtreatment.

Scenario 6. This scenario is proposed for patients with relapsing disease while receiving eculizumab therapy at doses targeted to levels of 50-100 µg/ml. This scenario may also apply to patients with ESRD due to aHUS, with a history of graft failure due to disease recurrence, CFH mutations, or other high risk factors. In these patients any risk of recurrence should be avoided.

*** These scenario's illustrate the treatment with eculizumab, as induction therapy for new-onset aHUS, either as first episode in incident patients or as relapse in prevalent patients. Prophylactic therapy with eculizumab in kidney transplant patients is not illustrated. We do not advise prophylactic therapy with eculizumab in each patients with aHUS. Still, we do not exclude the use of prophylactic therapy, in particular in children, in adult patient with a severe disease history, previous graft loss due to recurrence aHUS, genetic mutations in CFH, comorbidity (prior vascular events, known macrovascular disease), or highly sensitized patients. When prophylaxis is considered we suggest to start with the induction dose 7-10 days before the kidney transplantation with a second dose 0-3 days before transplantation as eculizumab through levels of 50-100 µg/ml (and CH50 <10%) may not yet be reached after the first dose. We would consider continued treatment with eculizumab after successful transplantation according scenario's 4-6.*

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Supplementary material

Supplementary Table 9.1 Prophylactic eculizumab therapy in patients with aHUS receiving a kidney transplantation

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Discontinuation of Ecu
[329]Nieto-Ríos et al.2018	No	38	DD	*CFH*: p. Arg257Cys	No
[279]Macia et al.2017	**No**	**38**	**LD**	**Negative**	**Yes**
[279]Macia et al.2017	Unknown	37	LD	*MCP*	No
[303]Krishnan et al.2017	**No**	**54**	**LURD**	***CFH***	**Yes**
[321]Manani et al.2017	No	45	DD	*THBD*: p.Pro501Leu *CFHR1-3* del with anti *CFH* ab	No
[302]Levi et al. 2017	Unknown	32	DD	*CFI*	No
[302]Levi et al. 2017	Unknown	44	DD	Anti *CFH* ab	No
[302]Levi et al. 2017	Unknown	37	DD	*CFI*: p.Ile357Met	No
[302]Levi et al.2017	Unknown	56	ABOi LD	Anti *CFH* ab	No
[302]Levi et al. 2017	Unknown	48	DD	*CFI*: p.Gly243Val	No
[302]Levi et al. 2017	Unknown	39	DD	*C3*: p.Arg161Trp	No
[302]Levi et al. 2017	Unknown	18	DD	*CFH*: p.Asn767LysfsX7 *CFI*: p.His183Arg	No
[302]Levi et al. 2017	Unknown	40	DD	Unknown	Yes
[302]Levi et al. 2017	Unknown	38	LD	*CFH*: p.Gln81Pro	No
[302]Levi et al. 2017	Unknown	50	DD	*CFH*: p.Tyr1177Cys	No
[299]de Andrade et al. 2017	Unknown	29	DD	*CFH*	No

	Time (in months) on Ecu before discontinuation	Recurrence (time in months after Tx)	Restart of Ecu	Last FU (time in months after Tx)	Serum creatinine (µmol/L) (MDRD) at last FU	Remarks
	NA	No	NA	18	71	
	14	**Yes (26)**	**Yes**	**Unknown**	**Unknown**	[1]
	NA	No	NA	Unknown	Unknown	
	12	**Yes (17)**	**Yes**	**18**	**150**	
	NA	No	NA	7	81	
	NA	No	NA	4.4	124	
	NA	No	NA	11.7	93	
	NA	No	NA	14	157	
	NA	No	NA	14.7	133	
	NA	No	NA	20.2	121	
	NA	No	NA	21.9	97	
	NA	No	NA	27.4	187	
	28.7	No	NA	32.5	77	
	NA	No	NA	35	171	
	NA	No	NA	44.3	178	
	NA	Yes, recurrence while on eculizumab (4)	NA	4	Graft loss	Graft loss (while experiencing endovascular problems) due to aHUS while on maintenance therapy (1200 mg, every 15 days) despite additional dosage of Ecu.

Supplementary Table 9.1 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Discontinuation of Ecu
[299]de Andrade et al. 2017	No	17	DD	*CFHR5*: p.Arg356His	No
[265]Sheerin et al. 2016 ***8 pts***	Unknown	Unknown	Unknown	Unknown	No
[330]Sun et al.2016	No	42	DBD	*CFH*	No
[331]Akchurin et al.2015	No	10	LURD	*CFH*: p.Trp920Arg	No
[301]Kasapoğlu et al.2015	No	18	LRD	CFH: p. Asp268Asn CFH: p.Tyr271stop	Yes
[186]Mallet et al.2015	Yes	18	LRD	Negative	No
[332]Alasfar et al.2015	Yes	35	DD	*CFH*: int20br-CFHR3 *CFH*: CFHR1int4-CFHR4 block 7/2 prime	No
[332]Alasfar et al.2015	No	27	LURD	*CFH*	No
[296]Riddell et al.2015	No	33	LURD	*CFH:* p.Arg1251Gly	No
[298]Parikova et al.2015	No	43	LUR	*CFH*: p.Ser451Stop	No

	Time (in months) on Ecu before discontinuation	Recurrence (time in months after Tx)	Restart of Ecu	Last FU (time in months after Tx)	Serum creatinine (µmol/L) (MDRD) at last FU	Remarks
	NA	No	NA	4	84	
	NA	No	NA	Unknown	Unknown ("good transplant function")	[2]
	NA	No	NA	6	66	
	NA	No	NA	Unknown	Unknown ("stable renal function")	
	1	No	NA	12	67	
	NA	No	NA	29	116	
	NA	No	NA	Unknown	44	
	NA	No	NA	Unknown	97	
	NA	Yes, subclinical TMA in biopsy while on eculizumab (9)	NA	± 24	150	Incomplete complement blockade with 1200 mg Ecu every two weeks. Increase of dosage to 1500 mg every two weeks.
	NA	Yes, TMA in peripheral blood (0.5)	NA	18	150	TMA in peripheral blood on day 14 despite therapeutic through levels of Ecu (293 µg/ml). Treatment scheme was intensified

Supplementary Table 9.1 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Discontinuation of Ecu
[333]Ažukaitis et al.2014	No	6	DD	*C3*: p.Pro1640Leu	No
[334]Ardissino et al.2014	No	19	Type unknown	Anti *CFH* ab	Yes
[297]Ranch et al.2014	No	3	DD	*CFH*: p.Ser1191Trp	No
[335]Román-Ortiz et al.2014	No	9	DD	*CFH*: p.Ser890Ile *CFH*: p.Val1007Leu *CFH/CFHR1* hybrid gene	No
[300]Matar et al. 2014	Yes	51	LRD	Negative	Yes
[300]Matar et al. 2014	No	38	LRD	Negative	Yes
[300]Matar et al. 2014	Yes	40	LURD	Negative	Yes
[300]Matar et al. 2014	Yes	27	LURD	*CFH*: p.Ser1191L *CFH*: p.Val1197Ala	No
[336] Békássy et al.2013	Yes	11	DD	*CFI*: p.Gly261Asp *CFB*: p.Leu433Ser	No
[337]Pelicano et al.2013	No	27	LRD	*CFH*: p.Pro1116Leu	No
[152]Zuber et al.2012	No	17	DD	*CFH-CFHR1* hybrid gene	No
[152]Zuber et al.2012	No	10 months	DD	*C3:* p.Arg161Trp	No

	Time (in months) on Ecu before discontinuation	Recurrence (time in months after Tx)	Restart of Ecu	Last FU (time in months after Tx)	Serum creatinine (µmol/L) (MDRD) at last FU	Remarks
	NA	No	NA	9 days after Tx	Patient deceased	Patient deceased due to iCVA possibly extrarenal manifestation of aHUS, no signs of peripheral blood TMA.
	± 5.5	No	NA	31.4	118	
	NA	Yes, TMA in peripheral blood (2 days)	NA	Unknown	Unknown (*"stable kidney function"*)	Maintenance therapy with Ecu (dosage of 300 mg 11 days before and on day 0 of Tx). TMA in peripheral blood on day 2 after Tx for which treatment scheme was intensified.
	NA	No	NA	36	80	
	6	No	NA	12	88	
	6	No	NA	12	97	
	6	No	-NA	12	115	
	NA	No	NA	12	80	
	NA	No	NA	12	(79 ml/ min/1.73m^2)	
	NA	No	NA	15	76	
	NA	No	NA	14	87	
	NA	No	NA	4.5	44	

Supplementary Table 9.1 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Discontinuation of Ecu
[152]Zuber et al.2012	No	1	DD	*CFH:* Gln1137X	No
[152]Zuber et al.2012	Yes	3	DD	*CFH:* p.Ser1191Leu	No
[152]Zuber et al.2012	Yes	33	DD	*CFH:* p.Tyr1177Cys	No
[338]Xie et al.2012	No	31	LURD	*CFH*: p.Gluc625Stop	No
[295,326]Krid et al.2012	No	7	DD	*CFH-CFHR1* hybrid gene	No
[295,339]Nester et al.2011	Yes	12	LURD	*CFH-CFHR1* hybrid gene	No
[295,327]Weitz et al.2011	No	7	DD	*CFH:* p.Glu1189Stop	No
[295,340]Zimmerhackl et al. 2010	No	9	DD	*CFH:* p.Trp1183Cys	No

*Supplementary table 9.1 Case reports and case series found in literature with prophylactic use of eculizumab in aHUS patients receiving a kidney transplantation. In several case reports serum creatinine (or follow up time) is not described in text but depicted in a figure, the approximate values were used in this table and designated as ± . Eight patients discontinued eculizumab after transplantation and are indicated in **red**. The patients printed in **bold** had a recurrence after discontinuation.*

LRD; living related donor, LD; living donor, DD; deceased donor, DBD; donation after brain death, DCD; donation after cardiac death, LURD; living unrelated donor, ABMR; antibody mediated rejection, CFH; complement factor H, CFI; complement factor I, CFB;complement factor B, C3; complement factor 3, MCP; membrane cofactor protein, THBD; thrombomodulin, CFHR; complement factor H related protein, del; deletion, Tx ; kidney transplantation, MDRD ; modification of diet in renal disease, ABOi; ABO incompatible, anti CFH ab; anti CFH antibodies,

	Time (in months) on Ecu before discontinuation	Recurrence (time in months after Tx)	Restart of Ecu	Last FU (time in months after Tx)	Serum creatinine (µmol/L) (MDRD) at last FU	Remarks
	NA	No	NA	4	58	
	NA	No	NA	1 day after Tx	Graft loss	Graft loss due to arterial thrombosis, transplant nephrectomy at day 3.
	NA	No	NA	1.5	176	6 weeks after Tx mixed rejection.
	NA	No	NA	12	78	
	NA	No	NA	15	48	
	NA	No	NA	16	70	
	NA	No	NA	23	44	
	NA	No	NA	39	46	

Ecu;eculizumab, NA; not applicable, Bx;kidney biopsy, TMA ; thrombotic microangiopathy, PE ; plasma exchange, GL ;graft loss, ATN ; acute tubular necrosis, GI ; gastro-intestinal, IVIG ; intravenous immunoglobulins, Scr; serum creatinine, HD ; hemodialysis, iCVA ; ischemic cerebrovasculair accident, FU ; follow-up.

[1] after six months eculizumab was discontinued again, resulting in a recurrence 4 months later, possibly triggered by a urine tract infection. Eculizumab was restarted as lifelong therapy. The renal outcome is unknown.

[2] one patient with a CFH mutation showed deterioration of renal function, incomplete complement blockade and low-grade TMA on renal biopsy during treatment with eculizumab (1200 mg every two weeks). Eculizumab treatment was intensified to 1500 mg every two weeks. Trough levels of eculizumab are unknown. No details on the post-transplantation period of the other patients were reported.

Supplementary Table 9.2 Efficacy of eculizumab rescue therapy in patients with aHUS after kidney transplantation

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[341]Devresse et al. 2018	No	23	LRD	*CFI:* p.Pro50Ala *MCP CFHR1-3* del	40 days	± 4 months
[312]Zwang et al.2018 *	Unknown	39	DD	*CFI:* p. Ile398Leu *CFHR1-3* del	7 months	± 15 days
[342] Vondrak et al. 2018	No	13	DD	*CFH/CFHR1 / CFHR3* hybrid gene	2 months	5 days
[299]de Andrade et al.2017	No	30	LD	Negative	4 days	Max 2 days
[299]de Andrade et al. 2017	Unknown	20	DD	*CFH*: p.Asn1050Tyr *CFH*: del in exon 23 *CFRH1-3* del	30 days	Max 2 days
[299]de Andrade et al.2017	No	36	DD	Negative	2 months	Max 2 days
[299]de Andrade et al.2017	No	32	DD	*CFI*: p.Ile416Leu *CFRH1-3* del	1 month and 20 days	Max 2 days
[299]de Andrade et al.2017	No	44	DD	Unknown	1 day	Max 2 days

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	107	±486	177; ± 17	Yes	No	NA	
	300	1043	486; 10	No	No	NA	Poor renal function before aHUS recurrence due to persistent borderline rejection.
	Unknown	322	109; 84	No	No	NA	
	Unknown	[1]	141; 42	No	No	NA	
	Unknown	[1]	80; 42	No	No	NA	
	Unknown	[1]	185; 7	No	No	NA	
	Unknown	[1]	194; 9	No	No	NA	
	Unknown	[1]	141; 6	No	No	NA	Patient died with functioning graft 6 months after transplantation due to aspergillus infection.

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[302]Levi et al. 2017	Yes	26	DD	*CFH* *CFH/CFHR1* hybrid gene	Within 25 days	Unknown (Ecu started d25 after Tx)
[302]Levi et al. 2017	Yes	23	DD	*CFH*: p.Ser1191Leu *CFH*: p.Val1197Ala	Within 6 days	Unknown (Ecu started d6 after Tx)
[294]Munch et al.2017	Yes	58	DD	*CFHR1-3* del Anti *CFH* ab	21 days	Unknown (after 6 sessions of PT)
[314]Shochet et al.2017 *	No	45	DD (pancreas-kidney)	Unknown	7 days	17 days
[343]Asif et al.2017	No	37	LRD	Negative	8 days	Unknown

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	Unknown	Unknown	Graft loss; 55	Yes	Yes (56)	Unknown	Graft loss due to chronic graft dysfunction and sepsis, resulting in restart of HD. After subsequent withdrawal of Ecu aHUS recurrence.
	Unknown	Unknown	NA	No	No	NA	
	Unknown	± 270 (incomplete respons to PT)	±150; 3.8	Yes	Yes (4.7)	Yes	Scr after 2nd recurrence ± 140µmol/L 18 months after Tx.
	315	HD	267; ±2.3	Yes	Yes (±2.8)	Yes	Renal function compromised due to infectious complications before and after transplantation. Scr after 2nd recurrence ± 182µmol/L 6 months after Tx.
	80	HD	150; 6	No	NA	NA	-

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[305] Duineveld et al. 2017	Yes	41	LURD	CFH: p.Trp678Cys	64 days	1 day
[344]Dedhia et al.2017 *	No	28	LURD	*CFHR1-3 del*	± 40 days	0 days
[279,306]Legendre et al. Macia et al. 2017 25 pts	Unknown	Median 41.5 (17-69)	Unknown	13 (50%) with complement mutation or autoantibody	Unknown	1.25 months (range 0.03-36.7 months)
[324]Yamamoto et al. 2017	No	35	LD	Negative	15 days	22 days
[265]Sheerin et al. 2016	Unknown	Unknown	Unknown	Unknown	Early post-operative period	Unknown

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	94	145	110; 5	Yes	Yes (±5.2)	Yes	Possible recurrence after Tx with peripheral blood hemolysis without TMA in Bx or alternative cause TMA. Scr after 2nd relapse 143µmol/L 14 months after Tx. [2]
	221	486	132; 30	No	No	NA	Poor renal function before aHUS recurrence due to rejection and bacteremia.
	Unknown	Median eGFR 22.2 ml/min/1.73m² (range 10-72.3)	Mean eGFR 44 ml/min/1.73m² (SD 27); 18 months after start of eculizumab	Yes, 16 pt	Yes, in 3 patients (including 1 patient during a dose reduction)	Yes, restart in 1 patient	In patient who restarted Ecu renal function improved. Graft loss occurred in the other two patients due to aHUS. A forth patient died, not due to aHUS.
	± 136	Unknown	116; 36	Unknown	No	NA	Treated for ABMR before aHUS recurrence.
	Unknown	Unknown	Unknown (*"good transplant function"*)	Unknown	No	NA	Further details are missing.

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[265]Sheerin et al. 2016	Unknown	Unknown	Unknown	C3	29 months	Unknown
[265]Sheerin et al. 2016	Unknown	Unknown	Unknown	CFH	Early after Tx	Unknown
[265]Sheerin et al. 2016	Unknown	Unknown	Unknown	Unknown	Early after Tx	Unknown
[345]Ikeda et al.2016	No	27	LRD	Not investigated	1 day	2 days
[345]Ikeda et al.2016	No	66	LURD	Not investigated	1 day	5 days
[346]Okumi et al.2016	Nov	30	LRD	*CFH*: p.Arg1215Gln	3 weeks	± 8 months
[317]Salameh et al.2016	No	48	DD	CFH: p.Arg1215Gln	3 months	Unknown (after 7 session of PT)
[320]Juega-Marino et al.2016	No	46	DD (pancreas-kidney)	Negative	7 months	>10 days
[347] Fan et al. 2015	No	30	LRD	*CFH:* p.Arg1251Glu	21 days	Unknown
[313]Iqbal et al.2015	No	49	DD	*C3*: p.Asp1093Asn	29 months	±2 months
[186]Mallet et al.2015	No	40	DD	Negative	Within 2 days	2 days

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	Unknown	Unknown	Unknown (*"improvement in transplant function"*)	Unknown	No	NA	Further details are missing.
	Unknown	Unknown	Unknown (*"stable transplant function"*)	Unknown	No	NA	Further details are missing.
	Unknown	Unknown	Unknown (*"stable transplant function"*)	Unknown	No	NA	Further details are missing.
	Unknown	± 681	117; 8	Yes	No	NA	-
	Unknown	± 575	120; 12	Yes	No	NA	-
	133	Unknown	158; 60	No	No	NA	-
	133	>353	133; 9	No	NA	NA	-
	110	438	180; >10	Yes	No	NA	-
	Unknown	Unknown	199; specific time after Tx unknown	No	No	NA	-
	100	396	(±20 ml/min/ 1.73m2); ± 39	No	No	NA	-
	Unknown	620 (PT resistant)	Graft loss; 12	NA	NA	NA	Graft loss 1 year after Tx due to antibody mediated rejection, ongoing aHUS remission.

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[186]Mallet et al.2015	No	23	LRD	Unknown	10 months	4 months
[186]Mallet et al.2015	No	36	DD	Negative	5 years	9.1 months
[319]Garlo et al.2015	No	30	LURD	Unknown	2 years	Unknown (after 9 sessies of PT)
[318]Broeders et al. 2014	Yes	41	DCD	CFH: p.His860His	6 months	1 week

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	Unknown	450 (PT dependent)	328; specific time after Tx unknown	No	NA	NA	
	80-90	130 (PT resistant)	469; specific time after Tx unknown	NA	NA	NA	No renal or hematological respons to ecu. Visual and GI symptoms attributed to aHUS. Pt died 114 days after start of Ecu.
	71	618	71; specific time after Tx unknown (two week after start of ecu)	Yes	Yes	Yes	Patient presented 26 weeks pregnant with pre-eclampsia for which a caesarian section was performed. Two days later she developed aHUS recurrence. Scr after 2nd recurrence ± 106 µmol/L ± 3 years after Tx
	141	168 (respons to PT unknown)	141; 18	No	NA	NA	13 days after Tx also episode of TMA diagnosed as TMA due to ABMR, treated with steroids, IVIG and 3 sessions of PT leading to full recovery of kidney function

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[300]Matar et al.2014	Yes	38	LRD	*CFH*:p. Leu1189Phe	6 years	Unknown
[300]Matar et al.2014	Yes	33	LURD	Negative	3 months	Unknown
[300]Matar et al.2014	Yes	57	DD	*CFH*: p. Ile059Thre *CFH*: p.Gln1143Glu	3 months	Unknown
[348]Commereuc et al.2013	No	22	DD (combined lung-kidney)	Negative	3 days	12 days
[308]Sinibaldi et al.2013	Yes	19	DD	*THBD*: p.Pro501Leu	11 weeks	5 days
[322]Reuter et al.2013	No	24	LRD	MCP: p.Tyr54Cys	9 days	± 14 days

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	Unknown	Unknown	Unknown	Yes	Yes (84)	Yes	After 2nd recurrence life-long Ecu therapy. Graft is still functioning.
	Unknown	Unknown	Unknown	Yes	Yes (17)	Yes	After restart of Ecu respons was seen. At 23 months after Tx graft loss due to contrast nephropathy and ATN.
	Unknown	Unknown	Graft loss; 6	NA	NA	NA	Graft loss due to aHUS recurrence , no respons to Ecu.
	72	HD	Unknown *("hemodialysis could be stopped")*	Unknown	No	NA	Unknown recovery of renal function, yet hemodialysis could be stopped.
	± 88	± 177	± 110-120; 4.5	No	NA	NA	Graft loss 7 months after Tx due to cellular rejection unresponsive to steroids.
	159	± 290	159; 44	No	NA	NA	-

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[310]Alachkar et al.2012	Yes	32	LD	Negative	3.5 months	Unknown (after 8 sessions of PT)
[152] Zuber et al. 2012	Yes	Unknown	Unknown	*CFHR1-3* del Anti *CFH* ab	5 years	3 months
[152] Gueutin by Zuber et al. 2012	Yes	Unknown	Unknown	*CFI:* p. Gly101Arg	± 36 months	9 weeks
[152] Zuber et al. 2012	Yes	Unknown	Unknown	*CFH*: p.Ser1191Leu *CFH*: p.Val1197Ala	3 days	3 days

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	Unknown (*"excellent function"*)	HD	159; 8	Yes	Yes (13)	Yes	Scr after 2nd recurrence 282 µmol/L. Graft loss, 2 years after Tx, due to complicated endovascular procedure with ATN, no signs of TMA in peripheral blood.
	Unknown	89 (incomplete respons PT)	80; ± 72	No	Yes, relapse while on eculizumab therapy (± 86)	NA	During Ecu therapy fresh TMA lesions on renal biopsy, possible also due to chronic active ABMR. CH50 below detection threshold. Unknown renal function after 2nd relapse.
	± 156	190 (PT resistant)	Unknown	No	Yes, relapse while on eculizumab therapy		During Ecu therapy TMA in peripheral blood after interval prolongation. Scr after 2nd relapse 156 µmol/L ± 40,5months after Tx.
	Unknown	627 (PT resistant)	65; 17	No	NA	NA	-

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[152] Zuber et al. 2012	Yes	Unknown	Unknown	*CFH-CRFH1* hybrid gene	3 days	1 month
[349]Zlamy et al.2012	No	10	DD	*CFH*	6 days	4 days
[315]Hodgkins et al.2012	No	5	DD	Negative	4 years and 6 months	2 days
[152,350]Duran et al. 2012	No	32	DCD	*CFH:* p.Glu1172Stop	2.5 months	1 month
[152,325]Heyne et al. 2011	Yes	Unknown	Unknown	Not investigated	8 days	1 day
[351]Wilson et al.2011	No	46	DD (pancreas-kidney)	Negative	41 days	3 weeks
[316]Chandran et al.2011	No	34	DD (pancreas-kidney)	Unknown	10 days	4 days
[152,323]Al-Akash et al. 2010	Yes	15	DD	C3: p.Arg570Trp	9 weeks	3 weeks

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	Unknown	237 (incomplete respons PT)	Unknown	No	Yes, relapse while on eculizumab therapy (± 4)	NA	During eculizumab therapy fresh TMA lesions on renal biopsy, possible partly due to high tacrolimus trough levels. CH50 below detection threshold. Serum creatinine after 2nd relapse 204 µmol/L ± 15 months after Tx.
	Unknown	GFR 45 ml/min/m²	48; 27	No	NA	NA	-
	44-53	751	± 53; 84	No	NA	NA	-
	159	Hemo-dialysis	168; ± 20	No	NA	NA	-
	Unknown	176	114; specific time after Tx unknown	Yes	Yes (±11)	Yes	Scr after 2nd relapse 123 µmol/L; ± 25 months after Tx
	Unknown	Unknown	(40 ml/min/1.73m²); 24	Yes	No	NA	-
	88	HD	63; 4	Yes	No	No	-
	115	202 (incomplete respons PT)	115; ±42	No	NA	NA	-

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[152,309]Larrea et al. 2010	No	22	DCD	Negative	12 days	9 days
[295,352]Châtelet et al.2010	Yes	>36	DD	*C3:* p.Arg570Gln	4 years	5 weeks
[295,353]Davin et al.2010	Yes	17	Unknown	*CFH*: p.Ser1191Leu	4 months	6 months
[152]Ardissino et al. 2010	No	Unknown	Unknown	*CFH*	2 months	2 days

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	97	415 (PT resistant)	70; specific time after Tx unknown	Yes	Yes (11)	Yes	Discontinuation after 1 dosage of ecu; after 2nd relapse Scr of 175 µmol/L. After 4 months Ecu was stopped because of infection; GL loss due to ABMR.
	± 160	320 (PT dependent)	Unknown	No	Yes, 2 x mild relapse while on eculizumab	NA	First aHUS recurrence after current Tx was successfully treated with PE. Second recurrence is described here. During Ecu therapy TMA in peripheral blood after interval prolongation. CH50 below detection threshold. Serum creatinine after relapse 230 µmol/L 68 months after Tx.
	130	131 (PT dependent)	120; ± 49	No	NA	NA	-
	Unknown	442 (incomplete respons PT)	48; ± 27	No	NA	NA	-

Supplementary Table 9.2 Continued

Reference	aHUS in previous transplant	Recipients age (yrs) at Tx	Donor source	Reported genetic variant	Time from Tx to recurrence	Time from recurrence to start of Ecu
[152,311]Legault et al. 2009	No	34	LRD	Unknown	1 month	± 8 months
[152,307]Nürnberger et al.2009	Yes	37	DD	*CFH*: p.Tyr475Ser	6 weeks	5 days

*Supplementary table 9.2 Case reports and case series found in literature with the use of eculizumab in case of aHUS recurrence after kidney transplantation. In several case reports serum creatinine (or follow up time) is not described in text but depicted in a figure, the approximate values were used in this table and designated as ± . For the ten patients indicated in **red** a serum creatinine before the onset of aHUS and after the recurrence was available and these patients had started eculizumab therapy within 28 days after onset of aHUS. Three patients (indicated with *) were excluded as these patients had a poor renal function before the onset of aHUS due of infectious complications or a rejection. LRD; living related donor, LD; living donor, DD; deceased donor, DBD; donation after brain death, DCD; donation after cardiac death, LURD; living unrelated donor, ABMR; antibody mediated rejection, CFH; complement factor H, CFI; complement factor I, CFB;complement factor B, C3; complement factor 3, MCP; membrane cofactor protein, THBD; thrombomodulin, CFHR; complement factor H related protein, del; deletion, Tx ; kidney transplantation,*

	SCr (µmol/L) (MDRD) before aHUS	Scr (µmol/L) (MDRD) at start of ecu	Nadir Scr (µmol/L) after 1st recurrence; follow-up (in months) after Tx	Discontinuation of Ecu	Second recurrence (time in months after Tx)	Restart ecu	Remarks
	106	321 (incomplete respons PT)	238; ± 15	No	NA	NA	-
	±107	132 (PT resistant)	97; specific time after Tx unknown	Yes	Yes (21)	No	Discontinuation after 1 dosage of Ecu; second recurrence, not treated with eculizumab, led to graft loss 22 months after Tx.

MDRD ; modification of diet in renal disease, ABOi; ABO incompatible, anti CFH ab; anti CFH antibodies, Ecu;eculizumab, NA; not applicable, Bx;kidney biopsy, TMA ; thrombotic microangiopathy, PE ; plasma exchange, GL ;graft loss, ATN ; acute tubular necrosis, GI ; gastro-intestinal, IVIG ; intravenous immunoglobulins, Scr; serum creatinine, HD ; hemodialysis, iCVA ; ischemic cerebrovasculair accident, FU ; follow-up.

[1]individual improvement in kidney function is depicted in a figure in article by Andrade et al. The mean serum creatinine for all 5 patients was 362 µmol/L ± 62 µmol/L at start of eculizumab and 168 µmol/L ± 53 µmol/L after 6 months of treatment with eculizumab.

[2] Eculizumab was discontinued 170 days after the second recurrence. A third recurrence was suspected based on slow increase in serum creatinine and confirmed, almost a year after discontinuation, by kidney biopsy. No signs of peripheral blood TMA were seen. Eculizumab was restarted and improvement of kidney function was seen.

Supplementary Table 9.3 Characteristics and outcome of rescue therapy according to genetics in aHUS renal transplantation patients

Mutation	aHUS in previous transplant	Donor source (%)	Median Time from Tx to recurrence in days (range)
CFH mutation (including 3 patients with CFH/CFHR hybrid genes) (n=19) 265,295,299,300,302,305,307,317,318,342,346,347,349,350,353	9 (47%)	10 (53%) DD 4 (21%) LD 5 (26%) unknown	42 (0-2190)
Other mutation[1] (n=13) 265,294,295,299,308,312,313,322,323,328,341,344	6 (46%)	7 (54%) DD 3 (23%) LD 3 (23%) unknown	77 (9-1825)
Negative (n=13) 186,299,300,309,310,315,320,324,343,348,351	2 (15%)	8 (62%) DD 5 (38%) LD	41 (2-1825)
Unknown (n=11) 186,265,299,311,314,316,319,325,345	0 (0%)	3 (27%) DD 5 (45%) LD 3 (27%) unknown	7 (0-730)
P- value[4]	P=0.075	P=0.313	P=0.016

Supplementary table 9.3 Characteristics and outcome of rescue therapy according to genetic mutation. The patients are divided into groups: CFH or CFH/CFHR hybrid gene, other mutation, no mutation or unknown mutational status. LD; living donor, DD; deceased donor, CFH; complement factor H, CFI; complement factor I, CFB;complement factor B, C3; complement factor 3, MCP; membrane cofactor protein, THBD; thrombomodulin, CFHR; complement factor H related protein, del; deletion, Tx ; kidney transplantation, MDRD ; modification of diet in renal disease, ABOi; ABO incompatible, anti CFH ab; anti CFH antibodies, Ecu;eculizumab, Scr; serum creatinine. [1]Other mutation: 4 mutations in the gene coding for CFI, 2 mutations in the gene coding for MCP, 4 mutations in

Start of Ecu ≤ 7 days after recurrence	Time from recurrence to Ecu in days (range)	Nadir median Scr (µmol/L) after 1st recurrence [2] (range)	Discontin-uation of Ecu	Second r ecurrence after discontinuation	Graft loss (due to aHUS)
9 (47%)	5 (1-244)	110 (48-199)	4 (21%)	4 (100%)	3/19 (2) [3]
3 (23%)	21 (0-120)	154 (80-486)	2 (15%)	1 (50%)	1/13 (0)
4 (31%)	10 (2-279)	150 (53-185)	5 (42%)	3 (60%)	4/13 (0)
5 (45%)	5 (1-244)	120 (63-328)	6 (50%)	3 (50%)	1/11 (1)
P=0.889	P=0.628	P=0.156			

the gene coding for C3, 2 CFHR1-3 deletions with anti-CFH antibodies, 1 mutation in the gene coding for THBD and 4 patients with CFHR1-3 deletions without anti CFH antibodies. [2] Excluding the patients who died (n=1), suffered from graft loss (n=9) and the patients without a reported nadir serum creatinine (n=11).[3] Two patients lost their grafts due to aHUS. One patient had not responded to eculizumab treatment and the second patient discontinued eculizumab therapy after one dose, leading to a second recurrence, which was not treated with eculizumab. [265,307]
[4] *Statistical method: Kruskal-Wallis test*

Supplementary Table 9.4 Characteristics and outcome of prophylactic therapy according to genetics in aHUS renal transplantation patients

Mutation	aHUS in previous transplant	Donor source (%)	Discontinuation of Ecu [2]	Recurrence after discontinuation	Graft loss (due to aHUS)
CFH mutation (including 3 patients with *CFH/CFHR hybrid genes*) (n=25) [295-303,326,327,329-332,335,337-340]	4 (16%)	14 (56%) DD 11(44%) LD	2 (8%)	1 (50%)	2 (1)
Other mutation[1] (n=14) [192,279,295,299,302,321,332,333,336]	2 (14%)	11(79%) DD 2 (14%) LD 1 (7%) unknown	1 (7%)	0 (0%)	0[3]
Negative (n=5) [186,279,300]	3 (60%)	0 (0%) DD 5 (100%) LD	4 (80%)	1 (25%)	0
Unknown (n=9) [265,302]	0 (0%)	1(1%) DD 0 (0%) LD 8 (89%) unknown	1(11%)	0 (0%)	0
P- value [4]	P=0.000	P=0.000	P=0.000	P=0.761	P=1.000

Supplementary table 9.4 Characteristics and outcome of prophylactic therapy according to genetic mutation. The patients are divided into groups: CFH or CFH/CFHR hybrid gene, other mutation, no mutation or unknown mutational status.

LD; living donor, DD; deceased donor, CFH; complement factor H, CFI; complement factor I, CFB;complement factor B, C3; complement factor 3, MCP; membrane cofactor protein, THBD; thrombomodulin, CFHR; complement factor H related protein, del; deletion, Tx ; kidney transplantation, MDRD ; modification of diet in renal disease, ABOi; ABO incompatible, anti CFH ab; anti CFH antibodies, Ecu;eculizumab, Scr; serum creatinine.

[1]Other mutation: 5 mutations in the gene coding for CFI, 1 mutation in the gene coding for CFB, 1 mutation in the gene coding for MCP, 3 mutations in the gene coding for C3, 3 patients with anti CFH antibodies, 1 mutation in the gene coding for THBD and 2 other mutations in the genes coding for CFHR.

[2] The amount of patients who discontinued eculizumab (not according to donor source).

[3] One patient died of a cerebrovascular incident, possibly an extrarenal manifestation of aHUS.[333]

[4] Statistical method: Kruskal-Wallis test

Supplementary Table 9.5 Kidney transplantation protocol to minimize endothelial injury in aHUS patients

Treatment	Dosage / targets
Living donor kidney	
Basiliximab	20 mg day 1 and day 4
Tacrolimus	Starting dose 0.03 mg/kg twice daily; target blood levels of 4-5 µg/L first 30 days, thereafter 5-7 µg/L
Prednisone	Starting dose 100 mg/day on days 1-3, thereafter 25 mg/day and tapering to 0.1 mg/kg/day at 3 months after transplantation
Mycophenolate mofetil	Starting dose 1000 mg twice daily; target area under the curve 40-60 mg/mL/hour
Blood pressure	Target <130/80 mmHg
Early introduction of ACE inhibitor	
Early introduction of statins	

Supplementary table 9.5 Transplantation protocol for adults aHUS patients aimed to minimize endothelial injury. This protocol was used previously by Duineveld et al. and Verhave et al.[266,305] Transplantation from a living related donor can be considered if the causative genetic factor is identified in the recipient and is absent in the donor.[233] After transplantation close monitoring is advised: daily for the first two week, twice weekly for the next two to four weeks and weekly thereafter until four months after transplantation, followed by gradual extension of the interval to every six weeks at 12 months and every three months after two years. Home blood pressure measurements are advised.

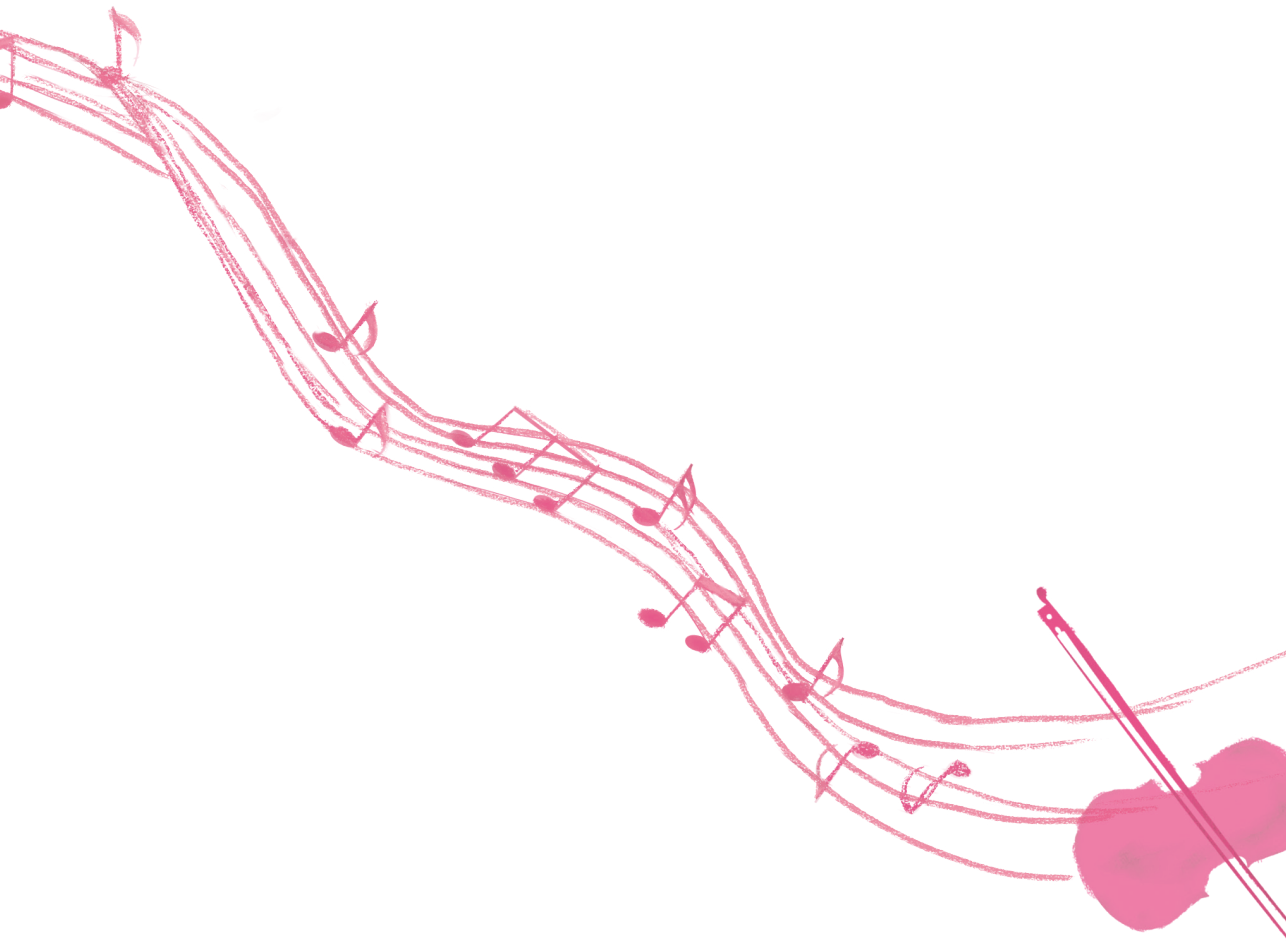
Supplementary Table 9.6 Proposed guidance for the selection of restrictive treatment scenarios based on patient characteristics

Characteristics		Points
Age	≤ 6	5
	6-12 years	1
	> 12 years	0
Native kidney		
First episode		0
Any relapse , never treated with eculizumab		0
First relapse >12 months after eculizumab withdrawal		1
First relapse >3- <12 months after eculizumab withdrawal		2
First relapse <3 months after eculizumab withdrawal		4
Multiple relapses after eculizumab withdrawal		3
Relapse while on eculizumab (with incomplete complement blockade)		4
Relapse while on eculizumab (with complete complement blockade)		14
Kidney transplantation		
First episode of recurrence > 1-3 months after transplantation		2
First episode of recurrence < 1-3 months after transplantation		3
Second relapse > 12 months after eculizumab withdrawal		3
Second relapse < 12 months after eculizumab withdrawal		4
Relapse while on eculizumab (with incomplete complement blockade)		4
Relapse while on eculizumab (with complete complement blockade)		14
Having received prophylactic treatment because of anticipated high risk		8
Genetic analysis		
No mutation		0
Mutation not in complement factor H		1
Mutation in complement factor H		2

Supplementary table 9.6 To assist in the selection of a certain scenariowe have tried to describe patient characteristics and attribute a score; points can be added for age, aHUS in a native kidney or allograft and for the presence of a genetic mutation. The total score (sum of points)provides guidance forthe selection of a scenario, however based on additional clinical characteristics and/or physicians or patients preference a different choice can be made. A total score of 1 points corresponds with scenario 1, a total score of 2 points corresponds with scenario 2, a total score of 3 points corresponds with scenario 3, a total score of 4 points corresponds with scenario 4, a total score of 5-13 points corresponds to scenario 5 and a score >13 points corresponds with scenario 6.

"I'm dancing in the dark with you between my arms.
Barefoot on the grass, listening to our favorite song."

Perfect - Ed Sheeran



Chapter 10

National observational study to monitor the new Dutch guideline concerning treatment of patients with atypical hemolytic uremic syndrome

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Abstract

Introduction

With the introduction of eculizumab, a new era began for patients with atypical hemolytic uremic syndrome (aHUS); a rare but severe form of thrombotic microangiopathy (TMA). Although eculizumab proved very effective, a worldwide debate started regarding optimal treatment strategy. Lifelong treatment with a standard dose (1200mg in adults) biweekly was advised, despite any evidence. However, treatment is not without risks. Moreover, high trough levels were measured in many patients, suggestive that we may need less drug for effective treatment in most patients. Finally, eculizumab is considered one of world's most expensive drugs, leading to both political and ethical discussions regarding reimbursement and affordability of gained life years in patients with aHUS. In the Netherlands, this led to the development of a new Dutch guideline, drafted by the National Working Group aHUS, which proposes a restrictive eculizumab regimen. When patients are stable and in remission, eculizumab therapy is tapered or withdrawn after three months. In a nationwide, observational prospective study, called CUREiHUS, treatment with eculizumab will be monitored and evaluated after four years.

Methods and analysis

In this national prospective, observational, cohort study, all pediatric and adult patients suspected of or previously diagnosed with aHUS ,who start treatment with eculizumab between 2016 and 2020 will be asked for informed consent. Participating centers are instructed to use the national guideline. At hospital visits, two blood tubes will be collected to study complement activation, biomarkers and eculizumab serum levels. Furthermore, every three months all patients will receive two questionnaires to assess quality of life and productivity losses to perform a cost-effectiveness analysis. Separate informed consent is requested for two add-on studies, one regarding pharmacokinetics and pharmacodynamics of eculizumab and the other on patient experience and satisfaction with expensive drugs like eculizumab.

Ethics and dissemination

This study will be conducted according the principles of the declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act. Medical ethical approval was obtained. After completion of the study the results will be submitted for publication in international peer-reviewed scientific journals. Registered in the Dutch Trial Registry with number NTR5988.

Introduction

Atypical hemolytic uremic syndrome (aHUS) is a rare, but severe form of thrombotic microangiopathy (TMA), that is characterized by the triad hemolytic anemia, thrombocytopenia, and acute renal failure. It is considered the consequence of dysregulation of the alternative pathway of the complement system.[5] Mutations in genes encoding complement (regulating) proteins can result in dysregulation of the complement system, eventually leading to TMA.[1,57] Atypical HUS has an estimated prevalence 1-2/1,000,000 cases per year in the Netherlands. The disease affects patients at all ages and is characterized by its recurrent character.[1,57] Until 2011, plasma therapy (PT) was the cornerstone of treatment in aHUS, yet with limited success. In the pre-eculizumab era aHUS was associated with poor outcome with mortality in 5-15% and development of end stage renal disease in 30-50% of the patients at 3-5 year follow up.[1,53,54,58] Moreover, the risk of recurrence in kidney transplant recipients was estimated as high as 80%, resulting in poor graft survival.[58,267]

With the introduction of eculizumab, the field of complement therapeutics experienced a watershed moment.[8,9,281,354] Eculizumab is a chimeric monoclonal antibody directed against complement component C5. It inhibits the cleavage of C5 into C5a and C5b, subsequently blocking formation of the terminal complement complex (TCC). Since 2011, eculizumab forms the cornerstone of treatment in aHUS and has improved morbidity and mortality significantly. Treatment consists of and induction phase of weekly infusions (up to four weeks) followed by a maintenance phase of eculizumab infusions every 14-21 days depending on the body weight of the patient, potentially lifelong.[8] Although eculizumab improves the health-related quality of life, the burden of this lifelong treatment is tremendous. Moreover, eculizumab is considered one of world's most expensive drugs with costs as high as €500,000 per patient per year. The high costs of such orphan drugs have stimulated political and ethical discussions regarding costs of healthcare.[354]

Therefore, the introduction of eculizumab as lifelong treatment for aHUS patients sparked a debate. Roughly, there are four important arguments to take into account. Historically, PT could be tapered and stopped in a significant proportion of patients, therefore questioning the need for lifelong duration of therapy. Since the introduction in 2011, numerous and often confliction opinions have been postulated concerning the proposed treatment scheme.[43,58,162,165,182,192,258,265] Secondly, recent studies and our own data indicate that we may sometimes exceed the recommended trough levels by a tenfold with the current treatment schedules. This offers hope that we may need less drug for effective treatment in most patients.[158,182] Thirdly, treatment is not without risks, with as most prominent risk factor the susceptibility of infections and especially meningococcal disease.[155] Lastly, since eculizumab is a highly expensive drug, the costs of lifelong treatment quickly exceed the willingness to pay thresholds per quality adjusted life year (QALY) thus impeding reimbursement policies and hereby patient benefit in the Netherlands.

To guarantee healthcare access for everyone in the Netherlands, specific arrangements were implemented. The aim was to promote efficient and purposeful usage of orphan drugs, to optimize (costs)effectiveness of orphan drugs like eculizumab. Three criteria have to be met before a drug can be (re)imbursed. The first criteria is the presence of an indication committee which has to give advice to physicians regarding start and withdrawal of treatment. Secondly, clear start and stop criteria have to be defined. Finally, patients have to be monitored in a registry to evaluate efficacy of the drug and to perform a costs effectiveness analysis.[60]

In the Netherlands this led to the development of a new Dutch guideline, drafted by the National aHUS Working Group, which proposes restrictive eculizumab regimen.[4] The national aHUS working group consists of one nephrologists and one pediatric nephrologists of each university center in the Netherlands and meet every three months to discuss all the patients. Patients are divided into different groups and will either receive PT or eculizumab depending on age and their risk profile. After three months therapy is accessed and in case patients are stable and shows no signs of active TMA, therapy is withdrawn (**Figure 10.1**). Only recently, the Ministry of Health, Wellfare and Sport of the Netherlands declined reimbursement of eculizumab as lifelong therapy. This decision derived mainly from the lack of clear insight in costs effectiveness as provided by the pharmaceutical company. Still, the introduction of the guideline offered hope for restrictive and costs-effective therapy. Therefore, the ministry allowed reimbursement of eculizumab, when treatment is started after approval by the committee, treatment is done according to the guideline, and patient data are monitored, with a planned evaluation after four years.

In the nationwide prospective study, called CUREiHUS, all pediatric and adult patients with aHUS treated with eculizumab between 2016 and 2020 will be included. This registry is independent of the pharmaceutical company as also suggested by Karpman et al.[354] In this prospective, observational, cohort study we will monitor eculizumab treatment of patients with aHUS in the Netherlands strictly adhere to the guideline and investigate the efficiency. Furthermore, we will perform a cost-effectiveness analysis.

Method and analysis

Trial design and setting

CUREiHUS is a national prospective, observational, cohort study. All pediatric and adult patients with a (suspected) diagnosis of aHUS and who are receiving treatment with eculizumab, in any of the eight Dutch academic centers, between 2016 and 2020 will be asked for informed consent to participate in this study. The study is coordinated by the research team instituted in the Radboud university medical center (Radboudumc).

Clinical evaluation at the start and during treatment of the patients will be performed by the local nephrologist or pediatric nephrologist, who participates in the national working group aHUS. The working group forms a platform for medical discussions and evaluation of individual cases concerning diagnosis and treatment of patients with TMA and suspected aHUS.

All patients with TMA and suspected aHUS, considered for eculizumab therapy are presented in the working group. Approval for start of therapy can be obtained within 24 hours after discussion by four representatives. **Figure 10.1** illustrates the treatment algorithm. In adults without a disease history of TMA, initial treatment consists of PT for a period of five days to allow proper diagnostics to exude secondary TMA. Eculizumab is started immediately in children, patients with a disease recurrence and in kidney transplant recipients. Eculizumab is given at the standard dose, as advised by the EMA and FDA for a period of three months. After three months treatment is stopped or continued at reduced doses, depending among others on age, previous disease history, underlying complement abnormality and kidney function. Of note, patients with TMA who do not receive eculizumab are not represented in the CUREiHUS study.

Eligibility criteria

A subject has to fulfill all of the following inclusion criteria without presence of any exclusion criteria before entering the CUREiHUS study. In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Patients of all ages, suspected of or diagnosed with aHUS, who are treated with eculizumab.
- Furthermore, subject and/or parents is/are able and willing to sign the informed consent before screening evaluations.

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Patients with other etiological forms of TMA than aHUS.
- Subject and/or his parents is/are not able or willing to sign the informed consent before screening evaluations.

If a patient does not want to enter the CUREiHUS study, this patient will still receive the restrictive treatment with eculizumab. Furthermore, separate informed consent is gathered for two add-on studies, however patients can still participate in the CUREiHUS study without informed consent for these two add-on studies.

Figure 10.1 Treatment algorithm following the new Dutch Guideline

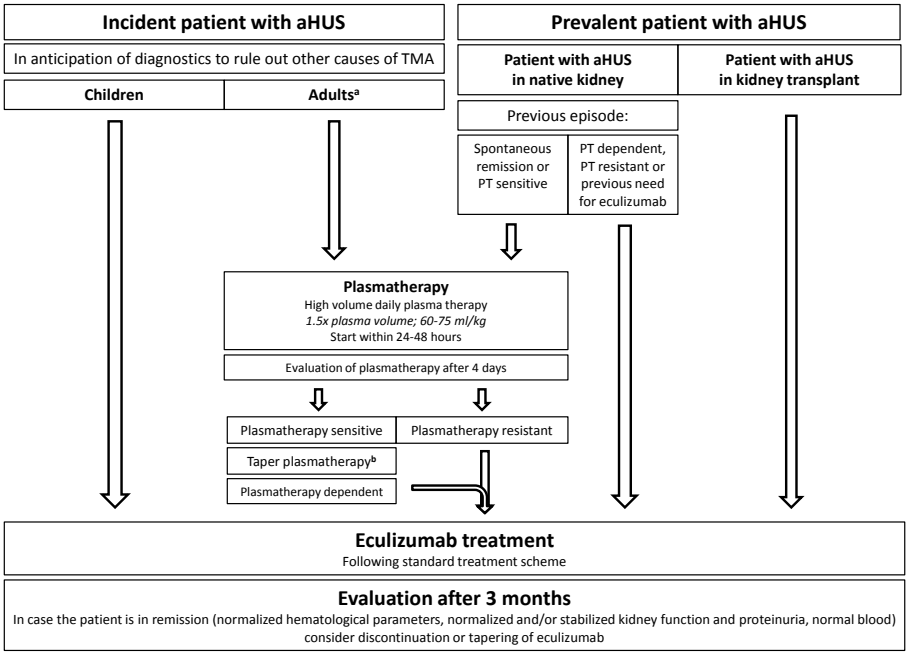


Figure 10.1 In 2016 a new Dutch guideline was implemented promoting a restrictive eculizumab regimen in patients with aHUS. Patients who present with first episode of TMA (incident patient) will receive plasmatherapy (PT) as first line treatment for four days to rule out other causes of TMA (e.g. STEC-HUS, TTP, secondary causes of TMA). In case no other cause of TMA is found and the patient does not respond to PT or is PT dependent, eculizumab treatment is started. In children with a strong suspicion of aHUS, eculizumab is the first choice of treatment. In patients who present with disease recurrence (prevalent patients) in native kidneys PT or eculizumab could be started depending on medical history. In patients with aHUS recurrence after kidney transplantation eculizumab should be started directly to preserve graft function. After three months, when the patient is stable and in remission, therapy is evaluated and when possible therapy is tapered or discontinued. Strict monitoring is essential to prevent chronic sequelae and for early detection of disease recurrence.

[a] Adolescents may be considered adults and treated accordingly.

[b] Taper PT 5x per week first two weeks, than, 3x per week for two weeks.

Study objectives

The primary study objective of the CUREiHUS study is to monitor and evaluate restrictive use of eculizumab in patients with aHUS according the new national guideline. An overview of the primary and secondary objectives is given in **Figure 10.2**.

National aHUS registry

Data from all aHUS patients treated with eculizumab will be gathered to evaluate outcome. Clinical information such as the first presentation, duration of illness, pathology reports, complement analysis and pathogenic mutations, family history, and treatment received in the past will be collected. The considerations and argumentations of the working group for start and/or stop treatment will be carefully described as well as laboratory values at time of presentation and during follow up. All data will be gathered in the online webbased database Castor. In addition to regular care, patients will be asked for additional collection of one serum tube (minimum of 1ml) and one EDTA plasma tube (minimum of 1ml) at every hospital visits for additional analysis. Samples are collected at time of venipuncture/intravenous access as part of standard care, so patients do not need extra invasive procedures. Directly after blood collection, the tubes will be placed on ice to prevent complement activation. Blood samples will be processed within 30-60 minutes within the university hospital where patient is treated. Plasma and serum aliquots will be stored on -80°C immediately after centrifugation (2000g, 10 min at 4 °C). Strict adherence to the protocol for sample collection is critical to prevent in vitro complement activation. All samples will be send to the department of Laboratory Medicine of the Radboudumc, where specialized assays will be performed. For a detailed overview of time points to collect blood see **Figure 10.3**.

Cost-effectiveness analysis

To perform a costs-effectiveness and budget impact analysis two questionnaires will be sent to all participants via email and automatically linked to the online Castor database. The first is to measure the quality of the health status of the patients, with a validated so-called health-related quality of life (HRQoL) instrument, the EQ-5D. This HRQoL instrument is available in a validated Dutch translation and will be completed by the patients. The EQ-5D is a generic HRQoL instrument comprising five domains: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. The EQ-5D index is obtained by applying predetermined weights to the five domains. This index gives a societal-based global quantification of the patient's health status on a scale that is (upper) anchored at 1 (perfect health). Patients will also be asked to rate their overall HRQoL on a visual analogue scale (EQ- 5D VAS) consisting of a vertical line ranging from 0 (worst imaginable health status) to 100 (best imaginable). For children between eight and 11 years of age the EQ-5D-youth will be used. Since the EQ-5D-youth is only available in 3

level (three answer options per question), we will use the EQ-5D-3L for children above 12 age and adults as well to generate as much as possible uniform data. After the start of inclusion patients will receive the EQ-5D-3L questionnaire every four weeks, for a period of three months, after which the patient will be asked to complete the questionnaire every three months. In case the patient experiences disease recurrence or in case of another serious adverse event the of the EQ-5D-3L will be sent every four weeks for three months again to fully document the effect on quality of life during these periods (**Figure 10.3**).

To measure health-care related productivity losses, we will use the Medical Technology Assessment Productivity Costs Questionnaire (iPCQ) questionnaire. All patients ≥ 18 years of age will receive the iPCQ and all parents of patients below 18 years of age will receive the iPCQ adapted to measure productively losses in families of patients with aHUS. Patients will receive the iPCQ questionnaire at start of inclusion and then every three months until the end of the study.

Figure 10.2 Study objectives

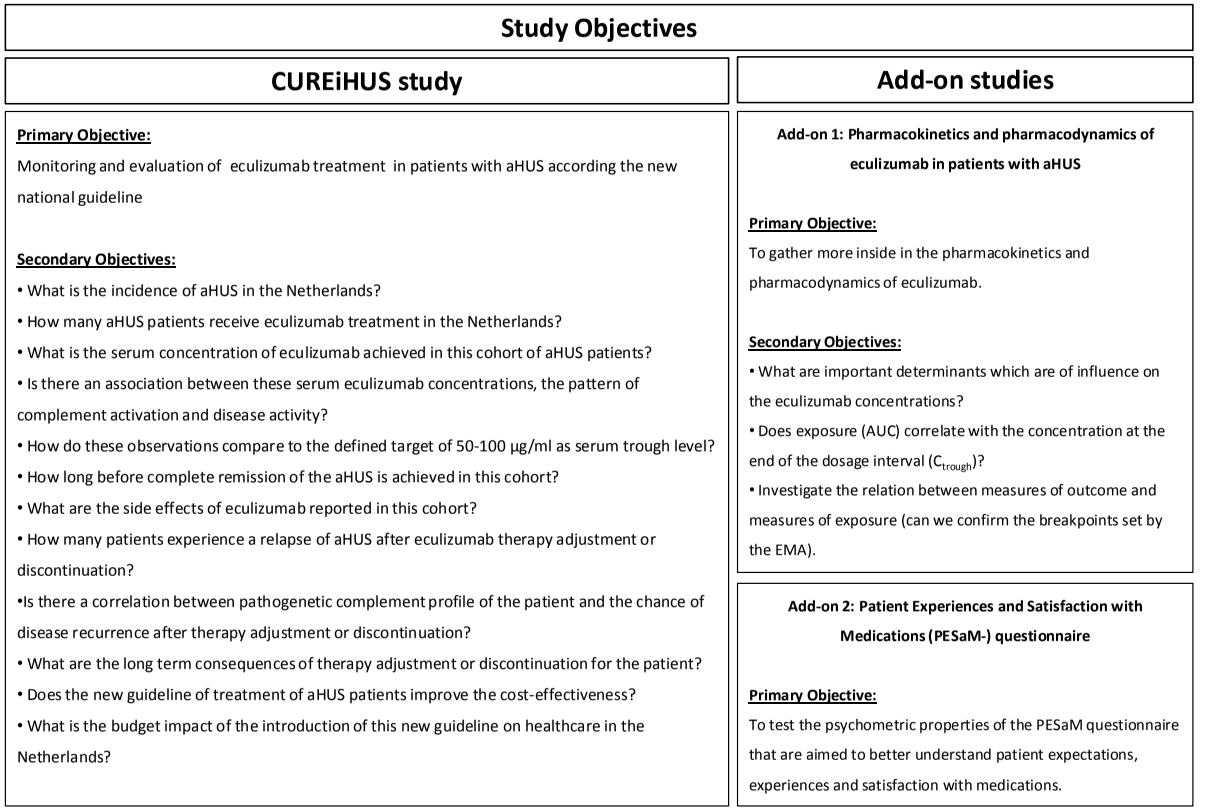


Figure 10.2 The primary and secondary study objectives of the CUREiHUS study are described. The study objectives for the two add-on studies, for which separate informed consent is given, are described separately.

Figure 10.3 Study proceedings algorithm

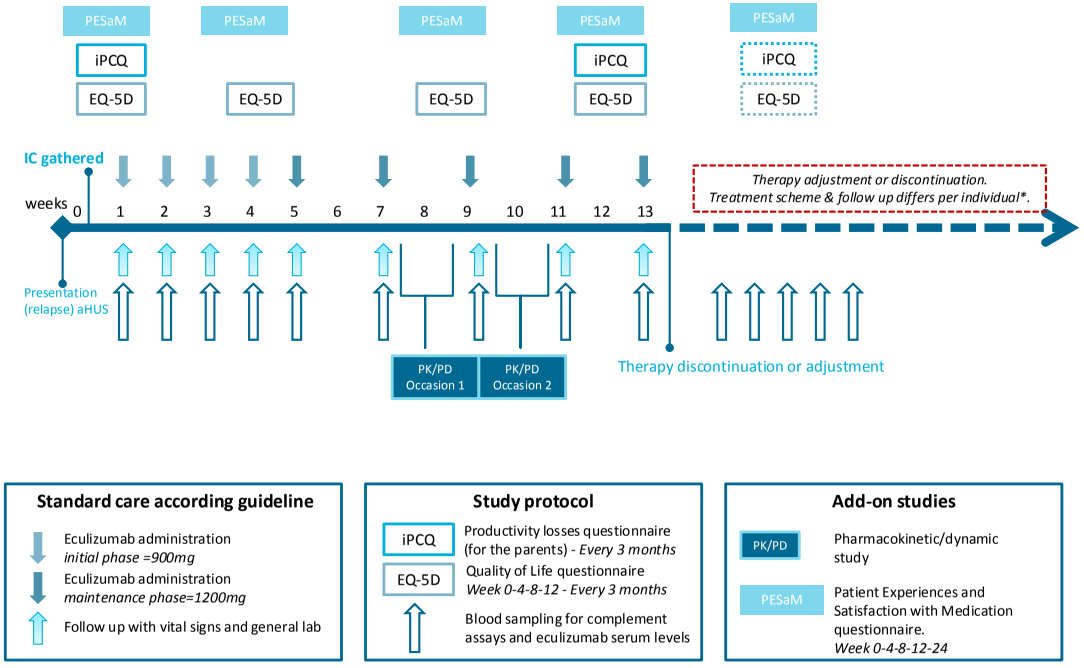


Figure 10.3 Depending on weight, patients receive eculizumab with different dosage and interval following pharmaceutical instructions. In this figure, the study scheme for patients with a body weight ≥ 40 kg has been depicted. After informed consent is obtained, patients receive questionnaires on set dates and additional blood is collected at time of hospital visits as part of standard care. In case informed consent is given for add-on study 1, patients have to return to the hospital in between eculizumab administrations. In case a participant is ≥ 15 years of age and separate informed consent is given for add-on study 2, PESaM questionnaires will be send in week 0-4-8-12-24. After three months, treatment will be evaluated and when a patient is stable and in remission, therapy will be tapered or discontinued. Patients will keep receiving questionnaires, and blood collection at the outpatient clinic will continue as long as the patient participates in the study. However, dates and amount of sampling will vary depending the individual care of the patient (depicted with dotted lines). aHUS; atypical hemolytic uremic syndrome, IC; informed consent

**Follow up: in case of discontinuation, follow up at least in week 16-24-32-52 after start of treatment with vital signs, general lab and gathering of study samples.*

Add-on studies:

Pharmacokinetics and pharmacodynamics of eculizumab

All participants will be asked separate informed consent to participate in add-on study one. To develop a protocol for therapeutic drug monitoring (TDM) in patients with eculizumab, a thorough understanding of pharmacokinetics (PK) and pharmacodynamics (PD) is required. In this study we want to investigate the PK in an actual clinical setting, under the tailored regimens and link this to complement inhibition, or PD in aHUS patients treated with eculizumab.

In consenting patients, additional blood samples are collected between two eculizumab administrations, during the maintenance phase (**Figure 10.3**). For this study, a patient will visit the hospital on four additional days for collection of blood. With the use of the program POPED which uses D-optimal approach, the optimal sampling design is determined, hereby maximizing the PK information gathered in this patient group.[355] PK of eculizumab depends on the clearance (CL) and volume of distribution (Vd).[240] The best moments to sample according our optimal sampling design, were respectively, 6, 16, 72, 100, 364 and 464 hours after eculizumab administration, to determine CL with 3% coefficient of variance (CV) and Vd with 4% CV. Taking into account the patient effort and the most convenient time schedule, we choose to spread the moments of sampling over two separate study periods between eculizumab infusions. Secondly, not all patients will receive eculizumab with a set interval of two weeks. Therefore, next to the optimal sampling design, we will determine the C_{trough} level, regardless of the dosing interval, instead of the time point set at 100 hours after administration, in all patients. Although, CV concerning the estimated CL and Vd shall increase when using these time points in patients in whom the interval between infusions exceeds 14 days, it will remain below 10%. The chosen time points to collect samples in study period one are: 2-4 hours after administration depending at the moment the patient will receive eculizumab, one day after infusion, three days after infusion and just prior to the new infusion. In study period two, samples will be collected one and five days after infusions.

Patient related outcome measures

All participants, aged ≥ 15 years, will be asked separate informed consent to participate in add-on study two: Patient Experiences and Satisfaction with Medications (PESaM-) questionnaire. In 2015, after literature review, focus group interviews, expert opinion, and face validity tests, a patient-reported outcome measure (PROM) was developed to gain insight into patient experiences and satisfaction with medications. The PROM consists of a generic module (for any medication) and a disease- specific module, specifically focused on experiences and satisfaction with eculizumab. In the PROM there are multiple items in the following domains: effectiveness, side-effects, ease of use, and overall satisfaction

(only for the generic module). Participants will receive the PESaM-questionnaire at four weeks after the first eculizumab gift; and every three months hereafter, either up to three months after the last eculizumab gift or when the maximum of five measurements are reached. In addition, participants in whom eculizumab is started or resumed will receive the expectation measure at baseline (week 0). If eculizumab is resumed (e.g. due to recurrence of disease) the participant will receive the questionnaire again following the scheme indicated above.

Assays

Eculizumab levels together with measurement of classical pathway activation (CH50) will be determined as described previously by Volokhina et al.[182] By quantifying the amount of the complement activation in our patients, we will be able to correlate serum concentrations of eculizumab to optimal inhibition of complement activity and subsequent optimal therapy. In addition to CH50, other measurements of complement will be evaluated. Methods to detect C3b/c, C3bBbP, and TCC have been developed previously and are available at the department of Laboratory Medicine, as is C3d assay.[158] Alternative pathway (AP) activity and C5 levels assays are commercially available (from Eurodiagnostica and Abcam, respectively). C3 and C4 concentration will be determined by nephelometry (BN™ II System of Siemens Healthcare Diagnostics).

Sample size

No official sample size calculation was performed since the CUREiHUS study is an observational study and it is intended to include all aHUS patients treated with eculizumab in the Netherlands. With an estimated incidence of 3 pediatric and 10 adult aHUS patients per year in Netherlands it is expected to enroll 52 patients until 2020.

Data analysis

For this descriptive study, clinical information such as the therapy followed, reported side effects, correlation between relapse and genetic profile are presented as qualitative data. Laboratory values are presented as quantative data. For continuous variables within normal distribution, values are expressed using the mean and standard deviation (SD), otherwise median and interquartile range 25-75 (IQR) is used. After four years of monitoring the guideline, the evaluation will take place. Differences will be measured using two sided t-test or mann-whitney test for non-parametric data as appropriate. For multiple analysis ANOVA with posthoc analysis or Kruskall Wallis followed by wilcoxon and bonferroni correction to control the alfa will be used.

Cost-effectiveness analysis

This study investigates the potential efficacy of a new safe and effective treatment regime of eculizumab in patients with aHUS from a societal perspective. The comparator is treatment as prescribed by the pharmaceutical company. The time frame of evaluation for the cost-effectiveness analyses (CEA) exceeds the follow-up of the cohort as it evaluates lifelong provision of eculizumab. Therefore, the CEA will follow an empirical approach as far as it concerns the cohort follow-up and a decision analytical approach to evaluate cost-effectiveness beyond the cohort follow-up period of four years. The design of the economic evaluation follows the principles of a cost-effectiveness analysis and adheres to the Dutch manual for costing research.[356]

Outcome measures specific for the CEA will be QALYs based on EQ-5D-3L. The direct (medical) costs of both eculizumab treatment regimens will be determined on a per patient basis. Further, on patient level, volumes of care will be measured prospectively using medical records and provider information systems. Resource utilization, such as medical specialist visits, general physician visits, medication, dialysis, and hospital admissions, as well as out-of-pocket expenses such as for over-the-counter drugs and travel costs will be collected. For units of care/resources where no guideline or standard prices are available, real prices will be determined. The effect of uncertainty in model parameters will be estimated through scenario analyses and a probabilistic sensitivity analysis Cost-effectiveness acceptability curves will be derived that are able to evaluate the probability that the new treatment regime is efficient against different thresholds (Willingness To Pay) for a QALY gained in different scenarios.

Productivity losses will be measured using the friction cost approach described in the Dutch manual for costing research.[356] All this input will be used in a decision analytic model with a lifelong horizon taking into account discounting. The discount rate follows the Dutch guidelines for pharmaco-economic evaluation.

Budget impact analysis

The budget impact analysis (BIA) will be conducted as outlined in Mauskopf et al.[357] The aim of this BIA is to assess the financial consequences of the introduction of the gender sensitive risk classification approach in the Dutch health care system in the short-to-medium term from the budget holders perspective. Also a more broad perspective i.e., the societal scenario (for example including productivity gains/losses) will be determined. It is obvious that if the new guideline is successful, the bulk of the budgetary savings comes from reduced eculizumab use. For the base case BIA perspective, within the health care domain, only costs associated with this perspective will be taken into account. Most of these cost are collected in the CEA. Deterministic uncertainty concerning BIA input such as perspective, pricing parameters, time horizon, uptake, etc. will be dealt with by generating the budget impact as a series of scenario analyses covering a relevant range

of costs. Both annual resource use (in terms of volumes consumed) and cost (volumes multiplied by prices) will be presented. The BIA will be assessed through more or less the same decision analytic model as the CEA.

Pharmacokinetic and pharmacodynamic properties
Individual and median plasma concentrations will be presented. Overlay presentations will be given to illustrate inter-subject variability. Descriptive statistics will be calculated for the plasma concentrations at each sampling time. The primary outcome measurement will be AUC0-tau values of eculizumab. PK parameters will be derived using a compartmental analysis with Phoenix version 7.0 (Pharsight Inc). Peak plasma concentrations (Cmax) will be directly observed from the data.

Patient reported outcome measure
The psychometric properties of the PESaM-questionnaire will be explored, including its construct validity and reliability. Existing standards and guidelines for evaluating reliability and validity of PROMs will be used.[358,359] In addition to testing psychometric properties, initial descriptive outcomes will be produced for the items and domains of the generic and disease-specific module.

Ethics and dissemination

Ethics approval

Ethical approval was obtained in the Netherlands from the medical ethical committee of Arnhem-Nijmegen at 15th of June 2016. The registration number of the CUREiHUS study is NL52817.091.15. This study will be conducted according the principles of the declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). Any substantial amendments and modifications will first be presented at the medical ethical committee before implemented in the CUREiHUS study protocol.

Consent

Patients will be approached for participation in this study by their physicians, mostly the head investigator at the site and member of the national working group aHUS. From all patients and/or their parents or legal representatives depending on age of the patient, informed consent will be obtained prior to the inclusion in the study. Patients have to give separate consent for all add-on studies. Patients and/or parents will receive an information letter and informed consent form. For the two add-on studies separate informed consent is obtained. Resistance of children to study procedures will be handled following the 'code of conduct involving minors' as established by the Pediatric Association of the Netherlands. Subjects can leave the study at any time for any reason if they wish to do so without any consequences. After withdrawal, subjects will continue treatment as prescribed by the physician. Data gathered up to point of withdrawal can be used for further analysis.

Confidentiality

All patients receive an unique encoded study ID as composed in Castor. The study ID is composed of the country and hospital where the patient presented, followed by a number, based on the order of inclusion, for example NL-Rad-001. The blood that will be collected after inclusion will be labeled with this study ID numbers together with time and date of acquisition. The investigators per academic centre safeguard the key of the study IDs assigned to the participants of that centre.

The hosting of Castor, the online database, is by ISO:27001 and NEN7510, certified datacenters. The system of the database enables an audit trail after each modification and login events. Login actions can only be with a personal (role and center specific) authorization with qualified passwords. Castor has a monitor function whereby data controls will be executed. The members of the national working group aHUS from the Radboudumc are responsible for this database. The investigator (together with their database manager) of each centre will enter the data in this online database. The handling of personal data is in accordance with the Dutch Personal Data Protection Act.

Data will be stored until fifteen years after publication. After the study is finished and scientifically published, subject material from the study will only be stored and possibly used for future studies when subjects agree with this, as included in the informed consent. Without agreement of the medical ethical committee, materials will not be used for future studies that will not have a direct relation to the current study and not for studies that involve the chance of finding additional outcomes. If subjects do not give permission to use their material for future studies, the material will be discarded after this study has been finished and scientifically published.

Safety

Based on the checklist risk classification of the Dutch Federation of University Medical Center (NFU) workgroup, there is no risk for participants of this study. For this observational study, the national working group aHUS will form the safety committee. The working group gathers every three month to discuss all patients with aHUS who receive eculizumab and the possibility of therapy adjustment and/or discontinuation. It forms a platform for medical discussions and evaluation of individual cases concerning the diagnosis, advising and interpretations diagnostic tests.

For this observational study, (serious) adverse events ((S)AEs), serious adverse reactions (SAR) and Suspected Unexpected Serious Adverse Reaction (SUSARs) as a consequence of our study are not applicable. However, information about side-effects or adverse reactions which occur at therapy adjustment/discontinuation of eculizumab during this observational study will be collected in the database.

Dissemination policy

Trial is registered with the Dutch Trial Registry NTR5988. After completion of this study the results will be published in (inter)national peer-reviewed scientific journals. A final report will be distributed to all stakeholders and the article and summary of results will be published on the website of the CUREiHUS study for all participants.

Trial status

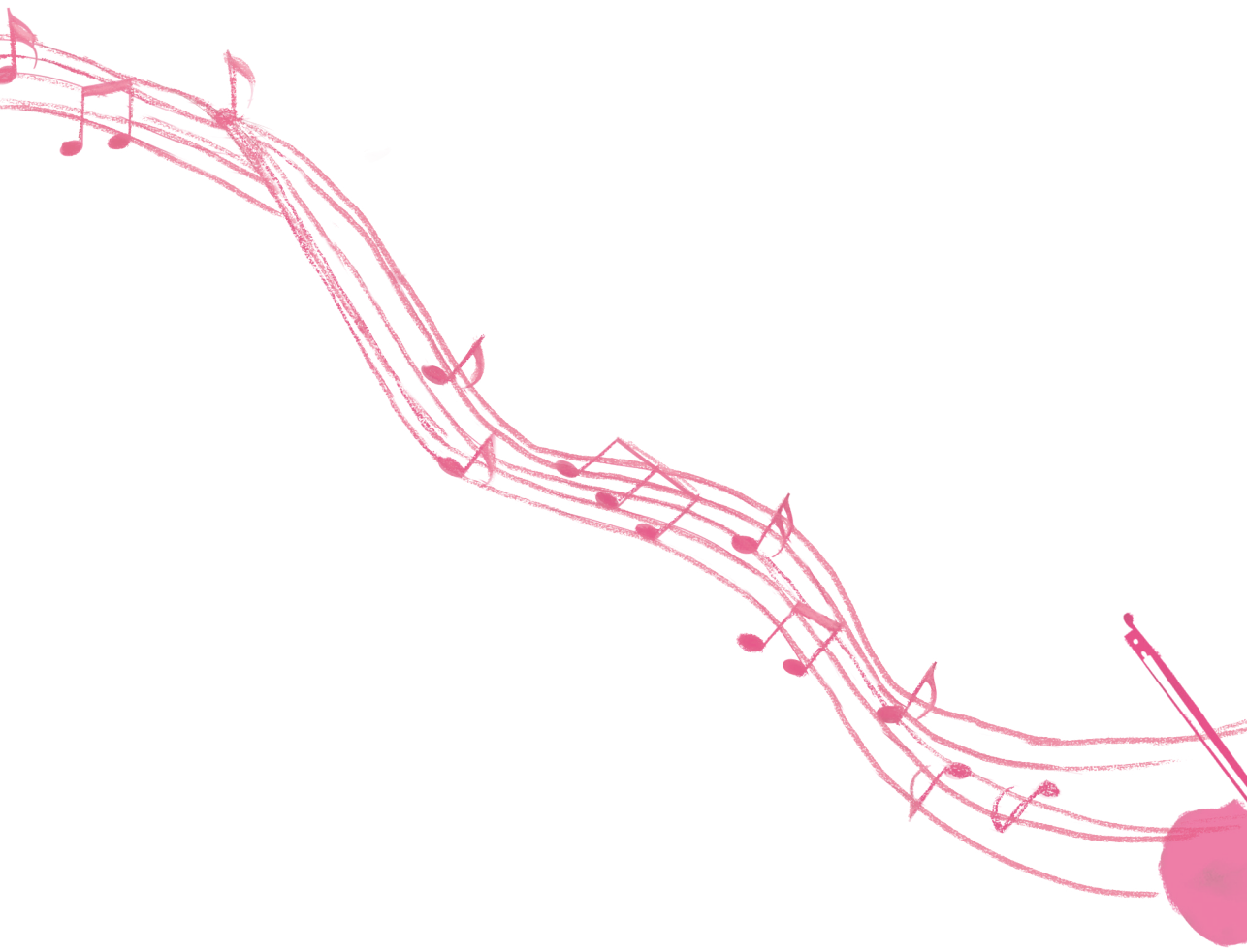
The study started recruitment in August 2016 and is currently still recruiting.

Acknowledgement

We would like to thank Maria Meulenbeek for her contribution in creating the study abbreviation: CUREiHUS. This work was supported by grants from ZonMw, 'Goed Gebruik Geneesmiddelen' (project number 836031008) and Zorgverzekeraars Nederland.

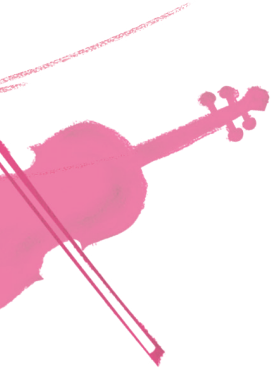
"Now this is not the end. It is not even the beginning of the end.
But it is, perhaps, the end of the beginning."

Winston Churchill



Chapter 11

Summary
and general discussion



Summary and general discussion

A new era has commenced for patients with a thrombotic microangiopathy (TMA). TMA is an umbrella term and characterized by hemolytic anemia, thrombocytopenia and ischemia of end organs. It can be a manifestation of a wide variety of diseases, often associated with significant mortality and morbidity. Both primary and secondary causes of TMA have been described (**Figure 11.1**). The introduction of eculizumab, the first drug to inhibit the complement system, not only provoked a renewed interest in complement involvement, but also increased the need to properly diagnose the underlying cause of TMA. The recent discovery of mutations in complement regulatory genes in presumed secondary causes of TMA, demonstrated that primary and secondary forms of TMA do overlap (**Figure 11.1**).[360,361] An accurate diagnosis to start the appropriate treatment is essential.

The scope of this thesis comprised two causes of TMA: Shiga toxin producing *Escherichia coli* (STEC) hemolytic uremic syndrome (HUS) and atypical HUS (aHUS). Over the last decade, increasing awareness for aHUS, has been raised by defining the role of complement dysregulation as the primary cause in aHUS.[5,57] By unraveling the pathophysiology, eculizumab became the first choice of treatment in patients with aHUS.[8] Thus far there is no proof that eculizumab is also effective in STEC-HUS, leading to divergent treatment strategies. Consequently, optimizing treatment requires accurate differentiation between STEC-HUS and aHUS. This may be difficult, since the clinical presentation shows a high degree of resemblance. Moreover, since aHUS is a diagnosis *per exclusionem* establishing STEC infections with high accuracy is indispensible.

Eculizumab as standard therapy for aHUS aroused a worldwide debate regarding the optimal treatment scheme and duration. Initial guidelines suggested lifelong treatment with a fixed dose for all patients. However, there is no evidence to support such strategy. Reducing the cumulative eculizumab dose is important, not the least in view of the enormous costs of this expensive orphan drug. In **part I** of this thesis we focused on STEC-HUS, aiming at determining new possible mediators of TMA and improving the diagnostics of STEC-HUS. In **part II** we focused on strategies to reduce eculizumab dosage, and implement individualized treatment strategies for patients with aHUS.

Part I: New aspects of pathophysiology and diagnosis in STEC-HUS

Diagnosis

The current gold standard to detect STEC infection involves fecal examination by detection of *Stx* encoding genes by polymerase chain reaction (PCR) and subsequent feces culture in case PCR is positive.[20,22,79,362] Of note, some laboratories use enzyme immunoassay (EIA) to detect Stx in the feces. However, since only a low inoculum is needed to cause disease, the detection of Stx can be difficult. Therefore it is strongly recommended to perform EIA only on enriched broth cultures.[19] Historically, feces culture was the gold standard to establish STEC infection and this was mainly based on the fact that the most common STEC serotype O157 could not ferment sorbitol, leading to colorless colonies on the Sorbitol MacConkey agar (SMAC) plate. Although STEC serotype O157 is still the predominant cause of HUS, non-O157 serotypes are increasingly associated with STEC-HUS.[30] Moreover, many non-O157 and even some O157 STEC strains are capably of fermenting sorbitol, making SMAC culture unreliable. Nowadays, only ˜40% of the feces samples which tested positive with PCR, can be cultivated on SMAC plates.[20] Hence, the detection of STEC in the feces is limited. Due to the natural course of the disease and low inoculums, the detection of STEC in the feces becomes increasingly difficult as the disease progresses. New and upcoming techniques such as molecular virulence and serotyping assays seem promising, however all rely on culture of the pathogenic strains.[20] Currently, solely use of fecal diagnostics is not yet sufficient to establish an STEC infection.[20]

In **CHAPTER 2** and **CHAPTER 3** we described the additional value of serology to detect antibodies against STEC O157. In **CHAPTER 2** we evaluated the role of a serological antibody assay to detect IgM antibodies against lipopolysaccharide (LPS) of STEC serotype O157 in addition to fecal diagnostics in a cohort of pediatric patients with STEC-HUS. In patients with suspected STEC-HUS, fecal diagnostics was positive in 54% of the patients. The LPS enzyme-linked immunosorbent assay (ELISA; LPS-ELISA), allowed establishing a diagnosis of STEC-HUS in 63% of the patients. This is an added value of 23% when serological antibody assay is used in addition to standard fecal diagnostics tests to confirm the diagnosis STEC-HUS. The added value of serology became especially apparent when the tests was performed more than seven days after the initial manifestation of the gastrointestinal symptoms.

Figure 11.1 Disease spectrum of thrombotic microangiopathy

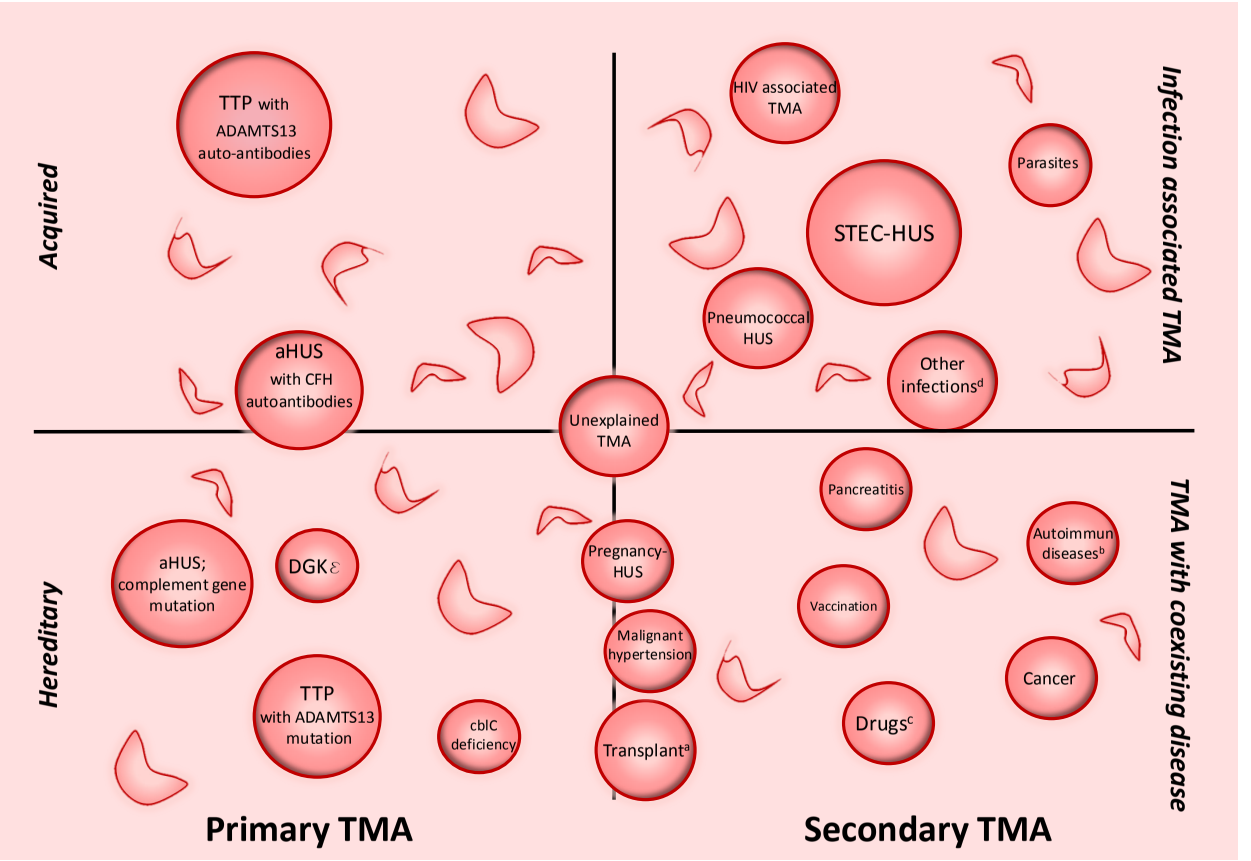


Figure 11.1 Thrombotic microangiopathy (TMA) has a broad differential diagnosis which can be divided into different subgroups. Primary and secondary causes of TMA are described and can be further divided into primary hereditary and primary acquired TMA and secondary TMA due to infection or with other coexisting diseases. Yet, the field of TMA keeps evolving and in an increasing amount of cases the subdivision appears to be ambiguous. In presumed secondary causes (e.g. malignant hypertension or pregnancy-HUS) pathogenic mutations in complement regulatory genes have been found. In the end leading to genetic predisposition to develop TMA concordant with atypical HUS. Adapted from Brocklebank et al.[361] (Clin J Am Soc Neph 2018;13:300-317) Copyright the American Society of Nephrology

aHUS; Atypical hemolytic uremic syndrome, cblC; cobolamine C, CFH; complement factor H, DGKε; diacylglycerol kinase e, HUS; hemolytic uremic syndrome, STEC; Shiga toxin producing E. coli, TMA; Thrombotic microangiopathy, TTP; Thrombotic thrombocytopenic purpura,

[a] TMA is described both after bone marrow transplant or solid organ transplant. Especially de novo onset of TMA after solid transplant is related to genetic predisposition for TMA such as aHUS.

[b] Various autoimmune diseases have been described in association with TMA such as systemic lupus erythematosus, antiphospholipid syndrome and scleroderma.

[c] Various drugs have been reported to induce TMA; e.g. calcineurin inhibitors, quinine, gemcitabine, oral contraceptives, heroin, cocaine

[d] A broad range of infectious agents have been described to cause TMA; e.g. Influenza H1/N1, Haemophilus influenzae, Coxsackie B virus, Epstein-Barr virus, Hepatitis A or C, Bordetella pertussis.

Although LPS-ELISA cleary improved the diagnosis of STEC-HUS, this conventional LPS-ELISA (in which ELISA plates are coated with purified LPS) has various drawbacks. The most important limitation is the presence of cross-reactivity with the conserved lipid A part of the LPS molecule.[29,31,81] The LPS structure consists of a lipid A part, an outer and inner core and an O-antigen, of which the latter has the highest immunogenic activity. Since the lipid A part is also present in other strains of STEC and even other gram-negative bacteria, this could lead to cross-reactivity. Potentially causing false positive results (in case of cross-reactivity with other bacteria than STEC) or incorrect determination of the STEC serotype causing HUS.[29,82,83] In **CHAPTER 3** we described a new ELISA technique (glyco-iELISA) to tackle the aforementioned issues. Via bacterial glyco-engineering, glycoproteins with engineered O-polysaccharides could be produced. In the glyco-iELISA recombinant serotype specific glycoproteins are used, consisting of the O157 polysaccharide attached to the protein carrier acceptor AcrA (O157-Acr). Due to the absence of the lipid A structure, potential cross-reactivity is counteracted.[81] We tested the added value of this new glyco-iELISA in two cohorts. The first cohort comprised pediatric patients with clinical suspected STEC-HUS (largely the same cohort as described in chapter 2). Of these patients 43% had a positive fecal diagnosis for STEC, 65% had positive serology based on the LPS-ELISA against O157, and 78% had positive serology based on the glyco-iELISA. Overall, the glyco-iELISA yielded 10 additional patients who tested negative with LPS-ELISA. Furthermore, the LPS-ELISA seemed false-positive in three patients in contrast to the glyco-iELISA. Fur-

thermore, by combining the glyco-iELISA with fecal diagnostics, STEC infection was detected in 89% of the patients. In a second nationwide cohort of 212 patients with TMA, in respectively 48 (23%) and 60 (38%) patients with TMA, confirmation of STEC O157 infection was found with LPS-ELISA and glyco-iELISA. Furthermore, the glyco-iELISA appeared highly sensitive, no cross-reactivity was observed with the glyco-iELISA and IgM could be detected for a long period of time.

STEC serotype O157 is still the most prominent serotype associated with STEC-HUS in the Netherlands. Yet, non-O157 serotypes are emerging as cause of HUS. Obviously, a diagnostic assay to determine anti-O157 antibodies will miss an infection with non-O157 STEC strain. Therefore, it remains highly important to perform both fecal diagnostics (PCR and culture) together with serology. With fecal diagnostics, one could establish STEC infection based on the presence of *Shiga toxin (Stx)* genes (and other virulence factors) in the feces with PCR. In case the pathogenic strain has been harvested with culture, molecular serotyping could help to further determine the STEC serotype and additional virulence factors. In **CHAPTER 4** the importance of thorough fecal diagnostics in the differential diagnosis of HUS is illustrated. We described the case of a 16-month old boy with an aggressive course of STEC-HUS due to a rare STEC serotype: *Stx*2d producing *E.coli* O80:H2. The clinical course of this patient mimicked aHUS due to the severity of the disease and the absence of a recent history of diarrhea. Consequently, eculizumab was administered. However,the next day PCR was positive for *Stx2* and intimin-encoding *eae* genes, confirming STEC infection as the cause of HUS. In addition, fecal isolates were used for molecular serotyping using whole genome sequencing (WGS) and revealed the rare STEC serotype O80:H2, which contained the *Stx*2d gene and the *eae* ξ gene variant, which is rarely seen in humans.[91] This report underlines the importance of STEC diagnostic tests in all children with HUS, including those with an atypical presentation, and emphasizes the importance of molecular and serotyping assays to estimate the virulence of an STEC strain.

Pathophysiology

Although STEC-HUS is one of the most common causes of acute kidney injury in children, the exact pathogenesis leading to TMA is still not well defined.[5,363,364] Besides the released Stx which undoubtedly plays a pivotal role, other virulence factors might contribute to the development of TMA, possibly explaining the broad range in clinical presentation and outcome. The development of anti-complement therapies has stimulated research into the role of complement activation in STEC-HUS.[39,41,42] As part of the host response to bacterial infections, the complement system is activated during the acute phase of disease in patients with STEC-HUS. Some patients exhibit low levels of C3 in combination with high levels of soluble C5b-C9.[39,41,42] Yet, it remains controversial if complement activation as seen in STEC-HUS contributes to the development

of endothelial damage in STEC-HUS. In rare cases, mutations in complement genes (both pathogenic mutations and mutations of unknown significance) have been found in patients who present with TMA associated with a STEC infection. Of note, many mutations need still to be confirmed by functional analysis. Most likely, these patients with STEC-associated HUS and pathogenic mutations might actually represent aHUS triggered by STEC infection.[42,365] Up till now, it seems highly unlikely that complement involvement could explain the wide variability in clinical presentation and outcome in STEC-HUS, ranging from no chronic sequelae to hypertension, proteinuria, chronic renal failure, end stage renal disease or even death.[5]

Several studies have been published regarding the role of the noxious molecule heme in relation to tissue injury in various forms of hemolytic anemias. During hemolytic anemia, heme is released and scavenged by hemopexin extracellularly under normal conditions . Yet, in case of massive hemolysis this system will be overwhelmed. Free heme is associated with endothelial activation and vaso-occlusion in among others patients with sickle cell disease.[100,104,129] We hypothesized that heme contributes to the development of TMA. In **CHAPTER 5** the contributing effect of heme in the evolvement of STEC-HUS is described. Significantly elevated heme levels were found in 48 STEC-HUS patients compared to controls. Moreover, the observed heme levels had a strong inverse correlation with hemopexin levels, making hemopexin a reliable biomarker for toxic levels of heme present *in vivo*. Furthermore, a pro-inflammatory and pro-thrombotic response was observed when primary human glomerular microvascular endothelial cells were incubated with heme. Moreover, upregulation of the cytoprotective enzyme heme oxygenase 1 (HO-1) was impaired by Stx. Consequently, heme as released during hemolysis could contribute, amplify and even drive the detrimental cascade leading to STEC-HUS.

Conclusions and future perspectives part I

The research field of STEC-HUS is constantly evolving. Although the role of complement in STEC-HUS is extensively studied, other factors like heme are relatively disregarded. With our study we showed the oxidative stress and pro-thrombotic effect caused by heme on glomerular endothelial cells. Moreover, STEC-HUS patients are more susceptible for these effects since Stx inhibits the production of the protective HO-1 enzyme. Furthermore, we have shown that serological assays have a place in the diagnostic work up of patients with TMA. We advocate to always combine fecal diagnostics together with serological diagnostics. Moreover, the optimal assay to determine serological antibodies against STEC serotype O157 is the glyco-iELISA. As discussed in chapter 2 and 3, children who present with HUS in the absence of positive fecal diagnostics should not be diagnosed as aHUS. One could even argue to exclude STEC infection in all patients (children and adults) who present with a first episode of TMA. Especially since the clinical presentation of STEC-HUS can be quite atypical in adults and is often accompanied by a delay in diagnosis.[366,367] Of note, in patients without stool production at time of presentation, the use of rectal swabs (performed on multiple occasions) is a sensitive alternative to detect STEC in the feces and therefore highly recommended.[368]

Until today it remains unclear why only ~15% of all patients infected with STEC develops HUS. Most likely, due to a complex interplay of host, pathogen and environment factors some patients are more prone to develop HUS. The susceptibiliy for heme toxicity could be one of these host factors. Heme is released during hemolysis, as present in various degrees in these patients. Subsequently, heme is degraded intracellularly by HO-1. The activity of this important degrading enzyme is influenced by known polymorphisms in the promoter region.[126] Moreover, one study even showed a relation between the length of repeats in the promoter of *HO-1* and the development of acute kidney injury.[369] Hence, future studies need to focus on the role of polymorphisms in *HO-1* in patients with STEC-HUS. Ultimately, leading to a better prediction and prevention model for patients who develop STEC-HUS.

The case as described in chapter 4 emphasizes the importance to rule out STEC infection, even in patients with an atypical presentation of HUS. Similar as in our patient, in 6-10% of the children with STEC-HUS there is no (bloody) diarrhea, whereas aHUS is preceded by diarrhea in 25% of cases.[44] Clinical features can thus be misleading. Furthermore, it underlines the importance to look for non-O157 serotypes as well as to estimate the virulence of an STEC strain, by molecular and serotyping assays using WGS. More research is needed to further characterize this emerging and highly diverse group of non-O157 serotypes which are increasingly detected due to improved diagnostics.[6,18,30,32,80] Molecular serotyping assays are also critical in public health as they can determine the source of an outbreak and establish transmission pathways.[20]

Speed is essential to rapidly distinguish between STEC-HUS and other forms of TMA. Future studies should focus on improving diagnostic assays for bedside use. For example, with the use of lateral flow technology one could develop a point of care test for patients presenting with TMA. This would be of great additional value since the current glyco-iELISA takes at least 24 hours to perform. Even with the new techniques like WGS, it takes at least 48 hours if not longer to complete the diagnostic workup since a cultivated STEC strain is necessary to perform WGS.[20] Recently, the role of metagenomic characterization of STEC is described, by applying shot gun sequencing on the collected feces (result within 24 hours).[370] Hereby excluding STEC culture as major limitation. Of note, due to the low inoculum needed to cause disease, also metagenomics can fail to detect STEC infection. Moreover, with the recent developement of a miniaturized sequence device (Oxford Nanopore Technology MiniION) bedside diagnosis of STEC-HUS comes within reach. Since these techniques will generate enormous amount of data, adequate bioinformatic analysis is needed to interpretate these data. Furthermore, with help of international genome databases for *E.coli* one could join forces to interpretate the large bulk of information. Although these techniques are most likely the future for STEC detection, for now PCR, culture and serology remain the gold standard.

Up till now, treatment of STEC-HUS is merely symptomatic and no specific treatment targeting STEC is validated yet. With temporary dialysis (in 50-60%) and erythrocyte transfusions (in over 70%), STEC-HUS usually is a self-limiting disease.[6,18] Furthermore, increasing evidence is emerging that early and generous intravenous fluid administration reduces the risk of development of oligo- or anuria, neurological complications and decreases the need for renal replacement therapy.[121,122] Overall, the use of antibiotics to treat STEC infection is discouraged as some antibiotics are associated with increased release of Stx.[96,371,372] A logical therapeutic approach would be to target Stx itself to neutralize the noxious effects. However, thus far no drug targeting Stx has proceeded past a phase II trial.[373] Urtoxazumab appeared safe and well tolerated in both healthy adults as pediatric patients with STEC-HUS. Yet, the effect of urtoxazumab on clinical outcome in STEC-HUS patients is not reported.[374] Furthermore, one could wonder if antibodies targeting Stx would be effective, since endothelial damage has already occurred when a patient presents itself with symptoms of HUS. Another approach could be to neutralize the noxious effect of heme and hereby increase the chance on a favorable outcome with minimized chronic sequelae. Hemopexin infusions would be the first choice as scavenger of heme. Although promising results with hemopexin infusions were obtained in animal models, the first human trials need still to be conducted.[129]

The use of the complement inhibitor eculizumab is still controversial in STEC-HUS. Simultaneously with the implementation of eculizumab as standard treatment of aHUS in 2011, a catastrophic outbreak with STEC serotype O104 was reported in Germany. The decision to administer eculizumab during this outbreak was primarily funded on one

case report of three patients with STEC-HUS with serotype O157 and rapid (neurological) improvement after eculizumab treatment.[375] However, in a retrospective analysis of a large adult STEC-HUS cohort during the outbreak, no effect was observed from eculizumab treatment on renal and neurological outcome.[376] Since then, several small studies report better outcome in STEC-HUS patients with neurological involvement, after eculizumab treatment.[98,377,378] Of note, one study conducted with 11 pediatric STEC-HUS patients with among others neurological involvement, suggested a potential negative effect in patients with simultaneous multi-organ failure.[98] Since spontaneous recovery (both renal as neurological improvement) is seen in nearly all patients with STEC-HUS, placebo-controlled trials are needed to study the additional effect of eculizumab on recovery. At this moment, the ECULISHU trial (NCT02205541) in France and the ECUSTEC trial in the United Kingdom are the first randomized double-blind placebo-controlled trials to study the effect of eculizumab in STEC-HUS. The ECULISHU trial was completed in June 2018, however the results are not yet published. The expected end date of the ECUSTEC trial is March 2022. To date, STEC-HUS is still treated symptomatically and eculizumab remains only eligible to treat patients with an atypical form of HUS.

Part II Strategies towards personalized treatment of aHUS

In patients with aHUS due to complement dysregulation outcome has improved dramatically after the introduction of eculizumab. Eculizumab very effectively inhibits complement activation, thus allowing to restore endothelial cell integrity, and inducing hematological remission. Subsequently, renal function improves or stabilizes in the majority of patients, including patients who were plasma therapy dependent or resistant previously.[8,185,379] Overall, treatment is well tolerated with few side effects. The severity of the disease together with the underlying genetic defect in the majority of patients and the risk of disease recurrence, has stimulated the use of lifelong eculizumab therapy with standard dosages. However, the need for lifelong therapy is not evidence based, and debated not the least because of the enormous costs of the drug. One year of therapy with eculizumab in an adult patient would cost approximately €500,000. Thus, there is an unmet need to optimize eculizumab therapy, specifically by limiting treatment dose (discussed in chapter 6 and 7) or duration (discussed in chapter 8 and 9), and hereby further personalize treatment in patients with aHUS.

In **CHAPTER 6** we described 11 aHUS patients, both children and adults, who received eculizumab according the standard dosage regimen and with an extended interval varying between 3-8 weeks. Eculizumab serum trough levels were measured. We showed that eculizumab levels increased over time when given at weekly intervals in the induction phase (depending on the body weight and possibly initial soluble C5b-9 concentration). Eculizumab levels remained stable when dosed at two weekly intervals during the maintenance phase, and decreased when the intervals were longer. However, even with an extended interval up till four weeks, sufficient complement inhibition was reached in a considerable subset of pediatric and adult patients. Moreover, during the standard treatment regimen, eculizumab trough levels up to 772 μg/ml were detected, far exceeding the therapeutic target of 50-100 μg/ml. Hence, the current treatment scheme (as approved by the european medicines agency and food and drug administration) resulted in a large variation in drug concentrations per aHUS patient and an excess of eculizumab could be noted in the majority of patients.

Besides aHUS, eculizumab is registered as therapy for two other diseases. Furthermore, numerous cases have been reported regarding patients with a wide variety of diseases who received eculizumab off-label. In **CHAPTER 7** the pharmacokinetic and pharmacodynamic properties of eculizumab, both for reported on- (paroxysmal nocturnal hemoglobinuria, aHUS, generalized myasthenia gravis) and off-label indications (hematopoietic stem cell transplantation associated TMA), were reviewed. We concluded that there were large differences in measured serum trough levels between the various patients groups. As a consequence, both the advised trough level targets as the pursued

effects were different per indication. To provide the best tailored care, we advocated an individualized approach and highlighted the importance of therapeutic drug monitoring, as foundation for personalized medicine, in patients treated with eculizumab.

Next to tapering of eculizumab therapy, another option would be to treat patients with a restrictive eculizumab regimen; withdrawal of eculizumab when the patient is stable and in remission. In **CHAPTER 8** we described the course in 20 aHUS patients, both children as adults, who were treated with restrictive eculizumab therapy. Eculizumab therapy could be tapered in all patients. Furthermore, in 17 patients eculizumab was withdrawn. Subsequently, recurrence of aHUS occurred in five patients. Due to close monitoring, recurrence was detected early and eculizumab was restarted. No clinical sequelae such as proteinuria or progressive kidney dysfunction were detected. In total, eculizumab has been discontinued in 13 patients without aHUS recurrence, of which five were event free for over a year. With this strategy approximately €11.4 million has been saved.

An increasing, but still limited, amount of case reports has been published over the years describing aHUS patients who received a restrictive eculizumab regimen. In **CHAPTER 9** we reviewed all literature regarding a restrictive eculizumab regimen in patients with a first episode of TMA, patients with aHUS relapse in their native kidneys and transplant recipients who experienced disease recurrence. Overall, withdrawal of eculizumab seemed possible in a large group of patients with strict monitoring for disease recurrence. Approximately 27% of the patients experienced disease relapse, mostly within one year after therapy withdrawal. After rapid reinitiation of eculizumab, no chronic sequelae were detected. Strategies to taper and withdraw therapy were extensively discussed and a treatment algorithm was proposed.

Conclusions and future perspectives part II

Although eculizumab therapy has dramatically improved the prognosis of patients with aHUS, the optimal treatment regimen is yet unknown. Most importantly, there is no evidence to support the current treatment protocol which advocates lifelong therapy, leaving room to develop strategies to personalize treatment. Within this thesis, we have proposed an alternative and unique approach to treat patients with aHUS. This strategy is based on the hypothesis that a restrictive eculizumab regimen in aHUS appears safe and (cost) effective.

As described in chapter 6 and 7, trough levels often exceed the set target of 50-100 µg/ml required to block the complement system. As extensively discussed in chapter 7, by applying therapeutic drug monitoring one could adjust therapy and minimize unnecessary use of eculizumab. Moreover, it is unknown if a completely blocked complement system is required to halt disease activity, or that merely a suppressed complement system would be sufficient, as described by Ardissino et al.[259] Of note, potential side effects of eculizumab, like high susceptibility for meningococcal disease, could be reduced if patients are still able to form C5b-C9 complexes upon infection. Therefore, targeted therapy aiming at complete and/or incomplete complement blockade should be further explored.

The introduction of eculizumab in clinical care in the Netherlands has created awareness for the difficulties in discussing treatment for rare diseases with expensive orphan drugs. Upon market approval of eculizumab, a national aHUS working group was formed, including one nephrologists and one pediatric nephrologists of every university hospital in the Netherlands.[4] Together they implemented a new guideline to diagnose and treat patients who present with TMA. Moreover, a restrictive eculizumab regimen was proposed, in which aHUS patients are treated with eculizumab for three months after which therapy is evaluated. The study protocol for the nationwide prospective observational study to monitor and eventually evaluate this new guideline is described in **CHAPTER 10**. This study, called CUREiHUS, includes all aHUS patients who are treated conform the new Dutch guideline. Questionnaires are sent to assess quality of life and productivity losses to perform a cost-effectiveness analysis and budget impact analysis. Furthermore, blood is collected to study complement activation, biomarkers for disease recurrence and eculizumab serum levels. The study will close in October 2020.

Our study and other ongoing international studies will evaluate the best strategies to prevent, predict and treat disease recurrence. As discussed in chapter 9, approximately 27% of the aHUS patients in which eculizumab therapy is withdrawn will suffer disease recurrence. Although with close monitoring eculizumab can be reinitiated rapidly upon recurrence, hereby preventing chronic sequelae, it remains important to predict disease recurrence. Unfortunately, no biomarkers that predict disease recurrence are yet discovered. The use of routine complement assays do not allow differentiation since normal

C3 and soluble C5b-C9 levels are often reported in patients with aHUS. More specialized assays have been proposed, however need to be tested in prospective studies to prove their value.[269,282] Especially the *ex vivo* serum complement activation assay on human microvascular endothelial cells gained a lot of attention.[269,360] The intensity of C3 and C5b-C9 deposition on endothelial cells was a reflection of the amount of complement activation. This assay could have clinical implications to help to differentiate between active disease and remission, to monitor and adjust eculizumab therapy and finally help to predict relapses after eculizumab withdrawal. Although the results are promising of this assay, a specialized and dedicated laboratory to perform this assay is required. Hence, hereby decreasing the accessibility and availability of this assay.

One could question if merely one biomarker would be sufficient to monitor such a complex and heterogeneous disease as aHUS. Increasing evidence suggests that the pathogenic mutation underlying the development of aHUS could guide duration of treatment. For example, patients with complement independent forms of aHUS will not respond to this therapy. Not all mutations associated with aHUS are located in genes encoding for complement proteins. For example, mutations in thrombomodulin, DGKε and plasminogen have been described in relation to the development of aHUS.[308,380-382] However, all these mutations appear to comprises genes involved in the coagulation system and hereby leading to coagulation mediated TMA in contrast to complement mediated TMA. Although there is bidirectional crosstalk between these two systems, the exact mechanisms and clinical implications still needs to be unraveled.[364] By default, these mutations have been described in relation to aHUS. Yet, in this case aHUS is defined as non-typical (hence STEC-HUS) rather than complement mediated aHUS such as discussed in this thesis. Some pathogenic mutations in complement regulatory genes are thought to be associated with worse outcome such as genetic alternations in complement factor H including complement factor H related hybrid proteins. More research is needed to study the potential influence of pathogenic mutations on disease course and treatment outcome.

Furthermore, the aHUS patient population is highly heterogeneous with patients who present with a first episode, patients who have encountered previous episodes of aHUS and even some patients who reached en stage renal disease and received a kidney transplant. This poses a new dilemma, since the optimal treatment for one individual can change over time, depending the response to previous treatment episodes. Instead of merely looking at one aspect, an extended algorithm should be developed to include all above mentioned topics, ultimately guiding tailored patient care (**Figure 11.2**). As discussed in chapter 9, by developing for example a scoring system, including gathered data on therapeutic drug monitoring, one could integrate all this information.

Figure 11.2 Personalized treatment of aHUS

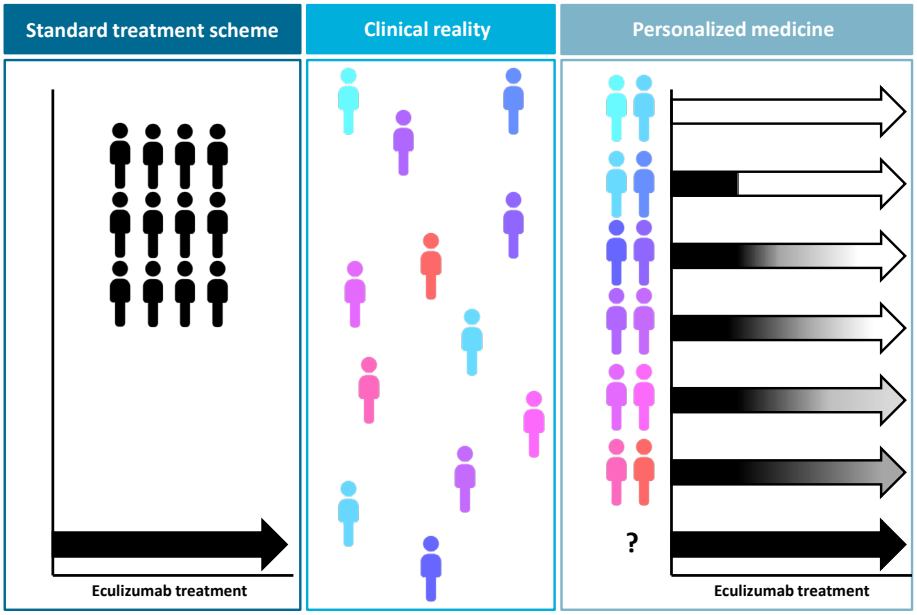


Figure 11.2 In the current standard treatment scheme as described by the pharmaceutical company, eculizumab dosage recommendations are depending on the body weight of the patient (categories: 5-10kg, 10-20kg, 20-30kg, 30-40kg, >40kg) and all patients receive a fixed dose with a set interval, potentially lifelong (black arrow). Yet, in clinical reality, some patients need less drug to achieve the target range of eculizumab in blood, or do not need lifelong treatment at all. Depending age, pathogenic mutation causing aHUS, medical history and previous episodes of disease relapse, each patient should receive a different and personalized treatment regimen. For example, in transplant recipients prophylactic eculizumab may not be necessary to prevent disease relapse (white arrow). Furthermore, patients could receive a short period of eculizumab after which therapy is withdrawn, or eculizumab therapy could be tapered before withdrawal. In only a small subset of patients, ongoing eculizumab therapy would be needed. Yet, instead of a fixed dose, therapeutic drug monitoring should be applied to achieve target levels.

The successful implementation of eculizumab let to a renaissance of complement-targeted drug developments. More than 20 drugs that target the complement system at some stage are nowadays being evaluated in clinical trials, with a dozens other agents in the pipeline.[9] Most candidate drugs in development to treat aHUS focus on blockade at C5 level, resembling eculizumab. For example, a phase III trial using a long-acting C5 inhibitor (Ravulizumab, or ALXN1210) produced by the same pharmaceutical company as eculizumab, is currently running. In this trial, intravenous administration of eculizumab is compared to ALXN1210 which has to be administered every eight weeks instead of every fortnight. Furthermore, with the patent protection of eculizumab expiring in May 2020 within Europe, various trials using a biosimilar of eculizumab are conducted (ABP959 and Tesidolumab).

In contrast to these antibodies who need to be administrated intravenously, Coversin is a small peptide blocking the splicing of C5 with the advantage of subcutaneous administration. Yet, no study results in patients with aHUS are published up till now. Blockade at the level of C3 would be less favorable considering the key role of C3 in all complement pathways. A phase III trial is currently running, with a novel therapeutic targeting the mannan binding lectin associated serine protease 2 (MASP2), an enzyme of the lectin pathway.[9] Although they report promising results in patients with aHUS, the interplay of the lectin pathway in the development of aHUS needs yet to be elucidated. Taking into account the pathophysiological mechanisms of aHUS with dysregulation of the alternative pathway of the complement system and excessive deposition of C5b-C9 on endothelial cells, C5 inhibition seems the most logical choice. Future studies should focus on blockade of the formation of the terminal complement complex (C5b-C9), preferably with drugs who have oral bioavailability or could be administered subcutaneously instead of parenteral administration.

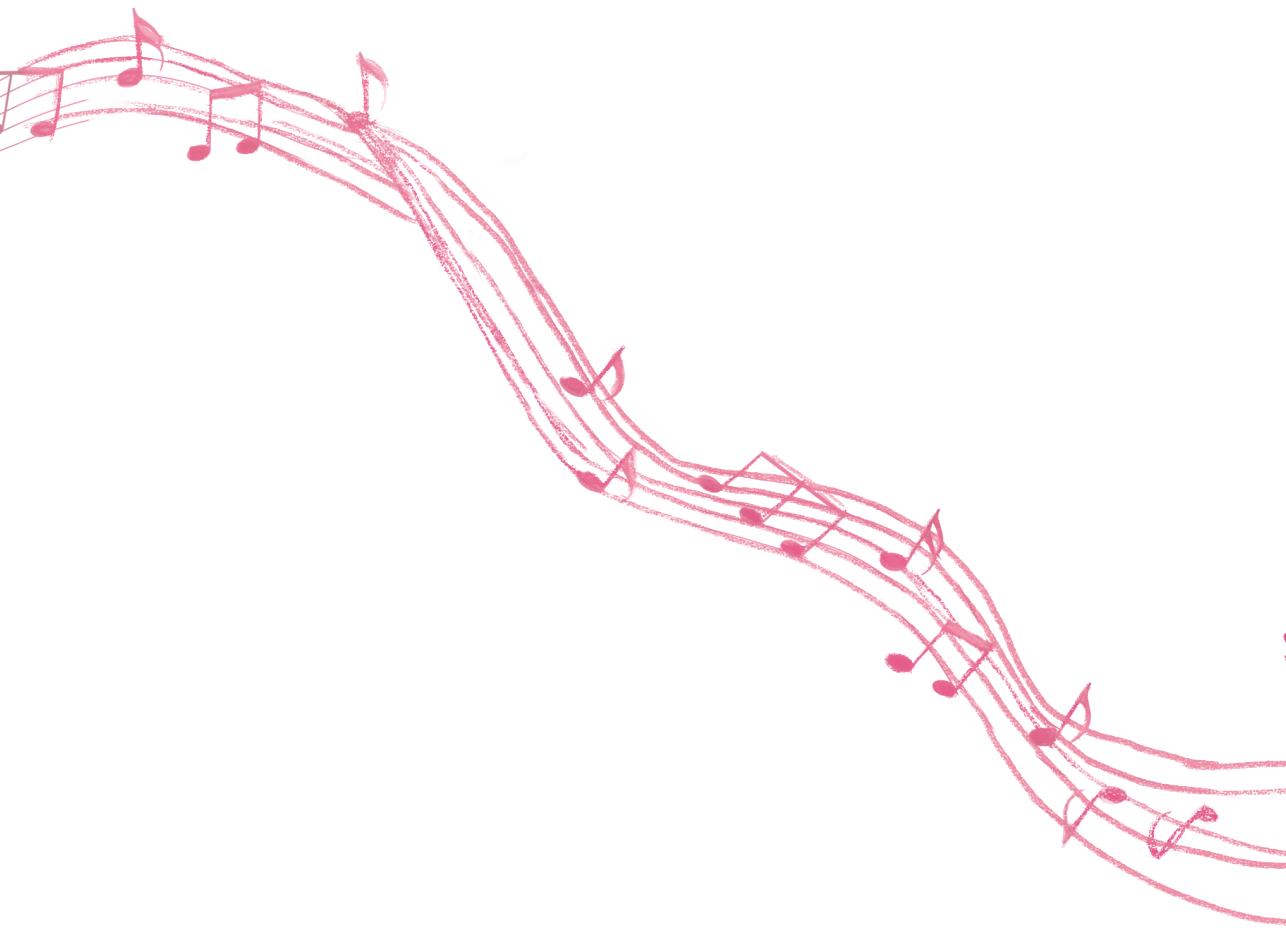
Eculizumab as treatment for the rare disease aHUS is just one of the many examples of a highly expensive orphan drugs which gained market approval the last decade. With the sharp increase in orphan drugs entering the market and even more in the development pipelines, various governments install interventions and regulations to control these expenses and secure economic health-care resources.[9,383] To smoothen the implementation process, a lot can be learned from our experience with eculizumab and the orphan drug arrangement as implemented in the Netherlands. The first step after market approval, would be to develop a guideline with clear start and stop criteria, drafted by experts in the field. Furthermore, studies have to be conducted regarding the optimal treatment regimen with each orphan drug. To facilitate policy making and translate the preclinical data into clinical benefits, monitoring of patients in a independent registry is imperative. With this gained knowledge, the proposed guidelines need to be re-evaluated after some years and adjusted accordingly. Imbursement policies vary from country to country, but overall countries tend to put a threshold on the affordability of drugs, despite its effectiveness. With the performance of an independent cost-effectiveness analysis decision making regarding reimbursement can be supported. Although informed consent is always obliged, one could argue on the moral duty of patients who receive such expensive treatment to participate in a registry study to monitor and evaluate (cost-) effectiveness. Ultimately, government, healthcare insurances, researchers, physicians and patients have to join forces to achieve optimal and affordable healthcare.

It remains important to optimize, evolve and innovate treatment of aHUS patients. With among others the results obtained from the CUREiHUS study, expected in the fall of 2020, the Dutch guideline regarding treatment of aHUS patients should be critically evaluated. With the learned experiences, further improvements should be implemented to pursue the best tailored care for patients with aHUS.

Appendices

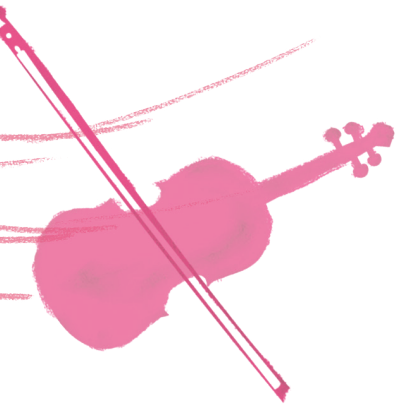
"The most important thing to do is really listen"

Itzhak Perlman



Appendices

References, data management, list of abbreviations



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Research data management

Research data presented in this thesis and obtained during this PhD at the department of pediatric nephrology were achived according the Findable, Accessible, Interoperable and Reusable (FAIR) principles. All studies were conducted according the principles of the declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO), Dutch Code of conduct, Dutch Personal Data Protection Act and the Medical Treatment Agreement Act. Research data management was conducted according Good Clinical Practice (GCP) requirements.

Both chapter 2 and chapter 3 contain data which were collected using the webbased database Castor. Furthermore, patient data gathered for the ongoing CUREiHUS study (as described in chapter 10) are stored in Castor. The hosting of Castor is by ISO:27001 and NEN7510, certified datacenters. The system of the database enables an audit trail after each modification and login events. Login actions can only be with a personal (role and center specific) authorization with qualified passwords. Castor has a monitor function whereby data controls will be executed.

All laboratory experiments were described in paper lab journals and stored appropriately. With the introduction of Labguru, a digital labjournal with backup facilities, all experiments were registered in Labguru. Remaining data is stored at the research server of the department of pediatrics in an encrypted subdirectory. Furthermore, all data regarding the CUREiHUS study are stored in the digital research environment (DRE) facilitating the FAIR principles. DRE offers researchers a safe and well equipped research environment for analysis, storage and sharing of data and is supported by the Executive Board of the Radboudumc. Both servers can be accessed by associated scientific staff members.

All data generated or analyzed in this thesis was included in published articles and its additional files are available from the associated corresponding authors on request. Data will be kept until fifteen years after publication. After the study was finished and scientifically published, subject material from the study will only be stored and possibly used for future studies when subjects agree with this, as included in the informed consent. Without agreement of the medical ethical committee, materials will not be used for future studies that do not have a direct relation to the current study and not for studies that involve the chance of finding additional outcomes. If subjects do not give permission to use their material for future studies, the material will be discarded after this study has been finished and scientifically published.

List of abbreviations

ABMR	Antibody mediated rejection
ABOi	ABO incompatible
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
aHUS	Atypical hemolytic uremic syndrome
anti-CFH	Autoantibodies against complement factor H
AP	Alternative pathway
AP50	Alternative pathway activity
AU	Arbitrary units
AUC	Area under the curve
BIA	Budget impact analysis
BSA	Bovine serum albumin
Bx	Kidney biopsy
CEA	Cost-effectiveness analysis
CFB	Complement factor B
CFH	Complement factor H
CFHR	Complement factor H related protein
CFI	Complement factor I
CH50	Total hemolytic complement activity
CI	Confidence interval
CL	Clearance
Cmax	Peak plasma concentration
CNI	Calcineurin inhibitor
CP	Classical complement pathway
Ctrough	Trough level
CV	Coefficient of variance
DAF	Decay acceleration factor
DBD	Donation after brain death
DCD	Donation after cardiac death
DD	Deceased donor
DGKε	Diacylglycerol kinase epsilon
eae	Attaching and effacing
Ecu	Eculizumab
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ESRD	End stage renal disease

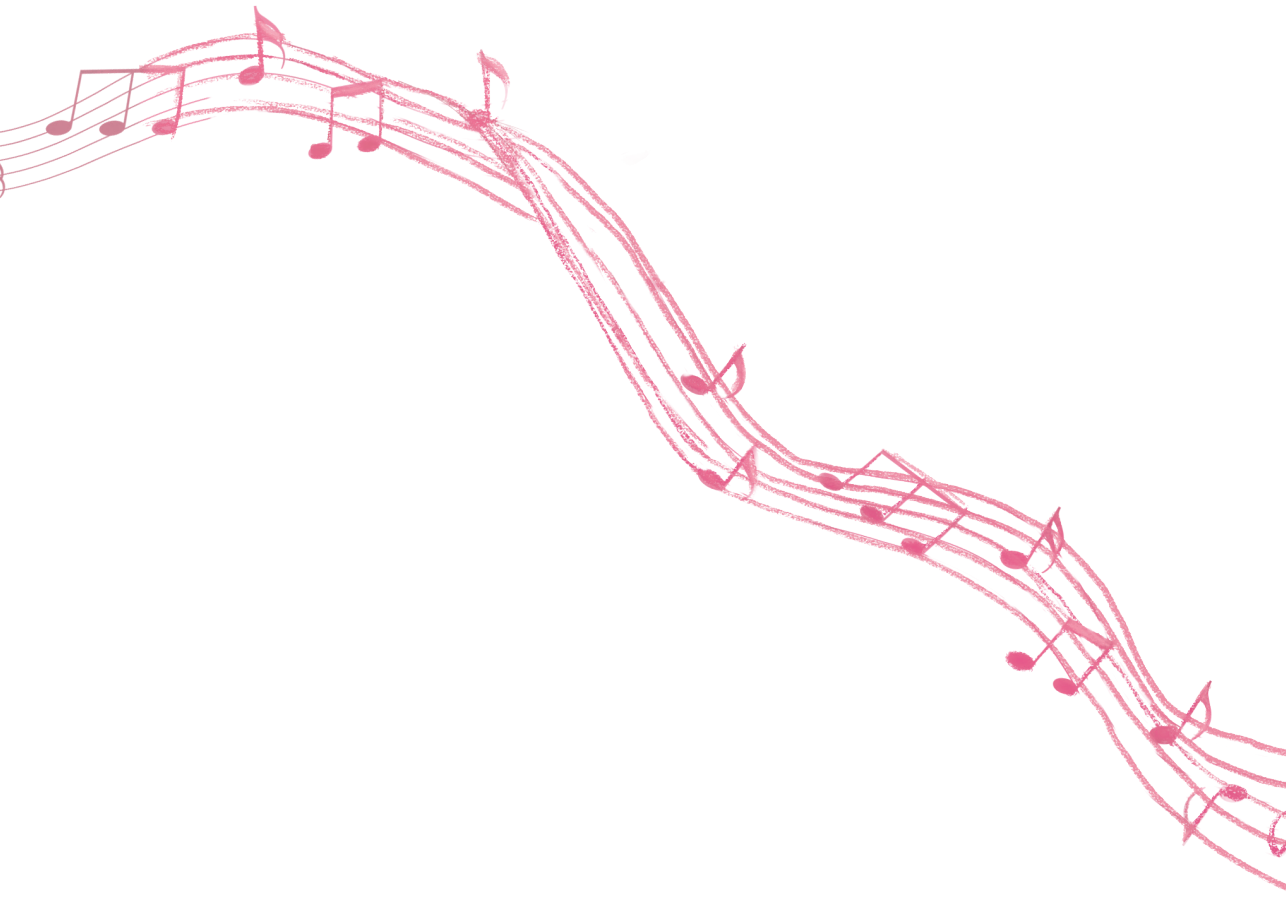
FDA	Food and Drug Administration
FStx	Free fecal shiga toxin test
FU	Follow up
Gb3	Globotriaosyl ceramide
glyco-iELISA	Glycoprotein based ELISA
gMG	Generalized myasthenia gravis
HAHAs	human antihuman antibodies
HGMVECs	Human glomerular microvascular endothelial cells
HO-1	Heme oxygenase 1
HRQoL	Health-related quality of life
HSCT	Hematopoietic stem cell transplantation
HUS	Hemolytic uremic syndrome
IC	Informed consent
ICU	Intensive care unit
IgM	Immunoglobulin M
IMG	Idiopathic membranous glomerulopathy
iPCQ	Productivity Costs Questionnaire
IQR	Interquartile range
IVIG	Intravenous immunoglobulin
KDIGO	Kidney Disease Improving Global Outcome
LD	Living donor
LDH	Lactate dehydrogenase
LP	Lectin pathway
LPS	Lipopolysaccharide
LRD	Living related donor
LURD	Living unrelated donor
MAC	Membrane attack complex
MASP2	Mannose-binding lectin-associated serine protease 2
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
RD	Modification of Diet in Renal Disease equation
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NA	Not applicable
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute for Health and Care Excellence
OD	Optical density
OR	Odds ratio

P/N ratio	Positive/negative ratio
P50	Median percentile for height and age
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline and tween
PCR	Polymerase chain reaction
PCR	protein-to-creatinine ratio
PD	Pharmacodynamic
PE	Plasma exchange
PESaM	Patient Experiences and Satisfaction with Medications
PI	Plasma infusion
PK	Pharmacokinetic
PLG	Plasminogen
PNH	Paroxysmal nocturnal hemoglobinuria
PREMs	Patient-reported experience measures
pRIFLE	pediatric Risk, Injury, Failure, Loss, End-stage renal disease
PT	Plasmatherapy
QALY	Quality adjusted life year
qPCR	quantitative polymerase chain reaction
RA	Rheumatoid arthritis
Radboudumc	Radboud university medical center
RIVM	Dutch National Institute for Public Health and Environment
ROS	Reactive oxygen species
RT	Room themperature
sC5b-9	Soluble C5b-C9
Screat	serum creatinine
SD	standard deviation
SLE	Systemic lupus erythematosus
SMAC	Sorbitol MacConky agar
SPC	Summary of Product Characteristics
STEC	Shiga toxin producing *Escherichia coli*
Stx	Shiga toxin
TCC	Terminal complement complex
TDM	Therapeutic drug monitoring
TF	Tissue factor
THBD	Thrombomodulin
TMA	Thrombotic microangiopathy
TMB	Tetramethylbenzidine
TTP	Thrombocytopenic purpura
Tx	Kidney transplantation

Vd	Volume of distribution
WGS	Whole genome sequencing
WMO	Medical Research Involving Human Subjects Act

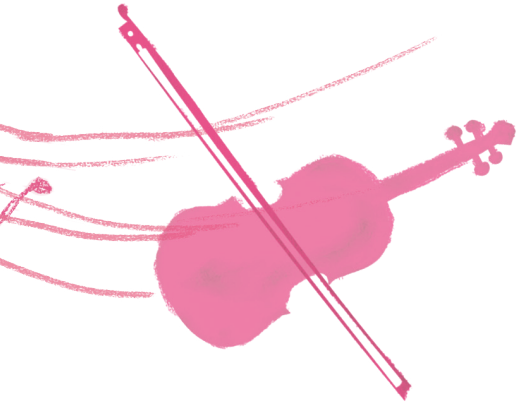
“Wanneer je je reis naar Ithaca aanvangt,
bid dan dat de weg lang mag zijn,
vol avontuur,
rijk aan stof tot kennis.
Wees niet bang.”

Konstantínos P. Kavákis



Appendices

Nederlandse samenvatting

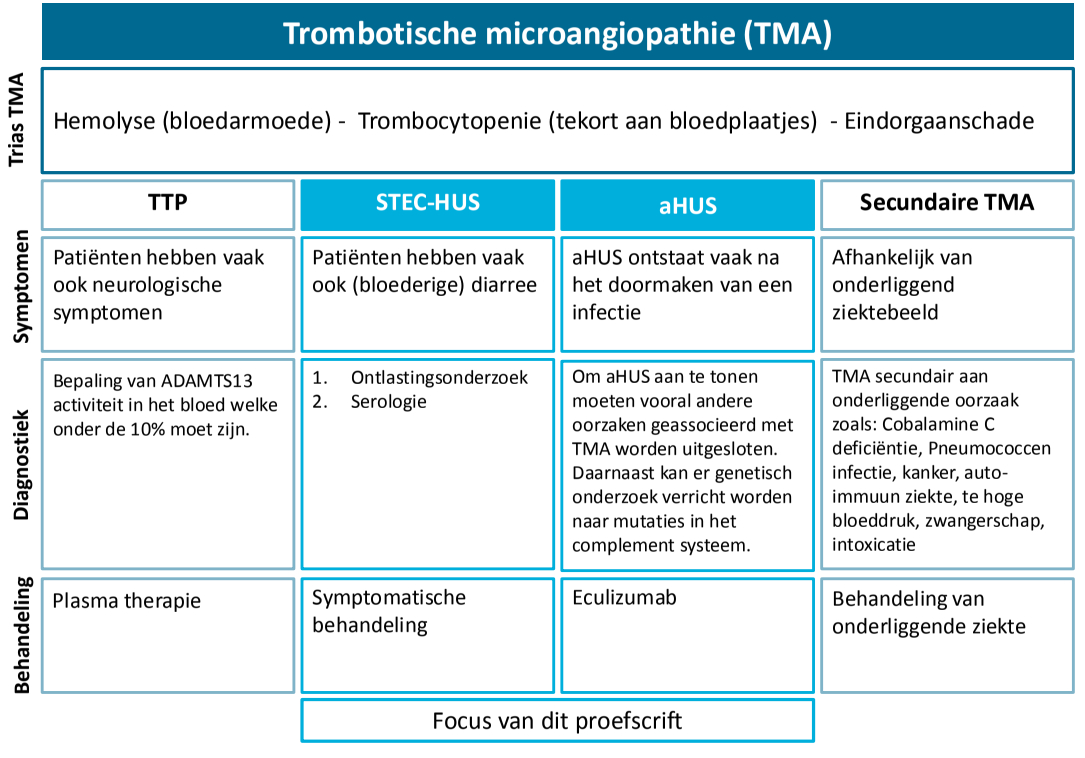


Nederlandse samenvatting

Het leven van de patiënten met een atypische vorm van het hemolytisch uremisch syndroom (HUS) veranderde drastisch met de implementatie van het weesgeneesmiddel eculizumab in 2011. Door de komst van dit geneesmiddel is de prognose van patiënten met atypische HUS (aHUS) enorm verbeterd: daar waar deze patiënten vroeger vaak niet herstelden met de standaard behandeling en vaak aangewezen waren op levenslange nierfunctievervangende therapie, treedt nu meestal herstel van de nierfunctie op, en hebben de patiënten een vrijwel normaal leven. Ondanks dat eculizumab een zeer effectief en potent geneesmiddel is, wordt eculizumab beschouwd als een van de duurste medicamenten ter wereld. Het beschikbaar komen van dit effectieve, maar ook dure geneesmiddel heeft geleid tot vele nieuwe vragen, o.a. met betrekking tot het diagnosticeren van aHUS en het optimale gebruik van eculizumab.

Atypische HUS is een zeldzame en ernstige vorm van trombotische microangiopathie (TMA). TMA is een verzameling van verschijnselen welke ontstaan als gevolg van vaatwandschade hetgeen leidt tot trombose in de kleine bloedvaten. TMA wordt gekenmerkt door een trias aan symptomen: hemolyse (afbraak van rode bloedcellen), trombocytopenie (tekort aan bloedplaatjes) en perfusie stoornis (zuurstoftekort) leidend tot eind orgaanschade. De nieren in het bijzonder zijn vatbaar voor deze effecten en TMA leidt dan ook vaak tot acuut nierfalen. Vele verschillende oorzaken kunnen ten grondslag liggen aan het ontwikkelen van TMA. Om TMA adequaat te kunnen behandelen is het van groot belang de onderliggende oorzaak te diagnosticeren. Grofweg wordt TMA in vier subgroepen onderverdeeld (**Figuur 1**). Echter is er veel overlap in symptomatologie tussen deze vier subgroepen, wat het klinisch onderscheid bemoeilijkt. De meest voorkomende vorm van TMA wordt bij kinderen veroorzaakt door een infectie met de shiga-toxine producerende *E.coli* (STEC), resulterend in STEC-HUS. Bij volwassenen wordt met name trombotische trombocytopenische purpura (TTP), veroorzaakt door verminderde activiteit van het ADAMTS13 eiwit, als oorzaak van TMA gezien. Snelle en goede diagnostiek is nodig om de juiste diagnose te stellen en adequate behandeling te kunnen starten. Het onderzoek zoals beschreven in dit proefschrift richt zich op twee subgroepen van TMA. In **sectie I** van dit proefschrift wordt de diagnose en pathofysiologie van STEC-HUS besproken. In **sectie II** van dit proefschrift wordt ingegaan op de behandeling van patiënten met aHUS.

Figuur 1. Verschillende oorzaken van trombotische microangiopathie



Figuur 1. Er zijn verschillende oorzaken die kunnen leiden tot een trombotische microangiopathie (TMA). Klinisch zijn deze ziektebeelden moeilijk van elkaar te onderscheiden doordat alle ziektebeelden dezelfde trias aan symptomen hebben passend bij TMA. Trombotische trombocytopenische purpura (TTP) en shiga-toxine producerende E.coli hemolytisch uremisch syndroom (STEC-HUS) zijn de meest voorkomende oorzaken van TMA. Daarnaast kan atypisch hemolytisch uremisch syndroom (aHUS) alleen worden aangetoond door andere oorzaken van TMA uit te sluiten. Omdat de behandeling sterk verschild is het van groot belang de juiste diagnose te stellen.

Sectie I Nieuwe inzichten in de pathofysiologie en diagnose van STEC-HUS

Meer dan 90% van de kinderen die zich presenteren met het beeld van HUS, heeft STEC-HUS. STEC-HUS komt met name op de kinderleeftijd voor (vooral tussen 1-5 jaar) en ontstaat na een darminfectie met de STEC-bacterie. Bij de overgrote meerderheid van de patiënten is er sprake van diarree die in de meeste gevallen bloederig is. Slechts een deel (15%) van de mensen met een darminfectie veroorzaakt door de STEC-bacterie ontwikkelt uiteindelijk HUS.

De shiga toxines welke geproduceerd worden door de STEC-bacterie veroorzaken vermoedelijk schade aan de vaatwand, resulterend in TMA. Er zijn verschillende serotypes van STEC die STEC-HUS kunnen veroorzaken. Ondanks dat STEC-O157 nog steeds het meest voorkomende serotype is bij patiënten met STEC-HUS, worden er steeds meer non-O157 serotypes (zoals O26, O103, O104 etc.) gevonden. Om STEC-HUS te diagnosticeren wordt ontlasting en bloed onderzocht. Het ontlastingsonderzoek bestaat uit een feceskweek en polymerase-kettingreactie (PCR) waarmee gekeken wordt naar de aanwezigheid van shiga toxine in de ontlasting. Helaas is vaak het ontlastingsonderzoek alleen niet toereikend om de diagnose STEC-HUS te kunnen stellen. Bij meer dan 30% van de patiënten met een sterke klinische verdenking op STEC-HUS kan geen STEC worden aangetoond in de ontlasting.

In het bloed kunnen antistoffen aanwezig zijn tegen het lipopolysaccharide, de buitenmembraan van de STEC-bacterie. Deze antistoffen kunnen worden aangetoond met behulp van een lipopolysaccharide enzyme-linked immuno sorbent assay (LPS-ELISA). Serologisch onderzoek in het kader van de diagnostiek van STEC-HUS wordt in Nederland alleen in het Radboudumc in Nijmegen uitgevoerd. In **hoofdstuk 2** hebben we onderzocht wat de toegevoegde waarde is van de LPS-ELISA voor detecteren van STEC-O157 infectie in relatie tot ontlastingsonderzoek. Bij een groep kinderen met een klinische verdenking op STEC-HUS werd de diagnose STEC-HUS in 54% van de gevallen bevestigd door ontlastingsonderzoek. De diagnose STEC-HUS werd in 63% bevestigd door de LPS-ELISA specifiek gericht tegen het meest voorkomende serotype bij STEC-HUS, serotype O157. Bovendien, wanneer ontlastingsonderzoek gecombineerd werd met serologie voor STEC-O157 (met de LPS-ELISA) kon in maar liefst 77% van de patiënten met verdenking op STEC-HUS, de diagnose worden bevestigd. Bij de overige 23% van de patiënten kan STEC-HUS veroorzaakt zijn door een ander serotype dan STEC-O157. Ook kan er sprake zijn van een andere oorzaak van HUS.

Echter heeft de LPS-ELISA ook enkele nadelen. Door de structuur van de LPS die gebruikt wordt in deze test kan er eventuele kruisreactiviteit ontstaan tussen verschillende serotypes van STEC (o.a. O157, O26, O103) en zelfs tussen verschillende gram negatieve bacteriën. Hierdoor kan de uitslag vals positief zijn. In **hoofdstuk 3** wordt

een nieuwe serologische assay beschreven die dit probleem verhelpt door gebruik te maken van recombinante glycoproteïnes (glyco-iELISA). In vergelijking met de oude test (LPS-ELISA) kon de glyco-iELISA STEC-HUS op basis van STEC-O157 vaststellen bij 10 patiënten waarbij de uitslag negatief was met de LPS-ELISA. Daarnaast bleek dat bij drie patiënten de test vals positief was met de LPS-ELISA. Door ontlastingsonderzoek te combineren met de glyco-iELISA voor STEC-O157 kon in 89% van de patiënten STEC-HUS worden gediagnosticeerd. Patiënten zijn alleen getest op de aanwezigheid van antistoffen tegen het serotype STEC-O157. Het plan is om de glyco-iELISA in de toekomst uit te breiden voor de meest voorkomende serotypes van STEC.

In hoofdstuk 2 en 3 ligt de focus vooral op STEC serotype O157, echter er worden steeds meer gevallen beschreven van patiënten met STEC-HUS die veroorzaakt worden door andere STEC-serotypes dan O157. In **hoofdstuk 4** wordt een casus van een 16 maanden oude jongen beschreven met een ernstig en atypisch beloop van STEC-HUS, veroorzaakt door het zeldzame STEC serotype O80. Initieel, mede gezien de afwezigheid van diarree, werd er gedacht aan aHUS, derhalve heeft de patiënt een gift eculizumab ontvangen. Echter de volgende dag werd door een positieve PCR uitslag voor de aanwezigheid van shiga toxine in de ontlasting de diagnose STEC-HUS bevestigd. Vervolgens is met behulp van moleculaire serotypering door middel van whole genome sequencing de STEC-bacterie verder getypeerd. Deze patiënt bleek STEC-HUS te hebben op basis van het zeldzame O80 serotype welke bekend staat vanwege de potente virulentie factoren. Deze casus onderstreept het belang van adequate diagnostiek en virulentie bepaling van de STEC-bacterie bij patiënten met HUS, juist ook bij patiënten met een atypische presentatie van HUS, om zo de juiste behandeling te kunnen starten.

Ondanks dat STEC-HUS een veelvoorkomende oorzaak is van acuut nierfalen bij kinderen is er nog steeds weinig bekend over de exacte pathofysiologie waardoor een STEC-infectie kan leiden tot HUS. Zo ontwikkelt slechts 15% van de patiënten met een STEC-infectie HUS. In **hoofdstuk 5** onderzochten we het effect van de toxische stof heem op het ontstaan en verergeren van HUS. Heem komt vrij als erytrocyten (rode bloedcellen) vervallen, bijvoorbeeld bij hemolyse zoals aanwezig bij patiënten met TMA. Heem is schadelijk als het in grote hoeveelheden tegelijk vrijkomt in de bloedbaan. In een groep van 48 STEC-HUS patiënten werd er een statistisch significant hogere heem-concentratie in het bloed gevonden in vergelijking met gezonde controles. Deze heem-concentraties werden vervolgens aangebracht op endotheelcellen (vaatwandcellen) *in vitro*. Toediening van heem op endotheelcellen cellen leidde tot een verhoogde stollings- en ontstekingsreactie, zoals ook aanwezig bij patiënten met HUS. Dit effect werd versterkt door het tegelijk aanbrengen van heem en shiga toxine op de cellen. Heem, wat vrijkomt bij patiënten met HUS door het verval van erytrocyten, kan dus bijdragen aan het ontstaan van STEC-HUS.

Conclusie sectie I

Het vaststellen van een STEC-infectie heeft vergaande behandelconsequenties voor patienten die zich presenteren met HUS. Aangezien aHUS niet direct middels een test aan te tonen is, wordt het onderscheid tussen STEC-HUS en aHUS gemaakt op basis van het aantonen dan wel uitsluiten van een STEC-infectie. Zoals aangetoond in hoofdstuk 2 en 3 is serologie, in combinatie met ontlastingsonderzoek, van essentieel belang in de diagnostiek van STEC-HUS. In de toekomst moeten deze serologische assays uitgebreid worden met andere STEC serotypes om zo de groeiende groep van non-O157 STEC-bacteriën te kunnen detecteren. Daarnaast worden moleculaire assays steeds belangrijker niet alleen voor de diagnostiek maar mede ook om virulentie factoren van de STEC-bacterie te bepalen. Dit is niet alleen van belang voor de behandeling en prognose van patiënten met STEC-HUS, maar geeft ook inzichten over de epidemiologie van STEC in Nederland.

Het is tot op heden onbekend waarom maar 15% van de patiënten een HUS ontwikkelt na een STEC-infectie. Waarschijnlijk heeft dit te maken met een complexe interactie tussen patiëntkarakteristieken, omgevingsfactoren en specifieke kenmerken van de STEC-bacterie. Een van de mogelijk factoren die een rol kan spelen is heem. Aangezien heem een pro-trombotisch effect heeft kan een verhoogde heem-concentratie een rol spelen in het ontstaan dan wel verergeren van TMA. Vervolgonderzoek moet aantonen of deze bevinding behandelconsequenties kan hebben voor patiënten met STEC-HUS.

Voor patiënten met STEC-HUS is er tot op heden geen specifieke behandeling, en worden patiënten alleen symptomatisch behandeld. STEC-HUS gaat bij de meeste patiënten vanzelf over mits de patiënt tijdig herkend wordt en op tijd symptomatische therapie (onder andere nierfunctie vervangende therapie, adequaat vochtbeleid en bloedtransfusies) ontvangt. Het gebruik van eculizumab als behandeling voor patiënten met STEC-HUS is tot op heden omstreden. Echter lopen er momenteel twee studies om het effect van eculizumab behandeling voor patiënten met STEC-HUS te onderzoeken. Deze studies zullen uitsluitsel geven of eculizumab behandeling van toegevoegde waarde is voor de behandeling van patiënten met STEC-HUS.

Sectie II Op weg naar een gepersonaliseerde behandeling voor iedere patiënt met aHUS

Atypische HUS is een ernstige en zeldzame vorm van HUS en kan voorkomen op alle leeftijden. In tegenstelling tot STEC-HUS kan aHUS een chronische ziekte zijn met een recidiverend karakter. Atypische HUS wordt ook wel complement gemedieerde HUS genoemd. Het complement systeem is een belangrijk onderdeel van het aangeboren afweersysteem en speelt een zeer belangrijke rol bij de afweer tegen micro-organismen. De belangrijkste functies van het complement systeem zijn opsonisatie door C3b, chemotaxis door C3a en C5a en het lyseren van cellen door de vorming van het zogenoemd membrane attack complex. Eigen lichaamscellen, zoals de vaatwand, zijn beschermd tegen het complement systeem door de aanwezigheid van verschillende complement regulatoren. Deze complement regulatoren spelen een belangrijke rol in het in balans houden van het complement systeem en voorkomen ontregeling. Veranderingen in de genetische codering van een aantal van deze eiwitten uit het complement systeem maken dat het complementsysteem te actief is en/of te actief blijft. Een overactief en niet goed gereguleerd complement systeem kan leiden tot vaatwand beschadiging, waardoor patienten aHUS kunnen ontwikkelen. Op dit moment wordt in 40 tot 60% van de patiënten met aHUS een erfelijke aanleg in het complement systeem gevonden, dan wel antistoffen gericht tegen de complement eiwitten.

Atypische HUS is een ernstig ziektebeeld met hoge morbiditeit en mortaliteit. Daarnaast ontstaat er vaak schade, waardoor een patiënt chronisch nierfalen kan ontwikkelen. Bij deze groep patiënten is dialyse noodzakelijk en in sommige gevallen een niertransplantatie. Indien geen specifieke maatregelen genomen worden, krijgt helaas de overgrote meerderheid van aHUS patiënten een recidief aHUS in de nieuwe getransplanteerde nier. Sinds 2011 is eculizumab de standaard behandeling voor aHUS en zijn deze lange termijn gevolgen sterk verbeterd. Eculizumab is een gehumaniseerd monoklonaal antilichaam gericht tegen complement C5 en blokkeert de splitsing van C5 in zijn actieve bestandsdelen: C5a en C5b. C5b is noodzakelijk is voor de vorming van het membrane attack complex. Door de remming van het membrane attack complex kan er geen schade meer door het overactieve complement systeem ontstaan aan de vaatwand. Volgens een vastgesteld schema (één dosis per twee weken in de onderhoudsfase bij volwassenen) wordt eculizumab via het infuus in het ziekenhuis toegediend. De farmaceut raadt aan het medicament levenslang te geven. Ondanks dat de ziekte meestal goed reageert op de behandeling met eculizumab, zitten er ook nadelen aan de behandeling. Zo hebben patiënten onder andere een sterk verhoogde kans op het ontwikkelen van meningitis en zijn er enkele studies die leverschade door eculizumab beschrijven. Daarnaast is er nog weinig bekend over het beste behandelschema en behandelduur, de precieze werking van eculizumab, en mogelijke overige risico's en bijwerkingen. Bovendien is eculizumab een

van de duurste medicamenten ter wereld, met kosten oplopend tot 500.000 euro per jaar per patiënt. Kortom, er is ruimte voor het optimaliseren van de behandeling van aHUS.

In **hoofdstuk 6** worden eculizumab spiegels in het bloed gerapporteerd. Deze spiegels zijn gemeten bij elf aHUS patiënten gedurende een standaard behandeling en tijdens het uitbreiden van het interval tussen de eculizumab toedieningen. Om een adequate onderdrukking van het complement systeem te waarborgen, wordt gestreefd naar een dalspiegel van 50-100 µg/ml. In deze studie lieten we zien dat de eculizumab spiegels vele malen hoger waren dan de streefwaardes (tot 772 µg/ml) bij patiënten die eculizumab elke twee weken ontvingen. Bovendien had een substantieel aantal van de patiënten die eculizumab ontvingen met een uitgebreid interval (tot vier weken) ook nog adequate dalspiegels. Hieruit kan worden geconcludeerd dat er minder eculizumab gegeven hoeft te worden om adequate spiegels te bereiken.

Om meer inzicht te krijgen in de optimale dosering en het effect van eculizumab, is er een uitgebreide literatuurstudie uitgevoerd welke staat beschreven in **hoofdstuk 7**. Hierin wordt alle literatuur besproken omtrent de eculizumab behandeling geïndiceerd voor verschillende aandoeningen waaronder aHUS en pleiten we voor therapeutische drug (medicatie) monitoring. Door zowel de spiegels als het effect van eculizumab te monitoren bij elke patiënt, kan de behandeling worden aangepast naar het individu.

Naast het aanpassen van het interval tussen de eculizumab toedieningen kan de behandeling ook gestaakt worden. In **hoofdstuk 8** worden twintig aHUS patiënten besproken waarbij de eculizumab behandeling bij zeventien patiënten werd gestaakt en bij drie patiënten aangepast werd door middel van een verlengd interval. Bij vijf patiënten (25%) resulteerde de aangepaste behandeling in een recidief aHUS, waarna de eculizumab behandeling is herstart zonder chronische schade. Naar schatting is er 11.4 miljoen euro bespaard dankzij deze aanpassingen.

Afgezien van onze studie, zoals beschreven in hoofdstuk 8, zijn er enkele andere studies gepubliceerd die het aanpassen en staken van eculizumab beschrijven. In **hoofdstuk 9** wordt alle literatuur betreffende het aanpassen en staken van eculizumab therapie bij aHUS patiënten besproken. Over het algemeen is het veilig en effectief om eculizumab behandeling te staken, mits er strikte monitoring voor een mogelijk recidief plaatsvindt. Ongeveer 27% van de patiënten beschreven in de literatuur ontwikkelde een recidief, meestal binnen het eerste jaar na staken. Er werd geen duidelijke chronische schade gevonden na het herstarten van eculizumab behandeling. In dit hoofdstuk worden verschillende strategieën beschreven om de behandeling aan te passen dan wel te staken.

Conclusie sectie II

De introductie van eculizumab heeft geleid tot een sterke verbetering van de prognose van patiënten met aHUS. In veel landen wordt eculizumab volgens een standaard schema voorgeschreven, in potentie levenslang. Echter is er weinig tot geen bewijs voor de noodzaak van deze levenslange duur. Door het toepassen van therapeutische drug monitoring kan de dosering van eculizumab worden geoptimaliseerd per patiënt. Daarnaast lijkt ~70% van de patiënten ziektevrij voor minimaal 1 jaar na het staken van eculizumab behandeling. Kortom, er is ruimte voor verbetering (**Figuur 2**). In dit proefschrift wordt een uniek en aangepast behandelschema besproken voor patiënten met aHUS.

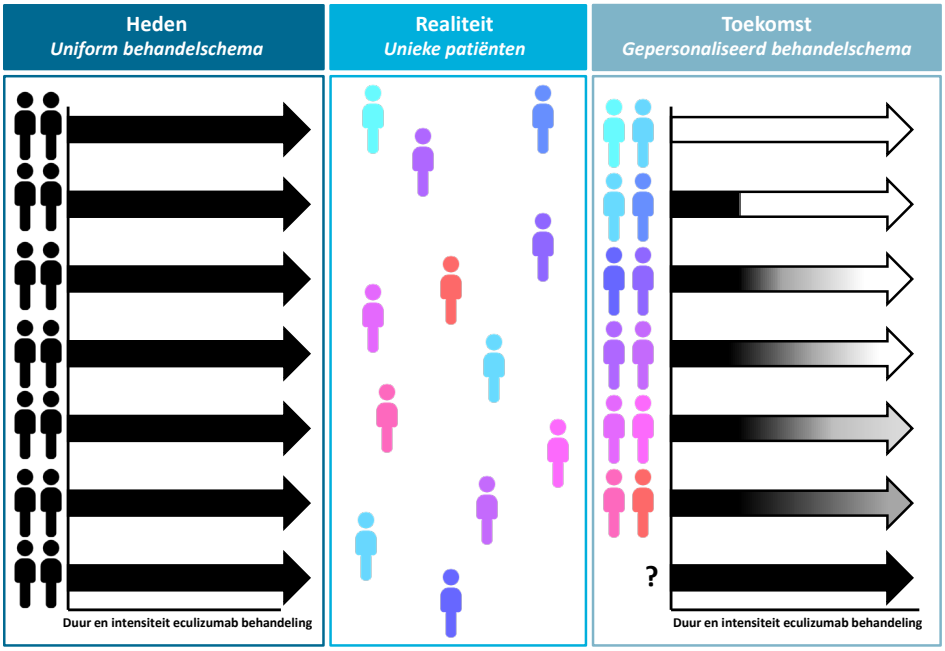
Om de zorg te optimaliseren, en om betaalbare en toegankelijke gezondheidszorg te waarborgen, is er in Nederland sinds januari 2016 een nieuwe richtlijn voor de behandeling van aHUS geïntroduceerd. Deze richtlijn is opgesteld door de landelijke werkgroep aHUS, bestaande uit een nefroloog en een kindernefroloog uit elk universitair medisch centrum in Nederland. In **hoofdstuk 10** beschrijven we het studieprotocol van de landelijke, observationele studie om de nieuwe richtlijn te monitoren en evalueren. Deze studie, ook wel CUREiHUS studie genoemd, includeert alle aHUS patiënten die behandeld worden volgens de nieuwe richtlijn. Patiënten worden gevraagd vragenlijsten in te vullen en er wordt bloed afgenomen om te kijken naar complement activatie, eculizumab spiegels en biomarkers voor het voorspellen van een recidief. Met behulp van twee vragenlijsten om de kwaliteit van leven en productiviteitsverliezen te meten zal aan het einde van studie een kosteneffectiviteitanalyse worden uitgevoerd. De CUREiHUS studie zal naar verwachting aflopen in oktober 2020. Aan de hand van de resultaten kan de behandeling van aHUS patiënten verder worden geoptimaliseerd.

Eculizumab is slechts één voorbeeld van de vele dure weesgeneesmiddelen die op de markt zijn gekomen. Door de scherpe toename in dure geneesmiddelen komt de toegankelijkheid en betaalbaarheid van de Nederlandse zorg onder druk te staan. Daarnaast is door het zeldzame karakter van veel ziektebeelden de beschikbare informatie omtrent de grootte van het effect, de kosteneffectiviteit en de klinische relevantie vaak beperkt. In Nederland is er een weesgeneesmiddelen arrangement geïmplementeerd door het Zorginstituut om zo de vergoeding van weesgeneesmiddelen te optimaliseren. Eculizumab was het eerste medicament welke volgens dit weesgeneesmiddelen arrangement werd geïmplementeerd. De CUREiHUS studie kan als voorbeeld dienen voor het opzetten en inrichten van dergelijke initiatieven om de (kosten)effectiviteit van weesgeneesmiddelen, onafhankelijk van de farmaceutische industrie, te evalueren.

Met het onderzoek gepresenteerd in dit proefschrift hoop ik een aanzet te geven tot het verder optimaliseren van de diagnostiek van STEC-HUS en de behandeling van patiënten met aHUS. Het is van groot belang om de zorg voor HUS patiënten te blijven evalueren en aan de hand hiervan passende en innovatieve maatregelen te bedenken én

te implementeren. Als artsen, patiënten, onderzoekers, politici, zorgverzekeraars en farmaceutische bedrijven de handen ineen te slaan, kunnen we samen bouwen aan goede, betaalbare en toegankelijke gezondheidszorg.

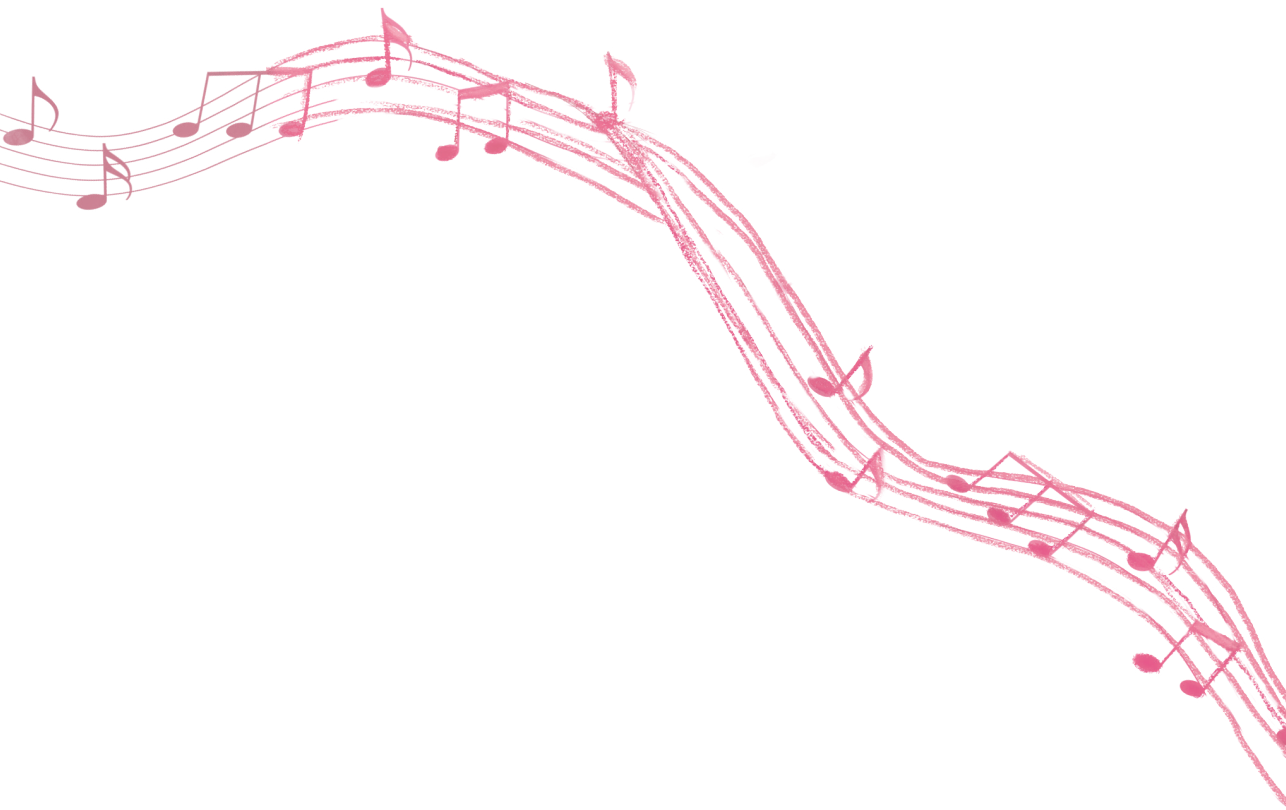
Figuur 2. Gepersonaliseerde behandeling voor patiënten met aHUS



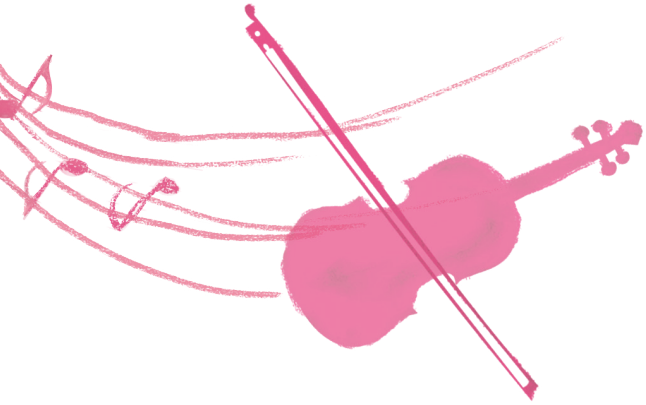
Figuur 2. Volgens het standaard behandelschema zoals beschreven door de farmaceut, ontvangt elke aHUS patiënt een vaste dosis eculizumab volgens een vast interval (zwarte balk). Echter de realiteit is weerbarstiger. Aan de hand van specifieke patiëntkarakteristieken (weergegeven door de verschillende kleuren) kan een gepersonaliseerd behandelschema worden opgesteld. Zo kan het mogelijk zijn om de eculizumab therapie te staken, dan wel de behandeling af te bouwen op geleide van spiegels. Naar verwachting zal maar in een beperkte groep patiënten levenslange therapie noodzakelijk zijn.

"Be yourself, everyone else is already taken"

Oscar Wilde



About the author



Curriculum Vitae

Kioa Lente Wijnsma, dochter van Maria Theresia Cecilia Meulenbeek en Pieter Wijnsma, werd geboren op 8 november 1990 te Utrecht waar zij samen met haar zusje Uzume Zoë opgroeide. In 2008 behaalde zij haar VWO diploma aan het Sint Bonifatius College in Utrecht waarna ze startte met de studie Geneeskunde aan de Radboud Universiteit Nijmegen. Naast haar studie heeft ze via het excellent student program van het Amalia kinderziekenhuis onderzoek gedaan naar kinderen met een respiratoir syncytieel virus infectie, onder begeleiding van Dr. I.M.L. Ahout en Dr. J.G. Ferwerda van 2010 t/m 2014 op het laboratorium kinderinfectiologie. In 2014 werd ze aangenomen voor het dedicated schakeljaar kindergeneeskunde bij de kindernefrologie in het Radboudumc Amalia kinderziekenhuis en bij de algemene kindergeneeskunde in het Canisius Wilhelmina Ziekenhuis te Nijmegen. Gedurende het schakeljaar heeft ze onder begeleiding van Dr. N.C.A.J. van de Kar onderzoek gedaan naar de diagnostiek van het hemolytisch uremisch syndroom, welke preliminaire resultaten ze mocht presenteren in december 2014 op het One Health symposium Focus on STEC-HUSEC te Groningen.

Na het succesvol afronden van haar studie Geneeskunde in februari 2015 startte Kioa haar promotieonderzoek omtrent diagnostiek en behandeling van het hemolytisch uremisch syndroom op de afdeling kindernefrologie in het Radboudumc Amalia kinderziekenhuis onder dagelijkse begeleiding van Dr. N.C.A.J. van de Kar, mede onder begeleiding van Prof.dr. L.P. van den Heuvel, Prof.dr. J.F.M. Wetzels en Dr. E.B. Volokhina. Dit onderzoek heeft geresulteerd in meerdere artikelen, verscheidende presentaties op (inter)nationale congressen en dit proefschrift. Gedurende haar promotieonderzoek heeft Kioa deelgenomen aan verschillende cursussen waaronder de Da Vinci Challenge en heeft zij meerdere studenten begeleid (zie ook PhD portfolio). Met hulp van het Sengers-Stipendium van Stichting Kindergeneeskunde heeft ze voor enkele maanden een student assistent kunnen aannemen om het onderzoek naar de rol van heem in kinderen met het hemolytisch uremisch syndroom voort te zetten.

Per 1 Januari 2019 is Kioa in opleiding tot kinderarts in het Canisius Wilhelmina Ziekenhuis (opleider dr. P.G. Voorhoeve) en Radboudumc Amalia kinderziekenhuis (opleider dr. A.A.E.M. van Alfen-van der Velden). Op 1 juni 2019 zal ze trouwen met Ties in San Gimignano, Toscane, Italië.

PhD portfolio

Name PhD student:	***K.L. Wijnsma***	**PhD period:**	***01-03-2015 until 31-12-2018***
Department:	***Pediatric Nephrology***	**Promotors:**	***Prof.dr. L.P. van den Heuvel,***
Graduate school:	***Radboud Institute for***		***Prof.dr.J.F.M. Wetzels***
	Molecular Life Sciences	**Co-promotors:**	***Dr. N.C.A.J. van de Kar,***
			Dr.E.B.Volokhina

TRAINING ACTIVITIES		Year(s)	ECTS
a)	**Courses & Workshops**		
-	Basic course on Regulations and Organisation for clinical investigators (BROK)	2015 2015	1.75 0.2
-	Writing a data management plan	2015, 2016,	2.25
-	IPNA-ESPN Master for Junior Classes, Leuven, Belgium	2017	2
-	Statistics course	2015	0.5
-	Economic evaluation and Health Technology Assessment (HTA)	2015	0.5
-	Winterschool Dutch Kidney Foundation	2016	3.0
-	Academic Writing	2016	0.5
-	Scientific Integrity	2016	1.75
-	PhD in the Lead	2016	0.5
-	ASN precourse glomerular diseases, Chicago, USA	2016	2
-	Participant of Da Vinci Challenge	2018,2019	
b)	**Seminars & Lectures**		
-	Radboud Research Rounds	2015-2018	1
-	Renal Disorders Theme Lunch*	2015-2018	1
-	Conference IQ healthcare	2016	0.25
-	VvAA Smposium medisch leiderschap	2016	0.1
c)	**(Inter)national Symposia & Congresses**		
-	New Frontiers symposium, Nijmegen, the Netherlands	2015, 2016	0.5
-	PhD retreat, Veldhoven, the Netherlands ##*	2015-2018	2.75
-	BeNeLux symposium, Eindhoven, the Netherlands	2015	0.25
-	Complement symposium, Lunteren, the Netherlands	2015	0.25
-	Young Investigator Day, TULIPS, Utrecht, the Netherlands	2015, 2016	0.5
-	PLAN scientific symposium, twice a year in an academic center, the Netherlands	2015-2018	1.5
-	New kids on the block (NfN), Amsterdam, the Netherlands	2015-2016	0.5
-	Dutch Nephrology days (NND), Veldhoven, the Netherlands*^**	2015-2018	3
-	HUS & related disorders, Innsbruck, Austria #*	2015, 2017	2.5
-	Pediatric Association of the Netherlands (NVK) symposium, Veldhoven & Papendal, the Netherlands **^	2015, 2017	2
-	European Society for Pediatric Nephrology (ESPN), Brussel, Belgium & Glasgow, Scotland *#	2015, 2017	2.5
-	Goed Gebruik Geneesmiddelen symposium, ZonMw, Amsterdam, the Netherlands	2015-2018	1

TRAINING ACTIVITIES (Continued)		Year(s)	ECTS
c)	**(Inter)national Symposia & Congresses (Continued)**		
-	Patient organization Nephrology (NVN), patient day, Lunteren & Bunnik, the Netherlands^^	2016, 2017	1
-	Amalia Science Day, Nijmegen, the Netherlands *#	2016, 2018	1
-	Dutch Federation of Nephrology (NfN) Fall symposium, Bunnik, the Netherlands #	2017	0.5
-	American Society of Nephrology, Chicago, USA #	2017	1.25
-	Bessensap, NOW, Amsterdam, the Netherlands *	2017	0.5
-	International symposium on VTEC, Florence, Italy*	2018	1.25
d)	**Other**		
-	Committee member of excellent student programme, among others chair of symposium yearly	2015-2018	2
-	PLAN (Platform for PhD students and postdocs within nephrology) board member and chair (2017-2018)	2015-2018	2
-	Organizing PLAN symposium in Nijmegen	2016	2
-	Travel grant, Stichting Kindergeneeskunde	2015, 2018	
-	Kidney STAR Award, American Society of Nephrology	2016	
-	Price for best poster presentation, NfN, Fall symposium	2016	
-	March of Dimes Award, IPNA	2016	
-	Sengers Stipendium, Stichting kindergeneeskunde, to set up project entitled "New kid on the block: free heme and its role in HUS"	2017	
-	Reviewing various scientific publications	2016-2018	
-	Best presentation of 2017-2018 Amalia Childrens Hospital Research Round	2018	
-	Price best abstract at conference of European Society for Pediatric Nephrology 2018	2018	0.5
TEACHING ACTIVITIES		Year(s)	ECTS
e)	**Lecturing**		
-	Transferable skills (weekly 1 hour education for students)	2017-2018	1
-	Education for interns; Hematuria & Proteinuria, Monthly	2015-2018	1
-	Education MM2TR	2016-2017	0.5
-	Capita Selecta	2018	0.5
f)	**Supervision of internships/other**		
-	Supervision of 6 students: medical biology first and second master internship, twice supervision of scientific research internship medical students for 3 months, supervision internship of student of Saxion, supervision of 2 excellent students for 10 hours per week	2015-2018	9
TOTAL			**58.55**

Invited, oral and poster presentations are indicated with a ^, * and # respectively after the name of the activity, respectively.

List of publications

Wijnsma KL, Duineveld C, van de Wetering J, Dorresteijn E, van der Heijden JW, van Wijk JAE, Gracchi V, Berger SP, de Vries APJ, Bouts AHM, Bemelman FJ, Keijzer-Veen MG, van Zuilen AD, Horuz FAPT, van Paassen P, Kimman ML, Adang E, Brüggemann RJ, Storm M, Volokhina EB, van den Heuvel LP, Wetzels JFM, van de Kar NCAJ. National observational study to monitor the new Dutch guideline concerning treatment of patients with atypical hemolytic uremic syndrome. *Unpublished*

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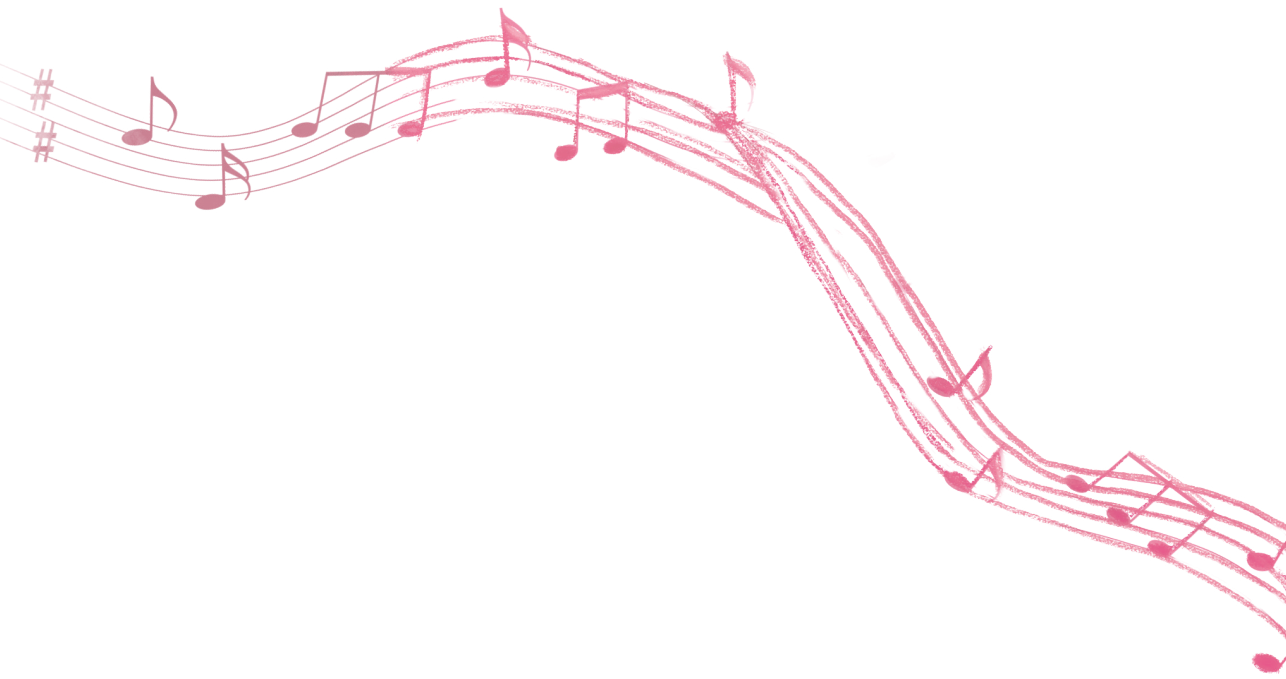
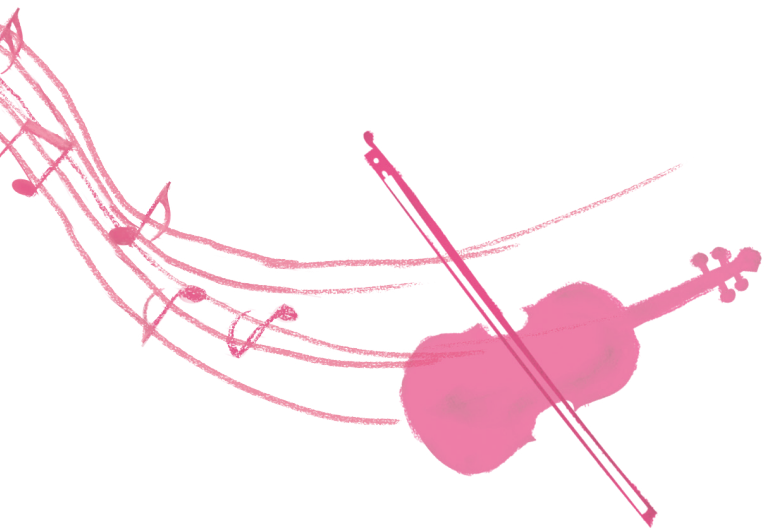
“Veni, Vidi, Amavi”



Appendices

Dankwoord



Dankwoord

In de afgelopen jaren heb ik me ondergedompeld in de onderzoekswereld. Met vallen en opstaan en vooral veel plezier heb ik een hoop geleerd en presenteer ik met trots dit proefschrift. Naast de inhoudelijke verdieping was mijn persoonlijke ontwikkeling ook een belangrijk thema in de afgelopen jaren. Graag wil ik iedereen bedanken die betrokken is geweest bij het tot stand komen van dit proefschrift en bijgedragen heeft aan mijn groei als onderzoeker én persoon.

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Lieve **Marlies, Linda, Charlotte, Jacqueline** en **Femke,** mijn jaren binnen het nefro team, als schakelco en promovenda, heb ik ervaren als een warm bad. Hopelijk eindigt onze samenwerking niet hier, maar mag ik nog wat langer genieten van jullie team! **Marlies**, het vertrouwen wat je mij schonk heb ik als zeer fijn ervaren. **Linda**, wat was ik trots als jij mij bestempelde als "complement expert" en om advies vroeg. **Charlotte**, je eerlijke en recht door zee houding waardeer ik erg. Ik hoop dat we tijd blijven vinden om af en toe bij te kletsen. **Jacqueline**, ik heb genoten van je hartelijkheid. Ties, en ik stiekem toch ook, zijn erg blij met jouw kattenconnecties, die ons Noet en Tibbie hebben opgeleverd!

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siasme en innovatieve blik ben je een aanwinst voor het team. Ik ben heel benieuwd naar de nieuwe farmacologische ontwikkelingen voor eculizumab en ongetwijfeld zal jij daar een sleutelrol in vervullen.

Beste **Eddy**, in de afgelopen jaren ben ik toenemend geïnteresseerd geraakt in Health Technology Assessment, en de kosteneffectiviteitanalyse zoals ook gepland staat voor de CUREiHUS studie. Ik kijk ernaar uit om me meer te kunnen verdiepen hierin en nauw betrokken te zijn bij de analyses aan het einde van de studie. Dank voor je inzet en betrokkenheid bij de CUREiHUS studie.

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De kracht van de CUREiHUS studie zit hem in de nationale samenwerking binnen de landelijke werkgroep aHUS. Dank jullie wel **Eiske, Jacqueline, Antonia, Frederike, Mandy, Arjan, Aiko, Ans, Joost, Valentina, Stefan, Flore en Pieter** dat ik een kijkje in de keuken mocht nemen tijdens de vergaderingen. Door jullie krachten te bundelen hebben jullie samen een unieke richtlijn opgesteld die veel invloed heeft gehad op de vergoeding van eculizumab. Ik wil jullie graag bedanken voor jullie inzet, flexibiliteit en geduld, waardoor we samen de CUREiHUS studie hebben kunnen opstarten. Gelukkig werd ieder van jullie ook gesteund door een groep databasemanagers/research verpleegkundigen. Superfijn om zo de studie achter te kunnen laten in de betrouwbare handen van **Katinka, Caro, Marja, Helma, Marieke, Anneke** en **Dorien.**

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Dear prof. dr. **Juan** Ugalde and dr. **Diego** Comerci, I want to thank you both for your hospitality to welcome Susan in your lab for several weeks and our fruitful collaboration. By combining our knowledge, patient material and the glycoproteins you so kindly supplied, we improved diagnostics in patients with HUS. Hopefully we will meet again the future.

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Susan, it is only appropriate that I write this in English. Although it seems that in the past couple of years your Dutch has become flawless, old habbits die hard. I want to thank you for all your hard work, insights, discussions in the cave and company. You did a great job and already have a successful career with two papers and a PhD position at out Pediatric Nephrology research group. Chapter 3 and 5 would not exist if it was not for you, we are a golden couple! It is with a smile that I think back at our years of collaborations with me as the big boss ofcourse. Hopefully you have forgiven me by now for all my" mierenneukerij". What do you think: shall we set up a new project together?

Gerben, na mijn avontuur als ESP student op jullie afdeling hebben we ons contact warm gehouden door jouw rol als mentor gedurende mijn promotietraject. Ondanks dat het niet nodig was om elkaar meer dan 1x per jaar te zien, staan een aantal van je rake opmerkingen mij nog scherp voor de geest. Zo heb ik je advies opgevolgd: niet wachten met dat doen waar je gelukkig van wordt, in het begin overal ja op zeggen en me (soms) niet tegen alles aan bemoeien. Ik hoop dat we elkaar in de toekomst nog eens tegenkomen.

Wouter, helaas zien we elkaar (te) weinig als mede PhDs op hetzelfde onderwerp. Maar zo af en toe krijg ik de kans even mee te genieten van jouw humor. Ons verblijf in Florence tijdens de VTEC staat nog in mijn geheugen gegrift (samen met de baas in een airbnb, de

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Per toeval werd ik bestuurslid van PLAN, maar mijn promotietijd zou zeker niet hetzelfde geweest zijn zonder jullie: **Hessel, Niki, Eliane, Maarten, Sjoerd, Laura, Tineke en Dominique.** Het was super om jullie op elk congres weer te zien en de befaamde borrels zijn natuurlijk onvergetelijk. **Dominique,** ik durf niet te zeggen wanneer wij hebben besloten dat het een goed idee was om naast PLAN ook eens samen af te spreken, maar een goed idee was het zeker! Openhartige gesprekken (en een heleboel speciaal biertjes natuurlijk) kenmerkt ons contact. Hopelijk weten we deze memorabele avonden te behouden.

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Lieve **Uzume**, ondanks dat ik weinig (lees:niks) snap van jouw promotieonderzoek (ik zat er schijnbaar grofweg 2000 jaar naast als ik iedereen trots probeerde te vertellen wat jij onderzoekt) vind ik het onwijs leuk en stoer dat wij beide een promotietraject doen!

Zoals weerspiegeld in onze promotieonderwerpen verschillen wij behoorlijk in karakter, maar juist de overeenkomsten brengen ons dichter bij elkaar. Zo hebben we dezelfde humor, bezitten we nul algemene kennis, houden we allebei van de winterse dagen met sinterklaas, kaarsjes en kerstmuziek, weten we goed wat we willen (a.k.a. eigenwijs en koppig) en zijn we gepassioneerd in datgene wat we doen. Ik geniet erg van onze zussen quality time, en wil die graag uitbouwen in de toekomst. En niet vergeten: als grote zus ben ik onwijs trots op je zumie.

Lieve **Papa**, zo vader zo dochter, dat blijkt maar weer. Vanaf jongs af aan was het al twee handen op een buik, maar de afgelopen jaren bleek dat we ook op werkgebied veel gemeen hebben. Ons debuut als de Wijnsma's hebben we gemaakt op de NND in 2016 waar we samen een workshop over kosten in de zorg hebben verzorgd. Daarnaast was het een nek aan nek race wie als eerst zou promoveren. Ik ben trots op je dat jij jouw jongensdroom hebt weten te verwezenlijken en vind het erg bijzonder dat Uzume en ik jouw paranimfen mogen zijn. Zoals Winston Churchill al eens zei *"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."*. Ik kijk uit naar je volgende boek.

Lieve **Mama**, als een trotse leeuwin waak jij over je dochters. Als eerste wist jij perfect aan iedereen uit te leggen waar mijn promotie over ging en heb je als kers op de taart ook nog de naam CUREiHUS verzonnen! Ondanks dat ik nu bijna doctor ben, zal ik altijd jouw meisje blijven die een knuffel krijgt ook al doe ik nog zo alsof ik hem niet nodig heb. Het meisje dat verwend wordt met een attent cadeautje als het even tegen zit en waar jij aan haar stem alleen al hoort dat het niet goed zit en degene voor wie je altijd in de bres zult springen, ongeacht wie er tegenover je zal staan. Ik hoop dat ik ooit net zo krachtig zal zijn.

Allerliefste **Ties**, wie zou ik zijn zonder jou? Je steunt me, je maakt me aan het lachen, je luistert naar me, en je spreekt me streng toe als ik doordraaf. Ruim 9 jaar later ben ik nog steeds smoorverliefd op je. Vol verlangen kijk ik uit naar ons volgende avontuur in Italië: met niemand liever wil ik de rest van mijn leven delen. *"In your arms, where all my journeys end"*