The syndromes of thrombotic microangiopathy

The syndromes of thrombotic microangiopathy: towards a true etiology–based approach

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The syndromes of thrombotic microangiopathy: towards a true etiology–based approach

DISSERTATION

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by

Sjoerd Antonius Maria Elisabeth Gerardus Timmermans

Promotores
Dr. Pieter van Paassen
Prof. dr. Chris P. Reutelingsperger

Copromotor
Dr. Jan G.M.C. Damoiseaux

Beoordelingscommissie
Prof. dr. Hugo ten Cate, voorzitter
Prof. dr. Peter Heeringa (University Medical Center Groningen, Groningen)
Prof. dr. Jeroen P. Kooman
Prof. dr. Abraham (Bram) A. Kroon
Prof. dr. Johan van der Vlag (Radboud University Medical Center, Nijmegen)

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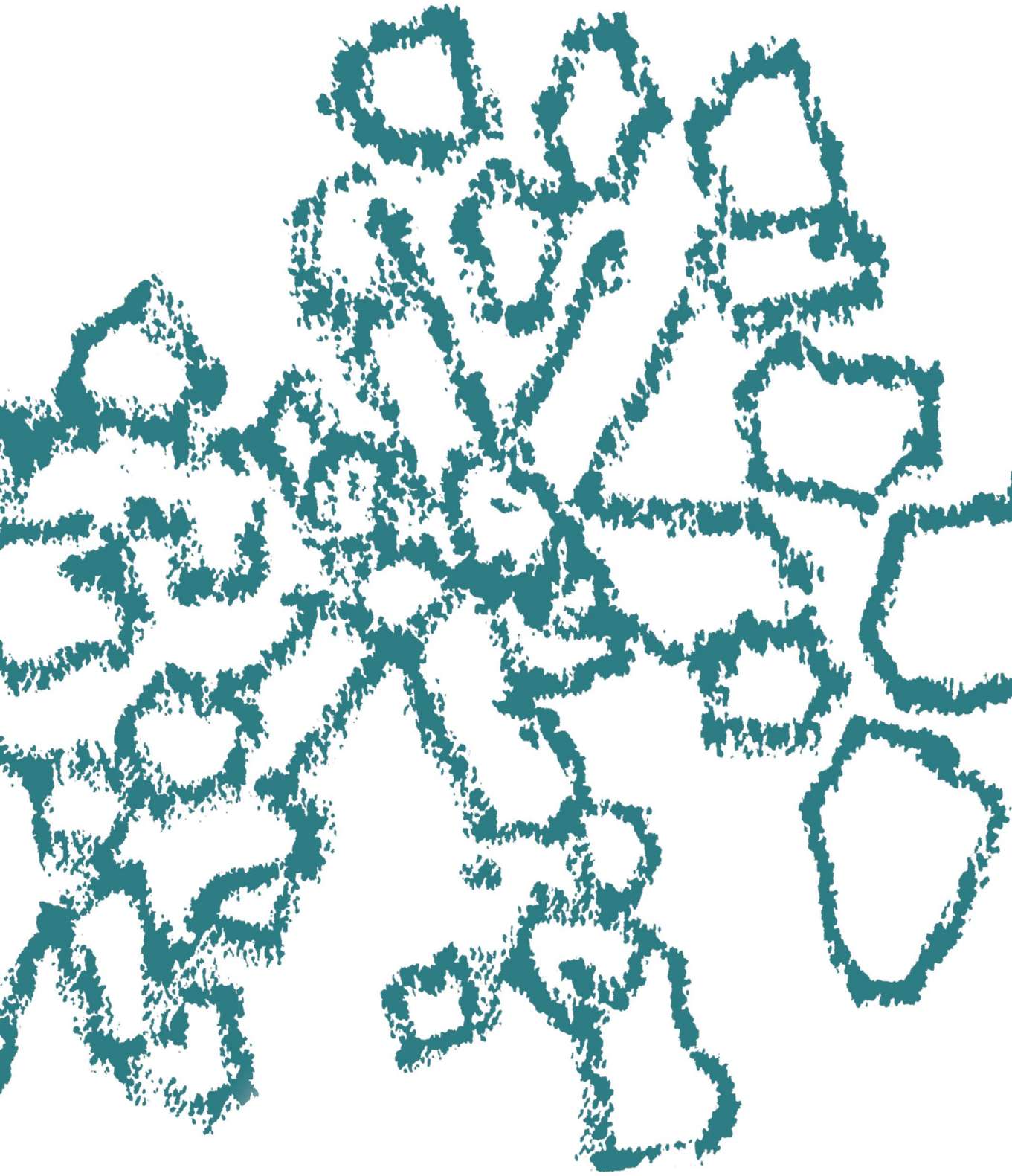
General introduction and aim of the thesis



1

The syndromes of thrombotic microangiopathy and their relation to the (deregulated) complement system

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Journal of Clinical Medicine, 2021;
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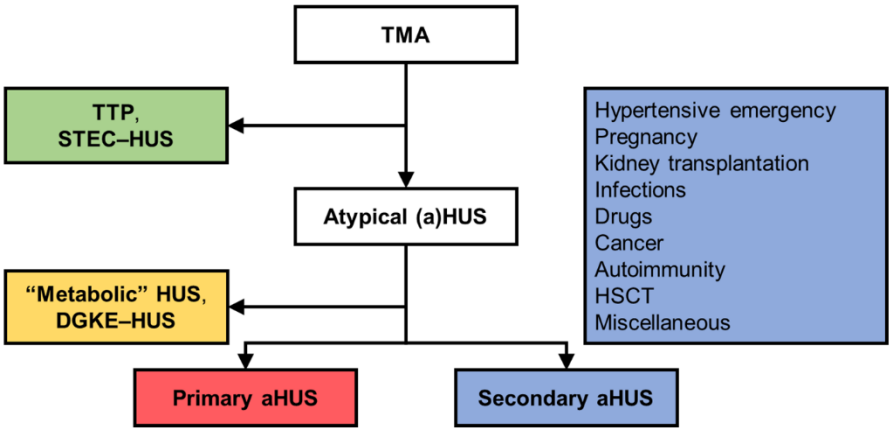
In the 1920s, Eli Moschcowitz reported on the first case of thrombotic microangiopathy (TMA), characterized by microvascular thrombosis in various organs, including the kidneys, and hemolytic anemia, resulting from a so–called powerful poison.[1] Ever since Moschcowitz's report, TMA appeared to arise from diverse, potentially life–threatening syndromes with identical morphologic features, reflecting a "common" final pathway of severe microvascular damage and thrombosis.[2] Clinically, TMAs translate into consumptive thrombocytopenia, mechanical hemolysis, and ischemic organ damage, often affecting the kidneys; in 1955, the term "hemolytic uremic syndrome" (HUS) was therefore proposed.[3]

The nomenclature on TMAs is continually evolving as new mechanisms are being discovered. Historically, patients with HUS were classified into diarrheal HUS associated with Shiga and verocytotoxin–producing bacterial infection or non–diarrheal HUS. In a subset of patients with non–diarrheal HUS, that is, thrombotic thrombocytopenic purpura (TTP), ultra–large von Willebrand factor multimers were found.[4] TTP typically presents with neurologic manifestations and more severe thrombocytopenia as compared to other forms of non–diarrheal HUS. The latter predominantly presents with acute kidney injury. The clinical presentation of these disorders, however, does overlap and it was therefore not possible to differentiate TTP from other forms of non–diarrheal HUS, leading to the introduction of the term TTP–HUS. Confusion between TTP and HUS persisted until the late 1990s, when a severe deficiency of von Willebrand factor cleaving protease (i.e., a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 [ADAMTS13]) was linked to TTP.[5] Ultra–large von Willebrand factor multimers accumulate without the proteolytic activity of ADAMTS13, inducing excessive platelet adhesion, aggregation, and TMA. The introduction of tests to assess ADAMTS13's enzymatic activity made it possible to differentiate TTP from other TMAs.

Around that same time, genetic studies pointed to complement (dysregulation) in another subset of patients with non–diarrheal HUS.[6,7] After 2000, our knowledge on the role of complement in non–diarrheal HUS increased and various studies showed that over half the patients with no coexisting condition present with hereditary and/or acquired complement dysregulation.[8,9] HUS International's nomenclature classified these patients as primary atypical HUS, indicating a diagnosis of exclusion (Figure 1).[10,11] Most patients (i.e., ~90%), however, present with coexisting conditions that are assumed to cause the TMA and hence, are classified as having secondary atypical HUS. Of note, a so–called second hit or trigger is required for primary atypical HUS to manifest.[12] Thus, these coexisting conditions, e.g., hypertensive emergency among others, may therefore reflect the second hit rather than etiologic factor of disease, having major impact on treatment and prognosis in the era of complement–specific drugs.[13-15]

Figure 1. HUS International's nomenclature on the syndromes of TMA.[10]



DGKE, diacylglycerol kinase epsilon. HSCT, hematopoietic stem cell transplantation. STEC, Shiga toxin–producing E. *coli*. TTP, thrombotic thrombocytopenic purpura.

Before proceeding to the aim of this thesis, the complement system in health and primary atypical HUS will be introduced.

THE COMPLEMENT SYSTEM

Jules Bordet, awarded 1919's Nobel Prize in Physiology or Medicine for his discoveries relating to immunity, demonstrated that lysis of bacteria is mediated by a heat labile fraction of serum, later identified as the complement system. This an–cient and conserved effector system of soluble and surface–bound proteins is involved in the defense against pathogens and homeostasis.[16] Many of these proteins are so–called zymogens, i.e., inactive precursors that require cleavage to become activated. After activation, the enzymes further cleave their substrates and activate an amplification loop.

The complement system can be initiated via the classical, lectin, and alternative pathway, converging to C3 (Figure 2). The former has been illustrated by Jules Bordet's experiments, in which he showed that complement proteins are downstream effectors of antibodies.[17] The Fc domain of antibodies and, in particular, IgM, IgG3, and IgG1, can bind C1q, leading to the activation of C4 and C2 and the formation of the C3 convertase C4bC2a. The lectin pathway, activated by proteins that bind to sugars expressed on the bacterial surface, also form the C3 convertase C4bC2a. The C3 convertase cleaves C3 into the anaphylatoxin C3a and C3b. C3a attracts leukocytes to the site of complement activation. C3's thioester domain located in C3b can bind to the cell surface for opsonization. Also, C3b can interact with C4bC2a to form the C5 convertase and activate the so–called terminal

pathway.

The experiments in the current thesis mainly focus on the alternative pathway. This is a spontaneously and continuously active surveillance system operating in the circulation (i.e., C3 tick–over) and on cell surfaces.[18] In the circulation, C3 associates with water to form C3(H_2O), which can bind factor B and factor D. Factor D cleaves factor B into Bb, the serine esterase that cleaves C3, and Ba. C3b, when bound to the cell surface, provides a platform to form the C3 convertase C3bBb that repeatedly cleaves C3, activating an amplification loop. Properdin, also known as factor P, can either stabilize C3bBb[19] or bind to microbes[20] to provide a platform for activation of the alternative pathway. Increasing density of C3b can shift C3bBb to the C5 convertase (i.e., C3bBbC3b), activating C5 and thus, initiating the terminal pathway. C5, similar to C3, can be cleaved into the anaphylatoxin C5a and C5b. C5a is more potent than C3a for the attraction of leukocytes. C5b can bind C6, C7, C8, and multiple C9 molecules on the cell surface to form the lytic C5b9 (i.e., membrane attack complex), eliminating opsonized cells, analogous to the lysis of bacteria as observed by Jules Bordet. The activation of the alternative pathway is tightly regulated to prevent damage to the self and, in particular, the exposed endothelium. Factor H, the most abundant complement regulatory protein in the circulation, can bind to surface–bound C3b and glycosaminoglycans via the C'–terminal domain (Figure 2); complement regulation, that is, decay accelerating and cofactor activities, is provided via the N'–terminal domain. Decay accelerating activity is achieved through the dissociation of the convertases. The cofactor activity is based on factor I–mediated cleavage of C3b into iC3b, C3c, and C3dg, preventing the C3 convertase to form and thus, activation of the terminal pathway. Also, surface–bound membrane cofactor protein (i.e., CD46) exhibits cofactor activity. The surface–bound proteins CR1 and CD55 have decay accelerating and/or cofactor activities, while CD59 attenuates C5b9 to form.

COMPLEMENT DYSREGULATION AND PRIMARY ATYPICAL HUS

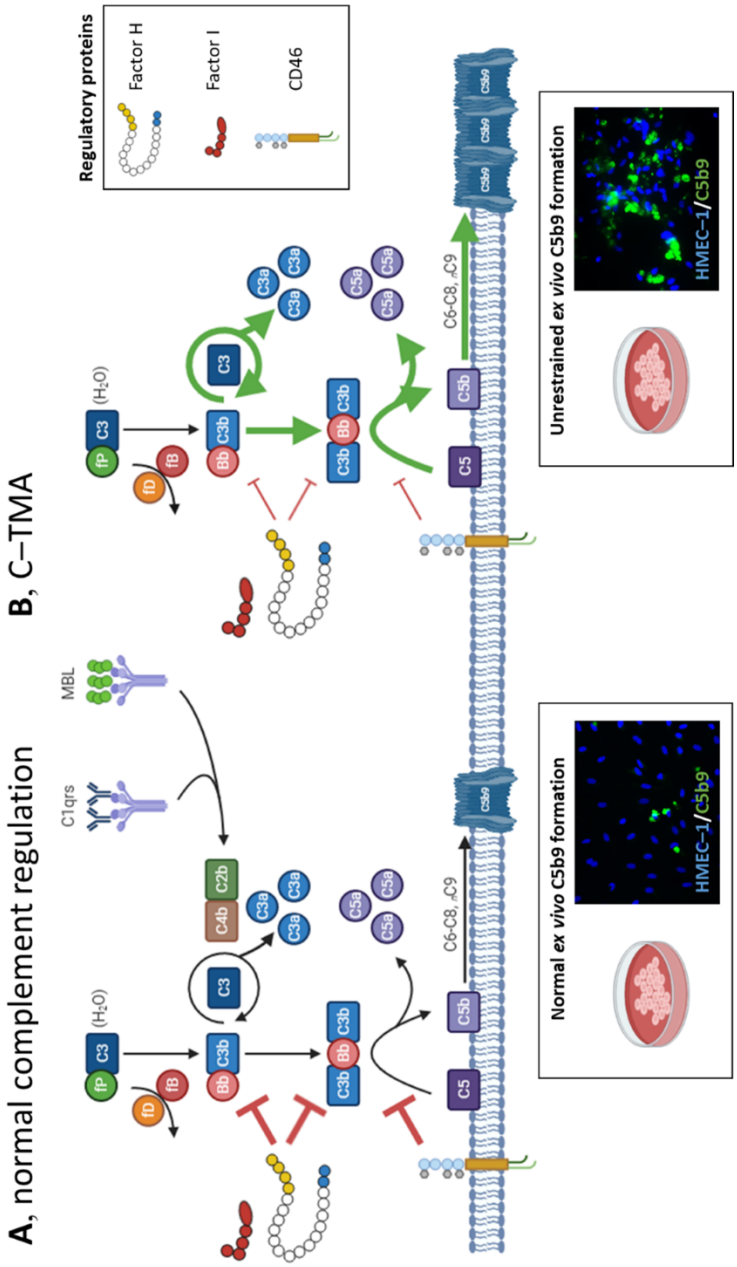
The endothelium, lining the blood vessels, is in permanent contact with the complement system and thus, C3's tick–over mechanism. The endothelium therefore needs an armamentarium of regulatory proteins to prevent damage to the self. In the 1980s, complement activation via the alternative pathway was found in two brothers with (primary atypical) HUS with a factor H deficiency, while their unaffected parents, who were first cousins, had half–normal levels of factor H.[21] Warwicker and colleagues performed a linkage study in three families with (primary atypical) HUS and found a variant in *CFH* (i.e., c.3643C>G) in one of the families but not in controls;[6] the arginine to glycine substitution at amino acid 1,215 in the C'–terminal domain reduced factor H's binding to the endothelium and C3b.[22] At present, >600 rare variants in complement genes have been identified in patients

►**Figure 2.** Schematic overview of complement in health and C–TMA.
(A) The complement system can be initiated via the classical (C1qrs), lectin (MBL), and alternative pathway (C3), converging to C3. The alternative pathway is a spontaneously and continuously active surveillance system operating in the circulation and on the cell surface. C3 associates with water to form C3(H_2O) and bind factor B (fB) and factor D (fD), the latter cleaves fB into Bb, the serine esterase that cleaves C3 into C3a and C3b, and Ba. C3's thioester domain located in C3b can bind to the cell surface and, in particular, microbes, providing a platform to form the C3 convertase of the alternative pathway (i.e., C3bBb); the C3 convertase repeatedly cleaves C3, activating an amplification loop. Properdin (fP) can either stabilize C3bBb or bind to microbes to provide a platform for activation of the alternative pathway. Increasing density of C3b can shift the C3 convertase to a C5 convertase, cleaving C5 into C5a and C5b and thus, activating the terminal complement pathway. C5a and, to a lesser extent, C3a attract leukocytes to the site of complement activation. C5b can bind C6, C7, C8, and various C9 molecules on the cell surface to form the lytic C5b9 and eliminate the opsonized cell. Host cells, including endothelium, are protected from the harmful effects of complement activation by factor I, factor H, and CD46; these proteins have decay accelerating and cofactor activities, leading to factor I–mediated cleavage of C3b into inactivated proteins. (Normal *ex vivo* C5b9 formation on perturbed human microvascular endothelial cells of dermal origin [HMEC–1] indicates normal complement regulation.) (B) Rare variants in complement genes and/or autoantibodies targeting complement regulatory proteins are often present in patients with C–TMA, causing unrestrained complement activation on the endothelium. (Massive *ex vivo* C5b9 formation on perturbed HMEC–1 indicates unrestrained C5 activation.)

with primary atypical HUS,[23] with a prevalence of >50%.[8,9]

Rare variants, that is, those variants with a minor allele frequency of <0.1%, in *CFH*, *CFI*, *CD46*, *CFB*, and *C3*, classified as (likely) pathogenic or uncertain significance are of particular interest.[24] *CFH*, *CFI*, and *CD46* variants lead to impaired protein synthesis or protein function, while *CFB* and *C3* variants cause a gain–of–function protein. Recombination between *CFH* and the *CFH* related genes *CFHR1–5* can form a hybrid gene linked to defects in complement regulation, while variants in one of the *CFHR* genes alone are not.[25] Patients with a homozygous deletion of *CFHR1* and *CFHR3*, however, can develop factor H autoantibodies.[26] These autoantibodies bind to the C'–terminal of factor H, preventing factor H to bind to the endothelium.[26] The presence of such abnormalities per se is not sufficient to cause primary atypical HUS, although combined abnormalities have been associated with a more severe clinical phenotype.[27] Thus, additional precipitants (i.e., second hit) are needed for the disease to become manifest. Of note, rare variants in genes encoding proteins involved in coagulation, e.g., *THBD* (thrombomodulin)[28] and *PLG* (plasminogen)[29] have been found in ~5% patients with primary atypical HUS. It remains unknown whether these variants affect complement regulation or not.

In vivo studies, using factor H knockout mice, demonstrated unrestrained C5 activation as the key factor for primary atypical HUS to develop.[30] C5b9, either with C5a or not, but not C5a alone induced TMA.[31] Similar data were obtained when using mice homozygous for a gain–of–function change in C3.[32] Thus, murine data


A, normal complement regulation
B, C–TMA
Regulatory proteins
Factor H
Factor I
CD46
MBL
C1qrs
C3
fP
fD
fB
(H2O)
C3b
Bb
C2b
C4b
C3a
C5a
C5
C5b
C6-C8, C9
C5b9
Normal ex vivo C5b9 formation
HMEC–1/C5b9
Unrestrained ex vivo C5b9 formation
HMEC–1/C5b9

underscored the role of complement dysregulation in thrombotic microvascular disease, pointing to a potential target for treatment.

Primary atypical HUS is characterized by the presence of fibrin thrombi rather than platelet–rich thrombi as seen in TTP, the latter resulting from the presence of ultra–large von Willebrand factor multimers. C5 activation on the endothelium leads to the expression and secretion of tissue factor via the insertion of sublytic C5b9[33] and the interaction of C5a with its receptor.[34] Induction of tissue factor, activation of the extrinsic pathway of coagulation, and assembly of the prothrombinase complex causes fibrin thrombi to form. Of note, thrombin[35] and plasmin,[36] upon activation of the fibrinolytic pathway, may accelerate C5 activation. Also, C5 products cause the release of Weibel–Palade bodies, containing von Willebrand factor, from the endothelium,[37] platelets, and leukocytes.[38] This provides a platform for platelets to adhere, enhancing coagulation.

THE RENAISSANCE OF THERAPEUTIC COMPLEMENT INHIBITION

The clinical course of primary atypical HUS is severe, with high rates of end–stage kidney disease (ESKD) despite plasma exchange. Historical cohorts showed kidney survival rates of 44% at 1 year.[9] Moreover, recurrent TMA after kidney trans–plantation is common and associated with graft failure.[39] Patients with primary atypical HUS often remained dialysis dependent, having significant impact on quality of life, morbidity, and mortality.

Eculizumab, an anti–C5 monoclonal antibody that binds with high affinity to C5 and blocks its activation, improved kidney survival rates to 90% at 1 year.[13] Patients who start treatment early have the best possible chance to recover kidney function.[14] Prolonged treatment further improved kidney function and prevented TMA recurrence.[11] Identical results have been reported after transplantation.[40] Ever since eculizumab's success, several complement–specific drugs have reached late–stage clinical development for the treatment of primary atypical HUS. Therapeutic complement inhibition that affects complement activation proximal to C5, e.g., factor D inhibition[41] among others, is promising for the treatment of primary atypical HUS and warrants exploration in men.

At present, eculizumab is one of the most expensive drugs in the world. In line with HUS International's nomenclature,[10] KDIGO therefore stated that only those patients with a strong suspicion of complement dysregulation, that is, when secondary atypical HUS has been excluded, should be selected for treatment.[11] Many patients with primary atypical HUS, however, require a second hit for TMA to become manifest.[12] For example, severe hypertension, either presenting as hypertensive emergency or not, can cause TMA on the background of shear stress but can also activate complement,[42] lowering the threshold for complement dys–regulation to occur. If one ignores complement dysregulation as a potential and

treatable cause of TMA in patients with secondary atypical HUS, a subset of patients may progress to ESKD without receiving optimal treatment, as is illustrated in the case vignette.

MOTHER AND CHILD REUNION IN "HYPERTENSIVE" ESKD

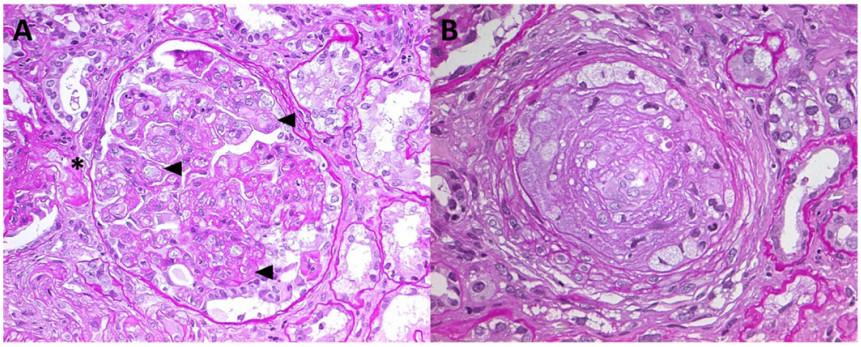
In 2006, a 40–year–old man with a history of controlled hypertension and mild urinary abnormalities was admitted to our hospital because of severe hypertension, progressive kidney failure, headache, and fatigue (i.e., patient no. M04306). He used ramipril and no other drugs. Blood pressure and pulse were 205/114 mmHg and 69 beats per minute, respectively; electrocardiography showed characteristic changes of left ventricular hypertrophy. Serum creatinine was 1,195 µmol/L and Coombs negative microangiopathic hemolytic anemia was present; 158,000 platelets per µL were found. Proteinuria of 2,300 mg/d but no hematuria or pyuria was found on urinalysis. Nine months earlier, serum creatinine was 106 µmol/L and platelet counts were 242,000 per µL. There was no known family history of kidney disease or hypertension. Malignant nephrosclerosis was clinically inferred and labetalol was i.v. administered, leading to rapid blood pressure control.

Because the lack of a renal response upon blood pressure control and the presence of normal–sized kidneys on ultrasound, a kidney biopsy was performed. The key findings on kidney biopsy were membranoproliferative glomerulonephritis with focal segmental glomerulosclerosis and typical features of severe as well as long–standing hypertension, that is, myxoid intimal alterations and arteriolar hyalinosis (Figure 3), indicating chronic TMA. Thrombi were not present. Testing for ADAMTS13's enzymatic activity, anti–nuclear and phospholipid antibodies, complement levels, and infections were unremarkable; the patient denied illicit drug use. Thus, chronic TMA on the background of hypertensive emergency was diagnosed.

Kidney function worsened and the patient became dialysis dependent. In 2007, he received an allograft from his mother with immediate function; tacrolimus, sirolimus, and prednisolone were started to suppress allograft rejection. Five years after transplantation when on tacrolimus monotherapy (trough levels, >4 ng/mL), non–hypertensive acute allograft failure (serum creatinine, 248 µmol/L) and proteinuria of 2,200 mg/d developed. Donor specific alloantibodies and infections (i.e., cytomegalovirus, hepatitis C virus, and parvovirus B19) were not found.

Because of a persistent and unexplained increase in serum creatinine, an allograft biopsy was performed. Membranoproliferative glomerulonephritis with moderate–to–severe interstitial fibrosis, tubular atrophy, and intimal fibrosis were present; thrombi, tubulitis, and peritubular capillaritis were not found. Neither C4d nor immune–complex deposits were found. Thus, recurrent chronic TMA was diagnosed.

Figure 3. Kidney biopsy findings.



Periodic acid Schiff stain showing double contours of the glomerular basement membrane (arrowhead), subtle margination of neutrophils, and hyalinosis (asterisk) of an arteriole (A, 400×); myxoid intimal alterations were seen in arteries, occluding the lumen (B, 400×). No features of acute TMA, that is, endotheliosis, thrombi, and mesangiolysis were found.

Most cases of recurrent TMA have been linked to rare variants in complement genes. Next generation sequencing for such variants revealed a heterozygous frameshift variant in *CD46* (c.811_816delGACAGT; p.Asp271_Ser272del) and a missense variant in *CFH* (c.2,850G>T; p.Gln950His). The ultrarare (i.e., minor allele frequency [MAF] <0.01%) variant in *CD46* has been classified as pathogenic,[43] while the variant in *CFH* is rather common (i.e., MAF ≥0.1%) and not associated with complement dysregulation. Also, the *CFH*–H3 haplotype and Δ*CFHR1–CFHR3* were found in heterozygosity. No variants were found in *CFI*, *CFB*, *C3*, *THBD*, and *DGKE*. Rearrangements in the *CFH–CFHR1–5* genomic region were not present.

TMA recurrence after kidney transplantation is uncommon in patients with variants in *CD46*,[44] since CD46 is expressed by the donor kidney's endothelium. The *CD46* variant, but not *CFH*–H3 haplotype was traced in the donor who is nowadays over 80 years of age and never developed TMA; chronic kidney disease stage 3b, however, developed after donation and remained stable ever since. Thus, the patient's diagnosis changed to primary atypical HUS on the background of a pathogenic variant in *CD46*.

The patient had been re–transplanted with a living–unrelated allograft and because the variant in *CFH* is not associated with primary atypical HUS, prophylactic therapeutic measures were not needed. Macroscopic hematuria developed, with stable allograft function. The urologic work–up demonstrated a urothelial carcinoma originating from the mother's kidney as confirmed by fluorescence *in situ* hybridization. Routine computed tomography scan of the abdomen showed no tumor mass in the donor kidney prior to re–transplantation. The patient died from dis–seminated disease 2 years later.

In conclusion, patients with TMA, a normal enzymatic activity of ADAMTS13, and coexisting conditions (i.e., those classified as secondary atypical HUS according to HUS International's nomenclature[10]) may present with complement dysregulation, challenging the concept that coexisting conditions exclude complement dys–regulation as the etiologic factor of disease.

"SECONDARY" ATYPICAL HUS: A NEW LOOK AT AN OLD ENTITY

In this thesis, we studied complement dysregulation and its potential implications on clinical outcomes along the spectrum of TMA. Also, we developed an *ex vivo* test to detect patients with TMA who may benefit from complement–specific drugs. Of note, the estimated incidence of TMA related to complement dysregulation is 0.2–0.4 per million persons per year,[9,45] indicating an orphan disease. The Limburg Renal Registry (i.e., regionale nierwerkgroep Limburg in Dutch) founded in the late 1970s by Peter van Breda–Vriesman to study the immunology/pathology of native kidney diseases[46] made it possible to test the hypotheses, as outlined below.

Primary atypical HUS can be missed in patients with TMA and coexisting hypertensive emergency, often referred to as hypertensive ESKD. In the era of therapeutic complement inhibition, the correct recognition of complement dysregulation is more important than ever before. In the first part of this thesis, we tested the hypothesis that complement dysregulation is linked to poor (kidney) outcomes in patients with TMA and coexisting hypertensive emergency by using genetic studies (**Chapter 2**, **3**, **4**). Also, the response to therapeutic complement inhibition was assessed. Patients with TMA on the background of complement dysregulation who start treatment early have the best possible chance to recover kidney function.[14] Because genotyping is time–consuming and lacks sensitivity, a specific serum–based *ex vivo* test to detect unrestrained complement activation on the endothelium in the earliest possible stage of disease was developed (**Chapter 3**). Morphologic and immunologic features on kidney biopsy that may distinguish patients with TMA on the background of complement dysregulation from those with hypertensive kidney disease were also studied (**Chapter 4**).

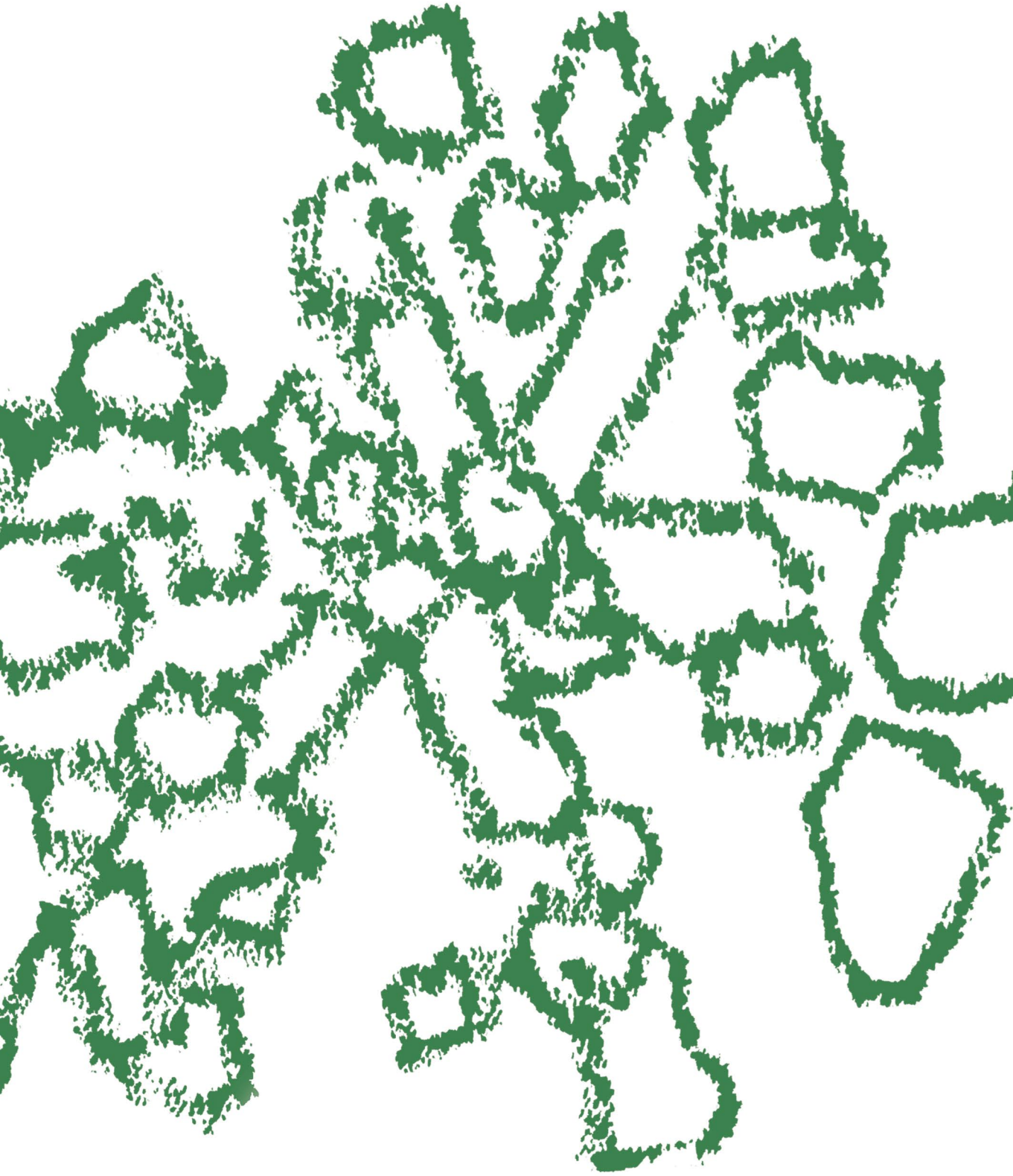
In the second part, we tested the hypothesis that complement dysregulation is common in patients classified as secondary atypical HUS according to HUS Inter–national's nomenclature, who present with severe kidney disease and/or relapsing disease by using an identical approach, including a large cohort of patients with secondary atypical HUS (**Chapter 5**) and TMA related to the antiphospholipid syndrome (**Chapter 6**). HUS suggests hemolysis and uremia (i.e., severe kidney disease), typically associated with low platelets. Profound hematologic ab–normalities, however, were uncommon in patients with TMA and coexisting hypertensive emergency (the first part of this thesis) and those patients with a prevalent gain–of–function variant in *C3*; the genotype–phenotype correlation of this

specific variant, often associated with chronic TMA, was studied (**Chapter 7**). This thesis, together with other provocative studies, provide a rationale for an updated nomenclature on the TMAs unrelated to TTP (**Chapter 8**). The term complement–mediated TMA, either hereditary or acquired, was introduced to improve the recognition of patients with complement dysregulation and thus, to replace the historical term primary atypical HUS.

In ~20% women predisposed to complement dysregulation, pregnancy appeared the precipitating factor for primary atypical HUS to occur.[9,47] Of note, adequate complement regulation is required for fetomaternal tolerance.[48,49] Robust clinical data on maternal and fetal outcomes in the setting of complement dysregulation are not available, making counseling of women predisposed to complement activation who wish to become pregnant difficult. In the last part, we studied the effects of complement dysregulation on maternal and fetal outcomes (**Chapter 9**).

Lastly, the integrated interpretation and discussion of the data, as described in this thesis, as well as future perspectives will be discussed in **Chapter 10** and **Chapter 11**.

PART I
Hypertensive emergency, thrombotic microangiopathy, and complement



“Pressure, if it is great enough, will eventually disrupt any structure. Obviously, this is also true of blood pressure. It is therefore not surprising that an experimentally induced great increase in pressure disrupts the integrity of the blood–vessel wall.”

Jan Möhring (1977)

2

Patients with hypertension–associated thrombotic micro–angiopathy may present with complement abnormalities

Sjoerd A.M.E.G. Timmermans,[1, 2] Myrurgia A. Abdul–Hamid,[3] Joris Vanderlocht,[4] Jan G.M.C. Damoiseaux,[4] Chris P. Reutelingsperger,[2] and Pieter van Paassen;[1, 2] for the Limburg Renal Registry.

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DOI: 10.1016/j.kint.2016.12.009.

Summary. Thrombotic microangiopathy (TMA) is a pattern of endothelial damage that can be found in association with diverse clinical conditions such as hypertensive emergency. Although the pathophysiological mechanisms differ, accumulating evidence links complement dysregulation to various TMAs and, in particular, primary atypical hemolytic uremic syndrome (HUS). Here, we evaluated the role of complement in 9 consecutive patients with TMA on kidney biopsy attributed to severe hypertension. Profound hematologic symptoms of TMA were uncommon. In 6 (67%) out of 9 patients, we found variants in *C3* (*n*=3), *CFI* (*n*=1), *CD46* (*n*=1) and/or *CFH* (*n*=2) either with (*n*=4) or without the risk *CFH*–H3 haplotype. Elevated levels of the soluble C5b9 and deposits of C3c and C5b9 along the vasculature and/or glomerular capillary wall, confirmed complement activation *in vivo*. In contrast to patients without genetic variants identified, patients with complement defects invariably progressed to end–stage kidney disease (ESKD) and TMA recurrence after kidney transplantation seems common. In conclusion, a subset of patients with hypertension–induced TMA fall within the spectrum of complement–mediated (C–) TMA, the prognosis of which is poor. Testing for genetic complement abnormalities is therefore warranted in patients with severe hypertension and proven TMA to adopt suitable treatment options and prophylactic measures.

Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Dept. Pathology, Maastricht UMC, NLD.
[4]Central Diagnostic Laboratory, Maastricht UMC, NLD.

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Correspondence (DOI: 10.1016/j.kint.2017.03.049);
Reply (DOI: 10.1016/j.kint.2017.03.048).

Severe hypertension may induce TMA within the kidneys associated with fibrinoid necrosis of arterioles and the glomerular capillary tufts. The exact mechanism remains to be established, but TMA may occur when autoregulation fails to counteract the hypertension–induced shear stress. In those patients with hypertension as the primary pathologic process, aggressive management of blood pressure is effective in resolving acute features of TMA and, at least in part, restoring kidney function.[50] However, numerous other processes may be relevant, particularly in those patients not responding to standard treatment and becoming dialysis–dependent.

During the last decade, the alternative pathway (AP) of complement activation has been linked to TMA and in particular to primary atypical HUS; a rare syndrome of microangiopathic hemolytic anemia, thrombocytopenia, and kidney injury. The AP is a continuously active immune surveillance and effector system operating in the circulation and on the cell surface, which is tightly regulated to prevent damage to the self. In primary atypical HUS, AP dysregulation can occur at the endothelial surface, leading to the formation of the terminal complement complex, i.e., C5b9, and subsequent endothelial cell damage.[30,51] AP dysregulation can be due to variants in genes that either regulate or activate the AP and/or autoantibodies that inhibit complement regulatory proteins.[8,9] The penetrance of primary atypical HUS is incomplete, indicating that a second hit, such as hypertension, is required for disease manifestations.[12] The prognosis is extremely poor,[9] but blockade of the terminal complement pathway has dramatically improved the clinical outcome.[13-15]

In clinical practice, it is often a diagnostic challenge to differentiate hypertension–associated TMA from complement–mediated disease. This is particularly the case in patients presenting without profound hemolysis and/or thrombocytopenia. If one refrains from a complete diagnostic work–up, including a comprehensive search for complement abnormalities, a subset of patients may progress to ESKD without receiving optimal treatment. Moreover, the correct diagnosis is of utmost importance to adopt suitable prophylactic measures prior to kidney transplantation. Here, we hypothesized that AP dysregulation is an often unrecognized, but treatable cause of hypertension–induced TMA. To test this hypothesis, we in retrospect thoroughly analyzed the AP in 9 patients with severe hypertension diagnosed with TMA on kidney biopsy. The study included 8 cases without profound hematologic signs of TMA. Furthermore, to explore whether indeed the prognosis of these patients is poor, we evaluated the long–term outcome, including the disease course after kidney transplantation.

MATERIAL AND METHODS

Patient cohort. Consecutive patients with TMA on kidney biopsy who presented with severe hypertension were recruited from January 2005 onwards. Severe

hypertension was defined as blood pressure levels of ≥180 mmHg systolic and/or 120 mmHg diastolic and evidence of impending or progressive target organ dysfunction secondary to hypertension.[52,53] Patients were screened for secondary causes of TMA such as autoimmune diseases, drugs, infections, and pregnancy. The enzymatic activity of von Willebrand factor cleaving protease (i.e., ADAMTS13) was tested in patients with microangiopathic hemolytic anemia and/or thrombo–cytopenia to rule out thrombotic thrombocytopenic purpura. Patients without a definite clinical diagnosis were diagnosed as hypertension–induced TMA; these patients were included. Patients with immune–complex glomerulonephritis were excluded.

Clinical and laboratory data were documented at the time of kidney biopsy and during follow–up. The information was specified in our Limburg Renal Registry and the patient's medical records.[54] The study was approved by the local ethics committee of the Maastricht University Medical Centre (UMC) and is in accordance with the Declaration of Helsinki.

***Genetic analysis and search for autoantibodies*.** Coding regions of *CFH*, *CFI*, *CD46*, *CFB*, and *C3* were amplified and screened for mutations and polymorphisms using DNA sequencing.[55,56] Rearrangements in the *CFH–CFHR1–5* genomic region were analyzed by multiplex–ligation probe amplification.[57] In selected cases, the presence of circulating factor H autoantibodies (FHAA) was assessed by ELISA.[58]

***Complement ELISAs*.** At the time of kidney biopsy, serum and plasma samples were obtained, processed, and immediately stored at –80 degrees Celsius to prevent *in vitro* complement activation.[59] AP and classical pathway functional assays (Eurodiagnostica, Malmö, Sweden) were completed.[60] Furthermore, plasma soluble C5b9 levels were determined using a capture ELISA (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

***Kidney biopsy*.** Kidney tissue was processed for light–, immunofluorescence–, and electron microscopy.[54] Also, 2 μm frozen sections were analyzed for deposition of C4d and C5b9 using mouse anti–C4d mAb (Quidel, San Diego, CA) and rabbit anti–C5b9 pAb (Calbiochem, San Diego, CA) as primary antibodies; FITC labeled anti–mouse Ab (Dako, Glostrup, Denmark) and Alexa488 labeled goat anti–rabbit Ab (Life Technologies, Carlsbad, CA) were used as secondary antibodies. The deposits were scored on a scale from 0 to 3.

RESULTS

***Baseline characteristics*.** Fourteen consecutive patients who fulfilled the inclusion criteria of hypertension–associated TMA were recruited from January 2005

Table 1. Baseline clinical features and laboratory evaluation.

Patient no.	Sex/ age	BP, mmHg	SCr, µmol/L	uP, g/d	ESKD	Hb, mmol/L	LDH, U/L	MAHA	Platelets, $\times 10^9$/L
M00105	F/38	184/140	1,730	N/A	Y	5.1	1,800	Y	224
M04306	M/40	205/114	1,195	2.3	Y	5.7	1,104	Y	158
M03307	M/37	200/120	586	3.9	Y	5.3	2,125	Y	100
M04010	F/32	180/120	1,138	N/A	Y	5.9	1,486	Y	142
M00915	M65	195/105	162	1.5	N	7.9	271	N	98
M01715	F/41	180/120	334	0.7	Y	7.5	291	N	285
M02715	F/28	224/122	1,065	1.6	Y	5.1	298	N	228
M01016	M/27	240/150	673	1.6	Y	7.9	165	N	133
M04516	F/44	220/120	649	0.4	Y	8.2	339	N	340

ESKD, end–stage kidney disease. MAHA, microangiopathic hemolytic anemia: hemolytic anemia and schistocytes on peripheral blood smear. SCr, serum creatinine. uP, proteinuria.

onwards; 5 patients were excluded because of secondary TMA (antiphospholipid syndrome, *n*=1; scleroderma renal crisis, *n*=1), immune–complex glomerulo–nephritis (*n*=2), or the lack of DNA material (*n*=1). Hence, 9 patients were included.

All of the 9 patients were evaluated at the Maastricht UMC, 6 of whom were referred from an outside institution. The baseline characteristics have been depicted in Table 1. At the time of presentation, a diagnosis of malignant nephrosclerosis was clinically inferred. In all patients, mild–to–moderate hypertensive retinopathy was found, while papilledema was observed in 1 case (no. M01016). Indeed, 7 patients had a known medical history of hypertension, including 2 patients with documented episodes of preeclampsia (no. M00105, M02715) and/or hypertensive emergency (no. M02715). Proteinuria and hematuria were found in patients not presenting with anuria. Kidney biopsies revealed characteristic lesions of TMA, including endothelial cell swelling, reduplication of the glomerular basement membrane, wrinkling of the glomerular capillary wall, and/or mesangiolysis. Fibrin thrombi were localized in the glomeruli of 6 and in the vasculature of 5 tissue samples. Prominent intimal fibrosis, myxoid intimal alterations, and/or fibrinoid necrosis of the arteries were also found, reflecting pre–existing severe hypertension. ADAMTS13 assays at presentation were normal in 5 patients tested for (no. M03307, M04010, M00915, M02715, M04516) and immunological tests were uniformly negative. Furthermore, iatrogenic causes, infections, and pregnancy were ruled out. Primary atypical HUS, however, was not suspected because profound hematologic signs of TMA were lacking in all but 1 patient (no. M03307); moreover, none of the patients had a family history consistent with familial primary atypical HUS. Thus, a diagnosis of hypertension–associated TMA was established.

***Complement abnormalities*.** DNA samples were tested; genetic AP abnormalities were identified in 6 (67%) out of 9 patients, most of which were found in heterozygosity (Table 2). The mutated genes included *C3*, *CFI*, *CFH*, and *CD46*. The *C3* and *CFI* mutations included the missense variants c.481C>T (p.Arg161Trp)

Table 2. Complement abnormalities.

Patient no.	Mutated protein(s)	*CFH*–H3	FHAA	CPFA, %	APFA, %	sC5b–9, ng/mL
M00105	C3 p.Arg161Trp	N	Negative	95	64	2,800
M04306	CD46 p.Asp271_Ser272del, CFH p.Gln950His	Y	Negative	97	107	1,000
M03307	C3 p.Arg161Trp	Y	ND	94	97	640
M04010	CFH p.Cys853Tyr	Y	ND	104	71	1,840
M00915	None	N	ND	99	99	1,800
M01715	CFI p.Asn151Ser	N	ND	97	87	4,200
M02715	C3 p.Arg161Trp, ΔCFHR1-CFHR3	N	Negative	110	62	1,840
M01016	None	Y	Negative	90	74	440
M04516	None	N	Negative	113	110	3,800

ΔCFHR1-CFHR3 was found in homozygosity.

APFA, functional activity of the alternative pathway; reference range, >40%. CPFA, functional activity of the classical pathway; reference range, >75%. FHAA, factor H autoantibodies. ND, not determined. sC5b9, soluble C5b9; reference range, <337 ng/mL.

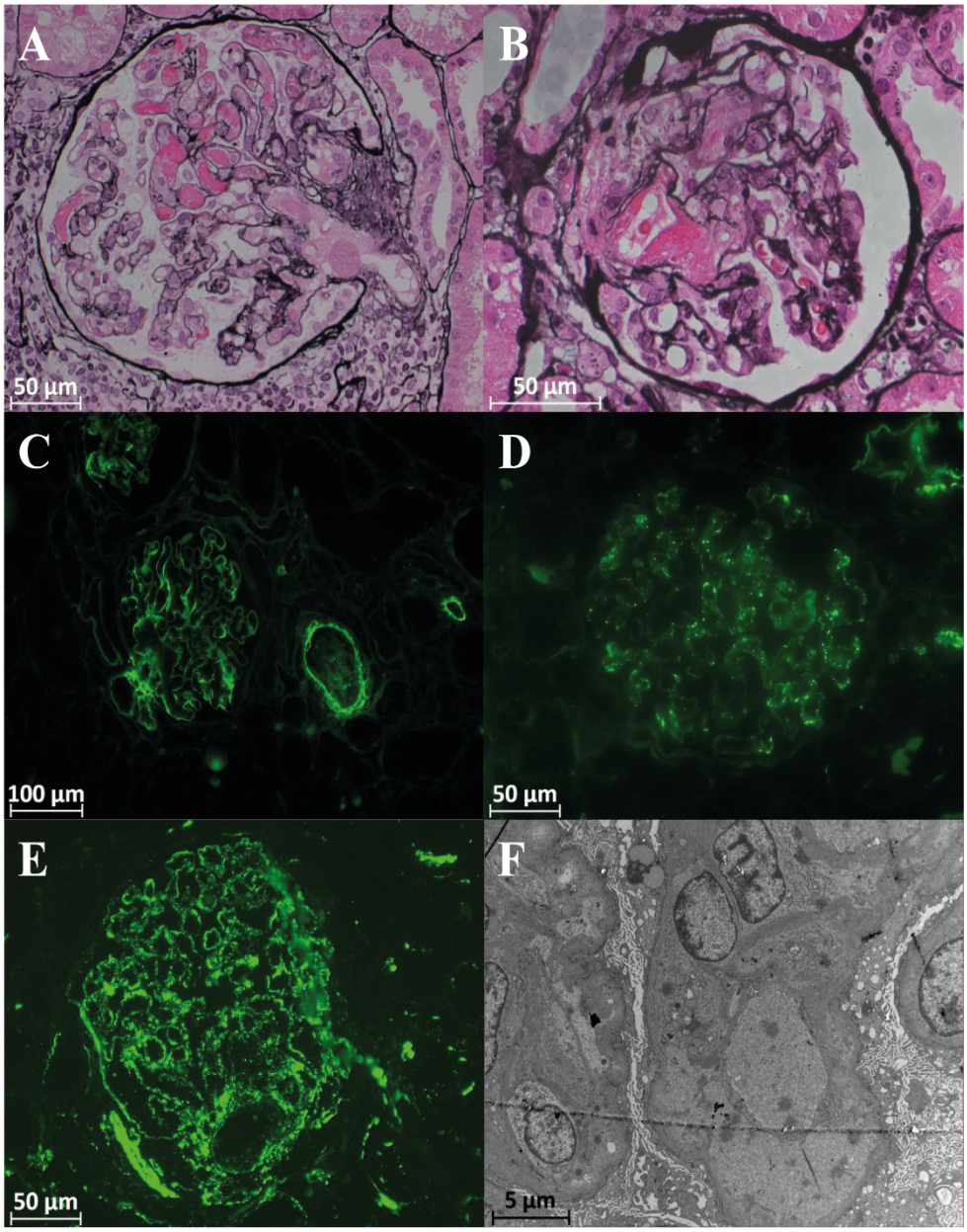
and c.452A>G (p.Asn151Ser), respectively. The *CFH* mutations included c.2558G>A (p.Cys853Tyr) and c.2850G>T (p.Gln950His). The *CD46* mutation included the 6 base pair deletion c.811_816delGACAGT (p.Asp271_Ser272del). Four patients carried the -331C>T and c.2808G>T single nucleotide polymorphisms that tag the *CFH*–H3 haplotype.[27,61] The homozygous genomic deletion of *CFHR1* and *CFHR3* was identified in 1 patient, while circulating factor H autoantibodies were not found.

Complement ELISAs. At the time of kidney biopsy, plasma soluble C5b9 levels were measured and although functional studies of the AP and classical pathway were unremarkable, increased soluble C5b9 levels were found in all patients. The ULN was set at 337 ng/mL (*n*=20 healthy controls, mean ±2 SD), while soluble C5b9 levels ranged from 440 to 4,200 ng/mL in our cohort (Table 2).

Complement activation in vivo. Kidney tissue sections of 8 patients were available. Deposits of C3c and C5b9 were found in patients with complement abnormalities along the vasculature and/or glomerular capillary wall (Figure 1), confirming complement activation. In addition, deposits of C4d, a biomarker for complement activation via the classical and/or lectin pathway, co–localized with C3c and C5b9. Six out of 8 tissue sections revealed entrapment of aspecific IgM along the vasculature and/or glomerular capillary wall, while staining for IgG and IgA was negative.

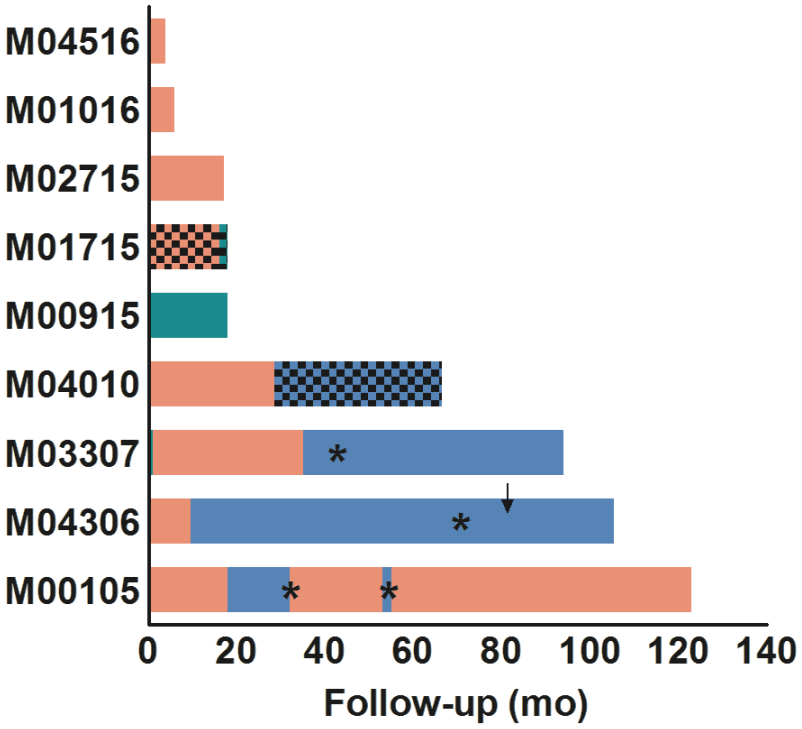
Clinical follow–up. Follow–up ranged from 0.2 to 9.3 years (Figure 2). Eight out of 9 patients progressed to ESKD despite aggressive management of hypertension and normalization of blood pressure. It is noteworthy that eculizumab was started in

Figure 1. Light–, immunofluorescence– and electron microscopic findings on kidney biopsy.



Fibrin microthrombi in arterioles and/or glomerular capillaries in a native kidney (A, 400×) and transplant biopsy (B, 400×), Jones methenamine silver. C4d (C, 200×), C3c (D, 400×), and C5b9 (E, 400×) deposits along the vasculature and/or glomerular capillaries. Subendothelial electron lucent material (F, 3,400×).

Figure 2. Clinical outcome.



Patient M04306 has preemptively been re–transplanted (arrow). Asterisk, TMA recurrence. Blue bars, allograft survival. Checkered bars, eculizumab treatment. Green bars, kidney survival. Red bars, dialysis.

patient no. M01715 when the *CFI* mutation was found after 3 months of dialysis. Kidney function recovered (estimated GFR, 38 mL/min/1.73m^2) within a 12–month treatment period.

Subsequently, 6 allografts (3 living–unrelated, 1 living–related, 2 cadaveric) were transplanted in 4 patients, all of whom received tacrolimus. TMA recurrence manifested in 4 grafts (Figure 2), either with (no. M03307) or without (no. M00105, M04306) microangiopathic hemolytic anemia and thrombocytopenia. Blood pressure was tightly regulated, whilst the presence of donor–specific alloantibodies and infections as endothelium–damaging events were ruled out. Thus, we linked TMA recurrence to AP dysregulation;[62] moreover, the allograft biopsies confirmed complement activation *in vivo* (Figure 1). Recurrent disease was associated either with variants in *C3* (no. M00105, M03307) or *CD46*/*CFH* (no. M04306). TMA recurrence, however, is uncommon among patients with mutated *CD46*. CD46 is a

transmembrane protein widely expressed in the kidneys and consequently, the expression of CD46 in the allograft is driven by endothelial cells from the donor. Therefore, we screened the living–related donor (no. M04306's mother) and traced the same variants in *CD46* and *CFH*.

Plasma exchange was initiated in all patients with established recurrent disease; however, graft loss occurred in 3 out of 4 disease episodes. At the time of transplantation, the genotype of patient no. M04010 was known and upfront eculizumab was started, which prevented disease recurrence.

DISCUSSION

We examined the AP in 9 patients who have been diagnosed with hypertension–associated TMA and although profound hematologic signs of TMA were uncommon, a high prevalence (67%) of genetic defects associated with impaired complement regulation was found. Elevated plasma levels of soluble C5b9 as well as deposits of C3c and C5b9 at the endothelial surface, confirmed complement activation *in vivo*. Also, both kidney survival and outcome after transplantation were poor. Therefore, we conclude that our patients fall within the spectrum of C–TMA, which is supported by the favorable response to therapeutic complement inhibition in 2 patients.

Most patients who present with severe hypertension and advanced kidney failure are not biopsied, because a diagnosis of malignant nephrosclerosis is clinically inferred. Here, we demonstrate that in these patients TMA can develop on the background of complement dysregulation even though profound hematologic abnormalities, as seen in primary atypical HUS, appeared uncommon. AP activation is a physiologic process that is tightly controlled by complement regulatory proteins. Factor I and cofactor molecules such as CD46 (i.e., membrane cofactor protein) and factor H are required to cleave C3b into inactive metabolites. CFI p.Asn151Ser,[63] CD46 p.Asp271_Ser272del,[43] and CFH p.Cys853Tyr[64] have been associated with a reduced expression of the respective proteins, affecting AP regulation at the endothelial surface. The functional consequence of CFH p.Gln950His, however, remains speculative.[65] C3 p.Arg161Trp has been associated with a hyperactive C3 convertase, enhancing complement activation on endothelial cells.[66] Also, the risk *CFH*–H3 haplotype was found,[61] lowering the threshold for TMA to manifest.[27] AP dysregulation and the formation of C5b9 can occur on activated but not on resting endothelial cells,[51] underlining the importance of a complement amplifying condition such as hypertension–induced shear stress.[42] In our cohort, severe hypertension occurred in patients with evidence of pre–existing hypertension and therefore, we propose that hypertension triggered complement activation, leading to over–activation of the AP and the development of TMA. No demonstrable genetic mutations, however, were found in 3 out of 9 patients who may have abnormalities in unscreened regions of complement–associated genes or genes that have not yet

been linked to TMA.

Recent data demonstrated that plasma levels of soluble C5b9 are elevated in the acute phase of C–TMA.[51,67,68] In line with these data, increased soluble C5b9 levels were found in all our patients, indicating activation of the terminal complement pathway. Kidney biopsies were also stained for complement components to analyze complement activation at the endothelial surface. C3c and C5b9 deposits were found, confirming complement activation of the early and terminal complement pathway, respectively. Furthermore, we validate the observations by Chua and colleagues that C4d deposits are a common denominator in TMA (data not shown);[69] although C4d is a biomarker for complement activation via the classical and/or lectin pathway, the significance of both pathways remains elusive. Hypertension–induced shear stress, however, might have been responsible for complement activation via the classical pathway.[42]

As compared to hypertension–associated TMA,[50] the kidney prognosis of primary atypical HUS is extremely poor, with high rates of ESKD and disease recurrence after transplantation.[44,51] We therefore questioned to what extent the disease course of our patients with hypertension–associated TMA has been affected by AP dysregulation. In our cohort, patients with genetically–confirmed complement abnormalities invariably progressed to ESKD despite aggressive management of blood pressure, whilst TMA recurrence after transplantation was common and linked to underlying complement abnormalities. Disease recurrence was associated with a *CD46* mutation in patient no. M04306, which is usually associated with a favorable graft survival and low recurrence rates.[70] Interestingly, the same *CD46* mutation was traced in the patient's mother who donated the kidney, explaining the discrepant disease course after transplantation. TMA, however, did not occur in the donor, providing evidence that additional factors, e.g., the *CFH*–H3 haplotype,[27] are key for the penetrance of disease. In patients with recurrent disease, graft survival was poor despite plasma exchange.

Taken together, our genetic and clinicopathological findings are consistent with those observed in primary atypical HUS, indicating that our patients fall within the spectrum of C–TMA. The lack of profound hematologic signs of TMA is remarkable, although exceptionally reported in primary atypical HUS,[71] reflecting a more gradual disease course, the progression of which can be affected by triggers such as pregnancy.[47] More modest complement activation has also been linked to TMA–like syndromes such as preeclampsia, which may have been the triggering event in 2 of our patients (no. M00105, M02715). This points out that ongoing damage to the endothelial cells within the kidneys can occur irrespective of hematologic signs of TMA. Therefore, novel techniques are needed to better determine the level of both the activation and regulation of the AP at the endothelial surface.

Landmark trials have demonstrated the dramatic effects of therapeutic comp–

lement inhibition, that is, eculizumab, in primary atypical HUS.[13-15] In our cohort, eculizumab was initiated in 2 patients. The kidney function of patient no. M01715 dramatically improved and dialysis could be stopped within a year. Furthermore, upfront eculizumab prevented disease recurrence in patient no. M04010 who carried a high–risk *CFH* mutation.[44] The favorable response to therapeutic complement inhibition confirms the pivotal role of AP dysregulation. Future studies, however, are needed to further examine the timing and duration of treatment with complement–specific drugs.

In conclusion, we believe that screening for abnormalities of the AP, both in kidney biopsies and by genetic testing, is mandatory in all patients with TMA attributed to severe hypertension. Our finding that AP dysregulation is the key causative factor of kidney failure in these patients has major impact on treatment and prognosis.

3

C5b9 formation on endothelial cells reflect complement defects among patients with thrombotic microangiopathy and severe hypertension

Sjoerd A.M.E.G. Timmermans,[1, 2] Myrurgia A. Abdul–Hamid,[3] Judith Potjewijd,[1] Ruud O.M.F.I.H. Theunissen,[2] Jan G.M.C. Damoiseaux,[4] Chris P. Reutelingsperger,[2] and Pieter van Paassen;[1, 2] for the Limburg Renal Registry.

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Background. Severe hypertension can induce thrombotic microangiopathy (TMA) in the kidneys, the occurrence of which has been linked to mechanical stress to the endothelium. Complement dysregulation may be the culprit of disease in patients who present with severe kidney failure and often progress to end–stage kidney disease (ESKD) despite blood pressure (BP) control.
Methods. We studied a well–defined cohort of 17 patients with hypertension–associated TMA to define the prevalence of complement dysregulation by a specific *ex vivo* serum–based microvascular endothelial cell assay.
Results. Compared with normal human serum and samples from patients with hypertensive arterionephrosclerosis, 14 (88%) out of 16 serum samples collected at presentation from patients with hypertension–associated TMA induced massive C5b9 formation on microvascular endothelial cells. We detected rare variants in complement genes in 8 (47%) out of 17 patients. ESKD occurred in 14 (82%) out of 17 patients, and recurrent TMA after kidney transplantation occurred in 7 (64%) out of 11 donor kidneys. Eculizumab improved the kidney function in 3 patients and prevented TMA recurrence in an allograft recipient.
Conclusions. These observations point to complement dysregulation as the key causative factor of ESKD and recurrent TMA after kidney transplantation in patients presenting with severe hypertension. Complement dysregulation can be identified by measurements of complement activation on microvascular endothelial cells, which should substantially influence treatment and prognosis.

Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Dept. Pathology, Maastricht UMC, Maastricht, NLD.
[4]Central Diagnostic Laboratory, Maastricht UMC, Maastricht, NLD.

Long–standing uncontrolled mild–to–moderate hypertension can induce kidney failure due to hypertensive arterionephrosclerosis with a slowly progressive disease course, whereas a more acute and potentially life–threatening TMA can occur in the setting of severe hypertension. The transition to TMA among patients with severe hypertension remains poorly understood, but it can arise from mechanical stress to the endothelium,[72] assuming that the kidneys are the victim rather than culprit of disease. Recently, we identified a clinically distinct phenotype in patients with severe hypertension and TMA on kidney biopsy who present with complement dysregulation as the driving factor of disease.[73] Most patients with severe hypertension achieve stable disease and do not require kidney replacement therapy following BP control,[50,74] whereas the prognosis of those with complement dysregulation is dismal.[73]

The complement cascade is an ancient and conserved effector system involved in the defense against pathogens and host homeostasis, which can be initiated via the classical, lectin, and alternative pathway (AP). AP is a continuously active surveillance system operating in the circulation and on cell surfaces, which is tightly regulated to prevent damage to the self.[75] AP dysregulation, however, has been linked to another syndrome of TMA, that is, primary atypical hemolytic uremic syndrome (HUS). AP dysregulation can be due to variants in genes encoding proteins that either regulate or activate the AP and/or autoantibodies that inhibit regulatory proteins.[8,9] TMA arises from an inciting trigger to the endothelium that exceeds the complement regulatory capacity, leading to unrestrained complement activation, consumptive thrombocytopenia, microangiopathic hemolytic anemia, and ischemic organ damage.[30] The pivotal role of complement dysregulation has been confirmed by prospective trials, showing the efficacy of eculizumab, an anti–C5 mAb that blocks C5, in primary atypical HUS.[13-15]

In contrast to primary atypical HUS, most patients with so–called hypertension–associated TMA do not present with systemic hemolysis, that is, thrombocytopenia and microangiopathic hemolysis, and thus, kidney biopsies are often needed to detect the TMA. In clinical practice, it is of utmost importance, although challenging, to identify patients with complement dysregulation as the causative factor of disease.[11] If complement dysregulation remains unrecognized, patients may progress to ESKD without receiving optimal treatment.[73] At present, however, no standard tests to detect the relevant complement defects are available. Noris and colleagues recently developed a specific serum–based test to detect endothelium–restricted complement dysregulation and activation in patients with primary atypical HUS.[51] Here, we questioned whether the test also can be used to detect complement dysregulation in patients presenting with severe hypertension and TMA on kidney biopsy. We hypothesized that abnormal test results can differentiate patients who develop TMA on the background of complement defects from those with

mechanical stress as the cause of disease. Furthermore, we extended our previous data regarding the presentation, long–term outcome, and prevalence of genetic complement defects in patients with TMA and coexisting severe hypertension.

MATERIAL AND METHODS

Patient population. From 1980 onwards, consecutive patients with hypertension–associated TMA were recruited from the Limburg Renal Registry.[54] Hypertension–associated TMA was presumed in patients with TMA and typical pathologic features of severe hypertension (i.e., myxoid intimal changes, hypertrophy of the arterial vessel walls, and/or fibrinoid necrosis of arterioles) on kidney biopsy, severe hypertension (i.e., BP levels of ≥180 mmHg systolic and/or ≥120 mmHg diastolic), and evidence of impending or progressive target organ dysfunction secondary to hypertension;[11,73] other causes of TMA were excluded according to recent guidelines.[10,11] Systemic hemolysis was defined as microangiopathic hemolytic anemia (hematocrit <30%, hemoglobin <10 g/L, lactate dehydrogenase >500 U/L, and schistocytes on peripheral blood smear) and thrombocytopenia (<150,000/μL).

Also, patients with severe hypertension and arterionephrosclerosis on kidney biopsy without morphologic features of TMA were included. Arterionephrosclerosis was defined as obsolescence of glomeruli, intimal fibrosis, and medial thickening of arteries without morphologic evidence of other kidney disease. Eight healthy controls without a relevant medical history who are not using any drugs were enrolled; serum samples were pooled and used as normal human serum (NHS).

At the time of kidney biopsy and during follow–up, sera and (heparin) plasma samples were obtained, processed, and immediately stored at –80 degrees Celsius to prevent *in vitro* complement activation.[59] Clinical data were obtained from the Limburg Renal Registry and the patients' medical records. The study was approved by the local ethics committee of the Maastricht University Medical Center and is in accordance with the Declaration of Helsinki.

Routine complement assays. Plasma C4 and C3 levels were determined using nephelometry. The functional activity of the classical pathway (Eurodiagnostica, Malmö, Sweden) was tested, according to the manufacturer's instructions.[60] Also, plasma soluble C5b9 levels were determined using a capture ELISA (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Complement activation on HMEC–1 cells. *Ex vivo* complement activation on human microvascular endothelial cells of dermal origin (HMEC–1; ATCC, Manassas, VA) was assessed as described with modifications.[51] Briefly, HMEC–1 were plated on glass culture slides and used when >80% confluent. Before serum incubation, HMEC–1 were activated with 10 μM adenosine diphosphate (ADP) for

10 minutes or not. HMEC–1 were incubated with serum diluted in test medium (1:2, final volume 300 µL) for 3 hours at 37 degrees Celsius, fixed in 3% formaldehyde, and blocked with 2% BSA for one hour.

To analyze complement activation, HMEC–1 were stained with FITC labeled anti–human C3c (1:20; Dako, Heverlee, Belgium) or rabbit anti–human C5b9 pAb (1:100; Calbiochem, San Diego, CA) followed by Alexa488 labeled anti–rabbit Ab (1:100; Life Technologies, Carlsbad, CA). In selected experiments, HMEC–1 were stained with FITC labeled goat anti–human IgG Ab (1:100; ICN Biomedicals, Irvine, CA) or mouse anti–human C4d mAb (1:200; Quidel, Alkmaar, the Netherlands) followed by FITC labeled rabbit anti–mouse Ab (1:60; Dako). Nuclei were stained with DAPI. Fluorescent staining was analyzed by using Image J (NIH, Bethesda, MD) on 15 systematically acquired fields and expressed as mean pixels per field; complement activation was compared with NHS run in parallel. Samples from patients with primary atypical HUS and dense deposit disease were used as positive and negative disease controls, respectively. The experimental design can be found in Item S1.

***Kidney tissue specimens*.** Sections from snap frozen kidney tissue specimens were stained with FITC labeled anti–human C3c (1:20; Dako) or rabbit anti–human C5b9 pAb (1:100; Calbiochem) followed by Alexa488 labeled goat anti–rabbit Ab (1:100; Life Technologies).

***Genetic testing*.** Coding regions of *CFH*, *CFI*, *CD46*, *CFB*, and *C3* were amplified and screened for mutations and polymorphisms using DNA sequencing.[55,56] Rearrangements in the *CFH–CFHR1–5* genomic region were analyzed by multiplex–ligation probe amplification.[57] In selected cases, the presence of circulating factor H autoantibodies was assessed by ELISA.[58]

***Statistical analysis*.** Continuous variables were presented as mean (± standard deviation [SD]) or median (interquartile range [IQR]) as appropriate. Comparisons were made for each patient comparing serum–induced complement deposits for the patient and NHS run in parallel by using the paired sample t test or Wilcoxon signed rank test as appropriate. Between group differences were analyzed by ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

***Patient population*.** Seventeen consecutive Caucasian patients who fulfilled the inclusion criteria of hypertension–associated TMA were recruited from January 1980 onwards. The baseline characteristics have been depicted in Table 1. Patients no. M04516, M01016, M02715, M01715, M00915, M04010, M03307, M04306, and

M00105 have been reported previously.[73] Female–to–male ratio was 0.8 and the median age at diagnosis was 38 (IQR, 34–45) years. Patients invariably presented with severe hypertension, acute kidney injury, and proteinuria; therefore, a diagnosis of hypertensive kidney disease was assumed. Kidney biopsies revealed characteristic lesions of TMA accompanied by prominent intimal fibrosis, myxoid intimal alterations, and/or fibrinoid necrosis of the arterioles, reflecting severe hypertension. Indeed, 12 (71%) out of 17 patients had a known medical history of hypertension, including 3 patients with documented episodes of preeclampsia and/or HELLP (hemolysis, elevated liver enzymes, and low platelets). No precipitating events were identified in the others. In 12 (71%) out of 17 patients, no systemic hemolysis was observed. The enzymatic activity of ADAMTS13 appeared normal in 8 cases; the other patients presented with platelet counts of over 95 $\times 10^9$/L, making thrombotic thrombocytopenic purpura unlikely.[76] Drug use, infection, autoimmune disease, and pregnancy as causes of TMA were ruled out.[10,11] Familial disease was not noted. Extrarenal manifestations included severe hypertensive retinopathy (*n*/*N*=12/15), cardiac (*n*/*N*=11/14), and/or neurologic disease (*n*/*N*=4/17). Thus, a diagnosis of hypertension–associated TMA was made.

Five patients with hypertensive arterionephrosclerosis were included as disease controls. They presented at older age with lower diastolic BP and less severe kidney failure as compared to those with TMA (Item S2).

Routine complement assays. Lower than normal C3 levels were found in 5 (31%) out of 16 patients with hypertension–associated TMA, while C4 levels and functional studies of the classical pathway of complement activation were normal in all but 1 patient. Plasma levels of soluble C5b9, however, were higher than normal in patients with hypertension–associated TMA (*n*/*N*=14/14), but did not differ from those with hypertensive arterionephrosclerosis (Item S3).

Ex vivo complement activation. At the time of presentation, sera from 14 (88%) out of 16 patients with hypertension–associated TMA induced massive C5b9 formation on resting HMEC–1 (25,926 ± 9,533 vs. 9,025 ± 3,144 pixels, $P<0.01$) as compared to NHS (*n*=8) run in parallel (Table 2, Figure 1A); of note, treatment naive serum from patient no. M99917 was not available. In additional experiments, HMEC–1 were pre–incubated with ADP to mimic a perturbed endothelium,[51] after which comparable results were found (Table 2, Figure 1B). The intensities of C5b9 deposits on ADP–activated HMEC–1 exposed to sera from patients with abnormal test results (*n*=12) were comparable with those exposed to primary atypical HUS sera (26,588 ± 8,229 vs.31,206 ± 2,866 pixels, respectively, Figure 1B). Also, C3c (*n*=5; 31,098 ± 12,690 vs. 8,421 ± 3,093 pixels [NHS], $P<0.05$) but neither C4d nor IgG were found on ADP–activated HMEC–1, indicating selective activation of the

Table 1. Baseline clinical features and laboratory evaluation.

Patient no.	Sex/ age	Relevant history	BP, mmHg	Hypertensive retinopathy	Heart	Brain	SCr, μmol/L	MAHA	Platelets, $\times 10^9$/L	ADAMTS13 activity, %
M09717	M/37	HT	220/130	Grade 3	Y	N	275	N	244	ND
M99917	M/47	HT	280/160	Grade 4	Y	Y	1,980	N	272	42
M01217	M/38	N/a	239/162	Grade 4	Y	Y	726	Y	62	78
M10216	M/47	HT	185/140	Grade 4	Y	Y	835	N	285	61
M04516	F/44	HT	220/120	Grade 2	N	N	649	N	339	76
M01416	F/72	N/a	225/125	Grade 3	N	N	356	Y	75	>10
M01016	M27	N/a	240/150	Grade 4	ND	N	673	N	133	ND
M02715	F/28	HELLP, HT	224/112	Grade 3	N	N	1,065	N	228	82
M01715	F/41	HT	180/120	Grade 3	Y	N	334	N	291	ND
M00915	M/65	HT	195/105	ND	Y	N	162	N	98	ND
M04010	F/32	PE, HT	180/120	ND	Y	N	1,138	Y	142	96
M03307	M/37	HT	200/120	Grade 3	Y	N	586	Y	100	>10
M04306	M/40	HT, CKD	205/114	Grade 2	Y	N	1,195	N	158	ND
M00105	F/38	PE, HT	184/140	Grade 2	ND	N	1,730	N	228	ND
M03802	F/37	HT	300/140	Grade 4	Y	N	1,030	N	204	ND
M05486	M/39	N/a	190/120	Grade 3	ND	Y	1,089	Y	101	ND
M06880	F/23	N/a	185/120	Grade 3	Y	N	645	H	179	ND

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13. CKD, chronic kidney disease. HELLP, hemolysis, elevated liver enzymes, and low platelets. HT, hypertension. MAHA, microangiopathic hemolytic anemia. PE, pre-eclampsia. SCr, serum creatinine.

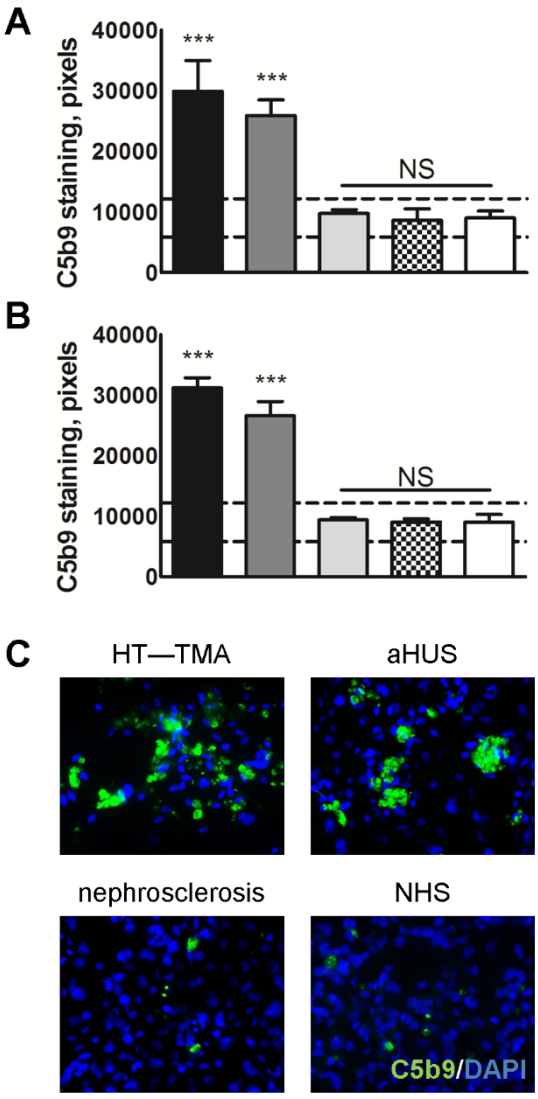
Table 2. Complement work–up at the time of kidney biopsy.

Patient no.	Mutated protein(s)	*CFH*–H3	Δ*CFHR1–3*	FHAA	C4, g/L	C3, g/L	CPFA, %	sC5b9, ng/mL	C5b9 formation on HMEC–1, % control	
									resting	ADP–activated
TMA and severe hypertension										
M09717	None	N	Y	ND	0.35	1.45	119	1,252	223%*	190%*
M99917	CFI p.Pro50Ala, THBD p.Thr478Ile	Y	Y	ND	ND	ND	ND	ND	ND	ND
M01217	None	Y	Y	None	0.36	1.40	ND	4,200	160%	117%
M10216	None	N	Y	None	0.24	0.81	113	740	353%*	205%*
M04516	None	N	N	None	0.35	1.56	113	3,800	198%*	204%*
M01416	None	N	N	None	0.23	1.17	ND	480	245%*	340%*
M01016	None	Y	N	None	0.26	0.82	90	440	224%*	ND
M02715	C3 p.Arg161Trp	N	Y†	None	0.14	0.72	110	1,840	373%*	246%*
M01715	CFI p.Asn151Ser	N	N	ND	0.27	1.20	97	4,200	395%*	252%*
M00915	None	N	N	ND	0.19	0.87	99	1,800	253%*	ND
M04010	CFH p.Cys853Tyr	Y	N	ND	0.21	0.63	104	1,840	339%*	253%*
M03307	C3 p.Arg161Trp	Y	N	ND	0.30	0.88	94	640	463%*	404%*
M04306	CD46 p.Asp271_Ser272del, CFH p.Gln950His	Y	Y	None	0.28	0.89	97	1,000	284%*	272%*
M00105	C3 p.Arg161Trp	N	N	None	0.20	0.69	95	2,800	310%*	325%*
M03802	None	N	N	None	0.25	1.10	105	1,800	140%	92%
M05486	C3 p.Arg161Trp	Y	N	ND	0.47	0.74	ND	ND	336%*	283%*
M06880	None	Y	N	ND	ND	0.64	50	ND	306%*	255%*
Hypertensive arterionephrosclerosis										
1	ND	ND	ND	ND	0.33	1.37	122	2,360	70%	ND
2	ND	ND	ND	ND	0.37	1.36	122	888	89%	71%
3	ND	ND	ND	ND	0.26	0.95	67	ND	76%	87%
4	ND	ND	ND	ND	0.27	2.00	110	780	ND	81%
5	ND	ND	ND	ND	0.38	1.22	103	1,168	67%	77%

*P value <0.05. †Genetic abnormality was found in heterozygosity.

C3; reference range, 0.75–1.35 g/L. C4; reference range, 0.11–0.35 g/L. CPFA, functional activity of the classical pathway; reference range, >75%. Δ*CFHR1–3*, deletion of complement factor H related genes *CFHR1* and *CFHR3*. FHAA, factor H autoantibodies. N, no. ND, not determined. sC5b9, soluble C5b9; reference range, <337 ng/mL. Y, yes.

Figure 1. Hypertension–associated TMA sera induced massive C5b9 formation on resting and ADP–activated HMEC–1 at the time of presentation.



(A) Resting HMEC–1 were incubated for 3 hours with serum from patients with primary atypical HUS (black bar; *C3* and/or *CFB* *n*=2, deficiency of CFHR plasma proteins and factor H autoantibody positive [DEAP] HUS *n*=1, no mutations *n*=1), hypertension–associated (HT–)TMA (dark grey bar; *n*=14), arterionephrosclerosis (light grey bar; *n*=4), dense deposit disease (black and white bar: *n*=5), and healthy controls (white bar; *n*=8). ***<0.001 vs. NHS run in parallel. (B) ADP–activated HMEC–1 were incubated for 3 hours with serum from patients with primary atypical HUS (*C3* and/or *CFB* *n*=3), HT–TMA (*n*=12), arterionephrosclerosis (*n*=4), dense deposit disease (*n*=4), and healthy controls (*n*=7). ***<0.001 vs. NHS run in parallel. (C) Representative immunofluorescence microscopy images of C5b9 (green) staining of ADP–activated HMEC–1 exposed to sera from patients with HT–TMA, primary atypical (a)HUS, (arterio)nephrosclerosis, and NHS (magnification, 400×). Dotted horizontal areas: control, mean ± SD. Patient no. M99917 and M03802's data have not been included.

AP. HMEC–1 exposed to ADP alone without serum or with heat inactivated patient serum (*n*=3, 30 minutes at 56 degrees Celsius) showed neither C3c nor C5b9 staining.

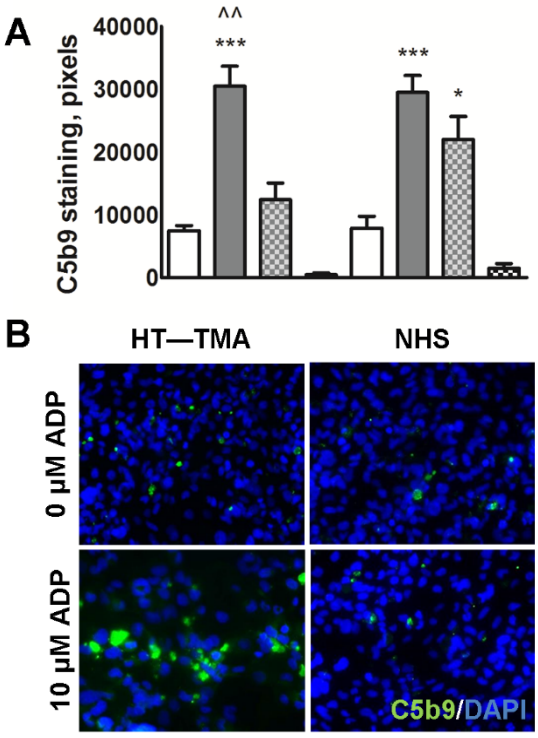
Follow–up samples from 9 patients with hypertension–associated TMA were available (Table 3). C5b9 formation normalized on resting but not on ADP–activated HMEC–1 (*n*=4 untreated patients; 21,972 ± 7,373 vs. 7,894 ± 4,136 pixels [NHS], *P*<0.05) at the time of quiescent disease (Figure 2), ruling out the possibility that complement activation was a secondary phenomenon triggered by acute TMA, that is, ischemic tissue injury and/or platelet activation. As expected, C5b9 formation on resting (*n*=5) and ADP–activated HMEC–1 (*n*=4), was attenuated when incubated with samples from patients on eculizumab treatment (Figure 2), underlining the specificity of C5b9 staining.

In contrast, sera from 4 patients with hypertensive arterionephrosclerosis induced normal C5b9 formation on resting HMEC–1 (9,753 ± 1,214 vs. 9,025 ± 3,144 pixels [NHS]); pre–incubation with ADP did not affect C5b9 formation (Figure 1B). Dense deposit disease samples also induced normal C5b9 formation on ADP activated HMEC–1 (*n*=4; 9,011 ± 941 vs. 8,989 ± 3,184 pixels [NHS]; Figure 1B). Dense deposit disease, however, has been associated with AP dysregulation in the circulation but not on the cell surface,[77,78] indicating that C5b9 formation on HMEC–1 reflects endothelium–restricted complement activation. The data of the disease controls can be found in Item S4.

***Complement deposits on kidney biopsy*.** Kidney tissue specimens of 10 out of 14 patients with hypertension–associated TMA and massive C5b9 formation on resting HMEC–1 were sufficient for analysis and revealed C3c as well as C5b9 deposits along the vasculature and/or glomerular capillaries, confirming *in vivo* complement activation (Figure 3A–B). Eight (80%) out of 10 tissue specimens revealed unspecific entrapment of IgM, while staining for IgG, IgA, and light chains was negative. Electron dense deposits were not found on electron microscopy, supporting the paucity of immune complex deposits (Figure 3D). In contrast, C3c was not found in the setting of arterionephrosclerosis (tissue specimens, *n*=5), excluding complement activation.

***Rare variants in complement genes*.** Rare variants in complement genes were found in 8 (47%) out of 17 patients with hypertension–associated TMA (Table 2, Item S5). The mutated genes included *C3* (*n*=4), *CFH* (*n*=2), *CFI* (*n*=2), and/or *CD46* (*n*=1). The *CD46* mutation was found in association with *CFH* c.2850G>T (p.Gln950His), the latter of which is a rare variant of unknown significance[65] present also in the normal population. Furthermore, a novel variant of unknown significance was found in *THBD*. Eight patients carried the -322C>T and c.2808G>T single

Figure 2. C5b9 formation normalized on resting but not activated HMEC–1 at the time of quiescent disease, whereas eculizumab attenuated C5b9 formation.



C5b9 formation on resting (left) and ADP–activated (right) HMEC–1 after incubation with serum from patients with hypertension–associated (HT–)TMA at the time of acute disease (dark grey bars; *n*=7), quiescent disease (dark/light grey bars; *n*=4), and/or during eculizumab treatment (black/white bars; *n*=5 and *n*=4, respectively); individual data are shown in **Table 3**. *<0.01, ***<0.001 vs. NHS (white bars) run in parallel. ^^<0.01 vs. quiescent disease samples. (B) Representative immunofluorescence microscopy images of either resting or ADP–activated HMEC–1 exposed to sera from patients with HT–TMA at the time of quiescent disease and healthy controls.

nucleotide polymorphisms that tag the *CFH*–H3 haplotype.[27,61] The homozygous genomic deletion of *CFHR1* and *CFHR3* was identified in 1 patient, while circulating factor H autoantibodies were not found.

Clinical outcome. Follow–up ranged from 4 months to 37 years. In all patients, intravenous administration of antihypertensive agents was started and although BP normalized, 14 (82%) out of 17 patients initially needed dialysis (Figure 4). Since 2015, eculizumab was started in 5 patients (no. M99917, M01217, M04516, M01416, M01715) not responding to conventional treatment. Patients were started on four weekly doses of 900 mg eculizumab, followed by a single dose of 1200 mg

Table 3. C5b9 formation on resting and ADP–activated HMEC–1 when incubated with follow–up serum samples from patients with hypertension–associated TMA.

Patient no.	Hb, mmol/L	LDH, U/L	Platelets, $\times 10^9$/µL	SCr, µmol/L	C5b9 formation on HMEC–1, % control: resting	C5b9 formation on HMEC–1, % control: ADP–activated
Eculizumab treatment						
M99917	7.2	135	199	297	21%*	ND
M04516	5.5	143	492	ESKD	0%*	0%*
M01416	6.3	154	298	243	17%*	14%*
M01715	5.5	179	291	315	0%*	24%*
M04010	6.8	138	216	132	8%*	29%*
No treatment						
M02715	7.0	239	333	ND	160%	231%*
M04010	6.8	197	174	205	163%	293%*
M03307	6.1	181	232	220	87%	171%*
M00105	7.5	ND	198	98	116%	248%*

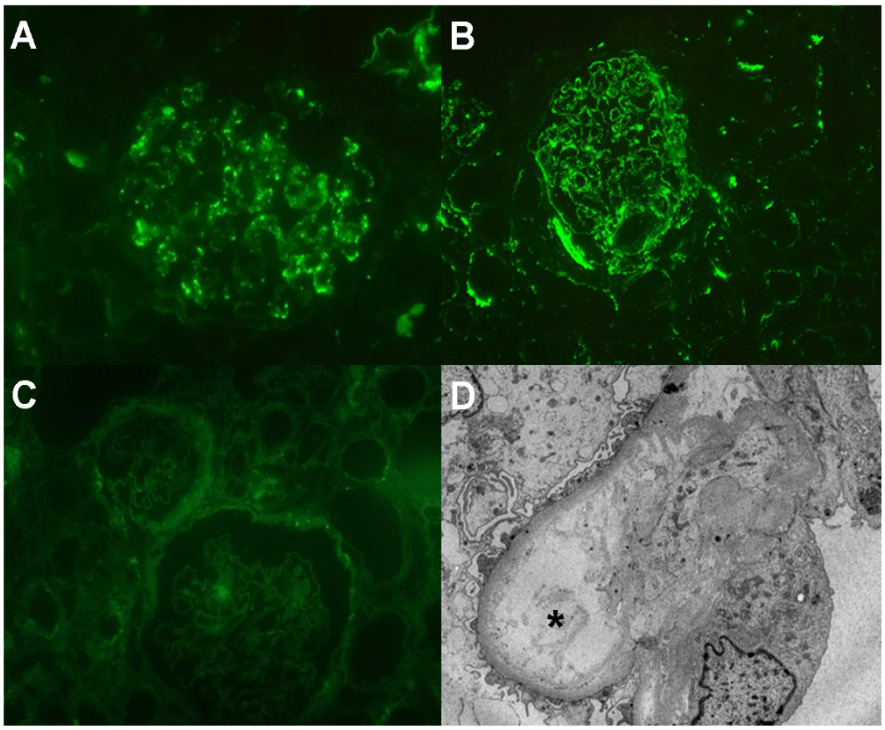
*P value <0.05 versus control serum run in parallel.

ESKD, end–stage kidney disease. ND, not determined. SCr, serum creatinine.

every two weeks (Item S6). Kidney function improved in 3 cases, whereas 2 patients remained dialysis dependent. At the time of quiescent disease, treatment was stopped (no. M04516, M01416) or tapered to 900 mg (no. M01715) every four weeks; during tapering, C5b9 formation on ADP–activated HMEC–1 remained suppressed (data not shown). Patient no. M99917's kidney function is still improving and the dosing regimen has not been tapered yet. No renal response, however, was observed in patient M01217 whose serum induced normal C5b9 formation; moreover, no genetic variants were found. He died from duodenal perforation probably due to pancreatic cancer, suggesting that TMA did not occur on the background of complement dysregulation.

Eleven allografts (cadaveric, *n*=5 and living–[un]related, *n*=6) were transplanted in 7 patients (Figure 5), all of whom received calcineurin inhibitors (tacrolimus, *n*=10 or cyclosporine, *n*=1) and steroids. TMA recurrence manifested in 7 grafts (recipients, *n*=5), either with (*n*=4) or without (*n*=3) systemic hemolysis. BP was tightly regulated and the presence of donor–specific alloantibodies as well as infections as endothelium–damaging events were ruled out, linking TMA recurrence to complement dysregulation. Recipients with recurrent disease showed massive C5b9 formation on HMEC–1 at the time of presentation. Four patients carried rare variants in complement genes (*C3*, *n*=3 and *CD46*, *n*=1). Graft loss occurred in 5 (71%) out of 7 disease episodes even though plasma exchange was initiated in 5 patients. At the time of transplantation, up–front eculizumab was started in a high–risk recipient (no. M04010), preventing disease recurrence. The recipients with a normal test result did not develop recurrent disease.

Figure 3. Representative immunofluorescence–, and electron microscopic findings on kidney biopsies.



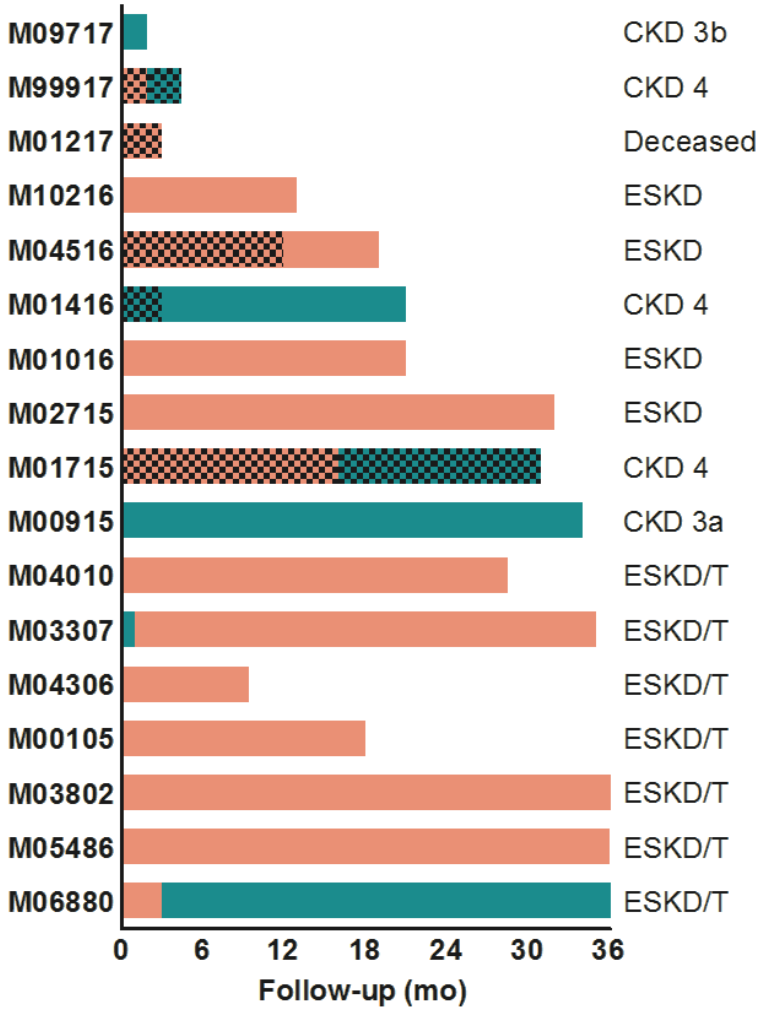
C3c (A, 400×) and/or C5b9 (B, 400×) were found along the vasculature in severely hypertensive patients with TMA, while electron dense deposits were not found (D, 1,900×); widening of the subendothelial space (asterisk) was often acknowledged. C3c (C, 200×) was not linked to arterionephrosclerosis.

DISCUSSION

Complement dysregulation has been linked to various syndromes of TMA, and has recently been acknowledged as the key causative factor of ESKD in a subset of patients with severe hypertension.[73] Many of these patients, however, do not present with systemic hemolysis and complement dysregulation may therefore remain unrecognized. This study showed that massive serum–induced C5b9 formation on microvascular endothelial cells can identify these particular patients, reflecting complement–mediated (C–)TMA. The high prevalence of rare variants in com–plement genes as well as the favorable response to therapeutic complement inhibition supports this premise.

This study supports our previous hypothesis that complement dysregulation is the key causative factor of ESKD in patients presenting with TMA in the setting of severe hypertension.[9] Serum samples from most patients with hypertension–associated TMA but not from those with hypertensive arterionephrosclerosis induced massive C5b9 formation on resting and ADP–activated HMEC–1 at the time

Figure 4. Clinical outcome of patients with hypertension–associated TMA who have been treated with eculizumab or not.

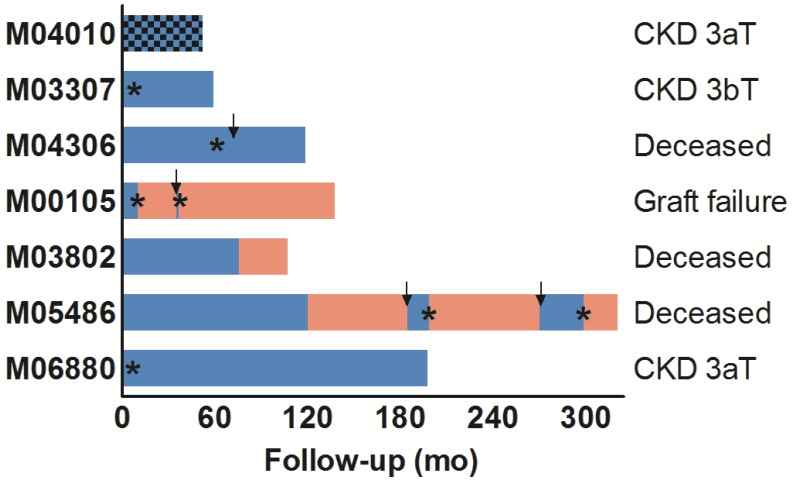


Green bars, kidney survival. Checkered bars, eculizumab treatment. Red bars, dialysis.

CKD, chronic kidney disease stage at last follow–up. ESKD, end–stage kidney disease. T, transplant recipient.

of acute TMA, reflecting unrestrained complement activation via the AP. C5b9 deposits along the vasculature and/or glomerular capillaries provided the *in vivo* counterparts of complement activation. At variance, C5b9 formation normalized on

Figure 5. Allograft survival.



Asterisk, TMA recurrence. Black arrows, re–transplantation; patient no. M04306 has preemptively been re–transplanted. Blue bars, allograft survival. Checkered bar, eculizumab treatment. Red bars, dialysis. CKD, chronic kidney disease stage at last follow–up.

resting but not on ADP–activated HMEC–1 at the time of quiescent disease, underlining that TMA developed on the background of complement dysregulation triggered by a concomitant condition. Rare variants in complement genes confirmed complement dysregulation in approximately half the patients. The variants in complement genes were considered pathogenic as based on proven functional abnormalities of the respective proteins. The presence of the *CFH*–H3 haplotype might have affected the penetrance of disease. In our cohort, most patients had a history of hypertension, suggesting that hypertension triggered complement activation,[42] leading to unrestricted AP activation and TMA to occur.

In line with previous studies focusing on severe hypertension, including our case series, systemic hemolysis appeared uncommon,[50,73,79] leading to a low suspicion of TMA. In these difficult cases, kidney biopsies are needed to detect the TMA, the presence of which should prompt screening for complement dysregulation.[73] Routine complement assays appeared normal in most of our patients and can therefore not be used to detect complement dysregulation. Elevated levels of soluble C5b9, however, can be found during the acute phase but lack specificity since soluble C5b9 concentrations overlap with conditions not linked to systemic complement activation,[67] including arterionephrosclerosis. At present, genetic

studies can be considered the reference method for the determination of complement dysregulation,[11] although suboptimal for diagnostic purposes because genetics are time consuming and lack sensitivity. Noris and colleagues recently demonstrated that massive C5b9 formation on microvascular endothelial cells is highly specific for primary atypical HUS.[51] In the present study, we validate their data and reproducibility in patients who present with TMA in the setting of severe hypertension, differentiating patients with complement dysregulation from those with mechanical stress as the cause of disease.

The prognosis of our patients with massive C5b9 formation on HMEC–1 and/or rare variants in complement genes, appeared dire with 80% of patients requiring dialysis at presentation, resembling historical primary atypical HUS cohorts.[8,9] These clinical observations recapitulate our previous data linking complement dysregulation to ESKD in an extended cohort of patients considered to have hypertension–associated TMA,[73] pointing to a new target for treatment. Indeed, eculizumab attenuated C5b9 to form on HMEC–1 and moreover, kidney function recovered and/or improved in all but 1 patient with complement dysregulation. Dialysis, however, could be tapered in the latter and further improvement in kidney function might have been expected upon extended treatment.[14] Yet, 2 additional cases concerning the use of eculizumab for the treatment of TMA in the setting of severe hypertension have been published with conflicting results.[80,81]

Thus, the question is how to diagnose C–TMA and when to consider treatment in patients presenting with severe hypertension? In our experience, complement dysregulation should be considered in patients who do not respond to BP control, that is, no decrease in serum creatinine of over 25%,[82] particularly when massive C5b9 formation is apparent. Furthermore, we feel that it is important to stress that most of our patients presented with fundoscopic lesions consistent with severe hypertension and thus, retinal lesions should not be used to exclude underlying complement dysregulation. Patients with complement defects appeared to benefit from treatment;[13-15] eculizumab should therefore be considered in these patients. Dosing schedule and treatment duration, however, remain controversial and prospective studies are needed to establish the optimal use of eculizumab for the treatment of TMA in patients with severe hypertension.[83] Also, it has to be proven whether longitudinal measurements of *ex vivo* C5b9 formation can guide treatment.

After kidney transplantation, recurrent disease appeared common and linked to massive C5b9 formation at baseline. TMA onset after kidney transplantation was associated with poor allograft survival, particularly among carriers of rare variants in complement genes. Thus, TMA can reoccur on the background of complement dysregulation. Patients who present with TMA and severe hypertension should therefore be screened for complement dysregulation prior to kidney transplantation to adopt prophylactic measures,[11] which is supported by the favorable outcome in a

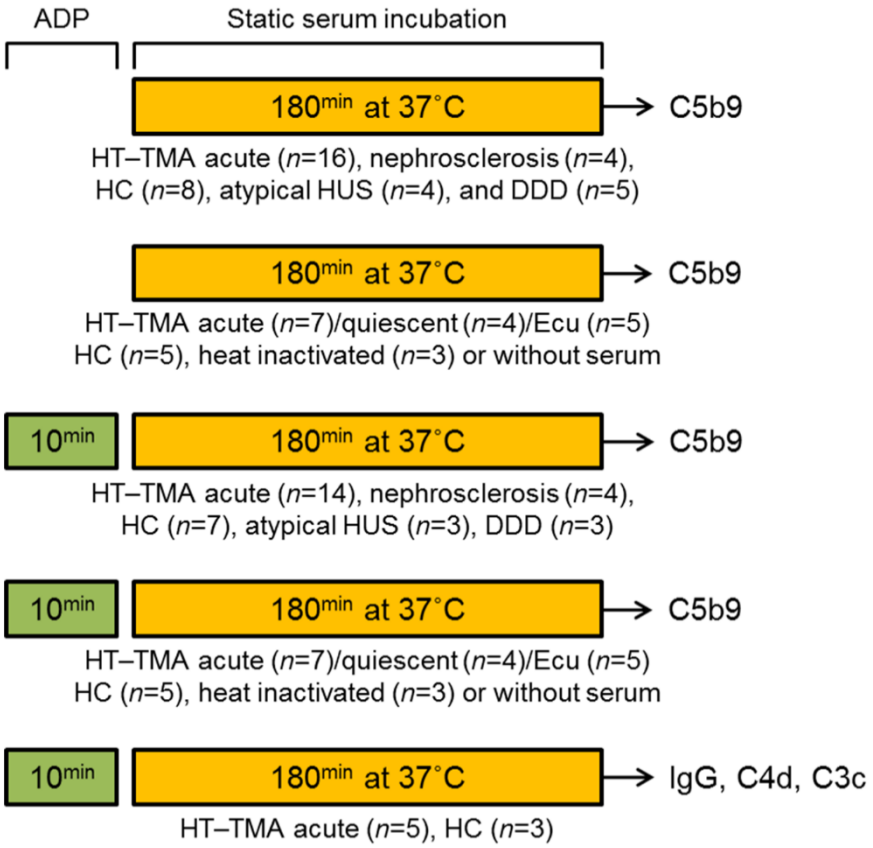
high–risk recipient who received preemptive eculizumab treatment.

The present study is limited by the number of included patients. Future validation in other, larger cohorts is therefore warranted. However, a strong aspect of our study is the fact that we studied a well–defined cohort of patients who have been classified according to clinical and pathological data.

Taken together, our data demonstrate that complement dysregulation is the key causative factor of severe renal sequelae in patients with TMA and severe hypertension at presentation, indicating that these patients should be classified as C–TMA even though systemic hemolysis appeared uncommon. Patients with complement dysregulation can be identified by massive C5b9 formation on microvascular endothelial cells, which is associated with a dismal prognosis. Finally, therapeutic complement inhibition should be evaluated as a novel approach to treatment of TMA in patients with severe hypertension.

SUPPLEMENTAL DATA

Item S1. Experimental design of *ex vivo* complement measurements.



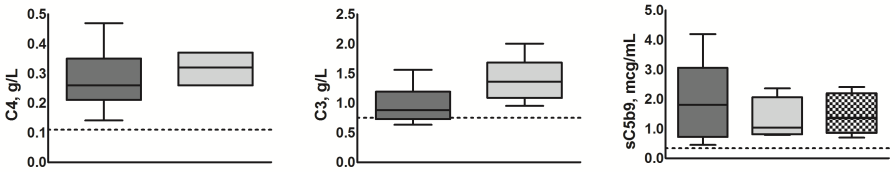
ADP, adenosine diphosphate. DDD, dense deposit disease. Ecu, eculizumab treated samples. HC, healthy control. HT–TMA, hypertension–associated TMA.

Item S2. Baseline characteristics.

	TMA (*n*=17)	**Arterionephro–sclerosis** (*n*=5)	***P* value**
M/F	9/8	5/0	N/a
Age, years	38 (34–45)	63 (52–68)	0.01
SBP, mmHg	212 (184–232)	185 (175–195)	NS
DBP, mmHg	126 (120–142)	100 (80–123)	0.03
Systemic hemolysis, %	5, 29	0, 0	N/a
Creatinine, µmol/L	835 (471–1,141)	193 (122–521)	0.01
Dialysis, %	14, 82	0	N/a
Proteinuria, g/d	1.6 (0.7–3.1)	2.1 (0.4–3.3)	NS
Low C4 (*n*/*N*)	1/16	0/5	N/a
Low C3 (*n*/*N*)	5/16	0/5	N/a

DBP, diastolic blood pressure. N/a, not applicable. NS, not significant. SBP, systolic blood pressure.

Item S3. Routine complement measures in hypertension–associated TMA (dark grey bars), arterio–nephrosclerosis (light grey bars), and C3 glomerulopathy (black and white bar).



Lower limit of normal: C4 0.11 g/L, C3 0.75 g/L, sC5b9 0.337 mcg/L.

Item S4. *Ex vivo* C5b9 formation in disease controls.

	C5b9 formation on resting HMEC–1			**C5b9 formation on ADP–activated HMEC–1**		
Patient	Absolute value, pixels	Control, pixels	% control	Absolute value, pixels	Control, pixels	% control
Primary atypical HUS, rare variants in complement genes and/or FHAA						
1, DEAP–HUS	34,148	10,349	330*	25,412	7,843	324*
2, C3†	27,170	9,918	274*	34,514	13,019	265*
3, C3† and CFB‡	40,967	9,057	452*	33,689	10,401	324*
4, None	17,237	4,707	366*	ND	N/a	N/a
Dense deposit disease						
1	5,531	4,707	118	4,026	4,945	81
2	4,970	4,707	106	ND	N/a	N/a
3	7,543	12,551	60	9,704	5,872	165
4	15,719	12,551	125	7,940	5,872	135
5	9,202	12,551	73	9,390	5,872	160

*P value <0.05. †C3 indicates p.Arg161Trp. ‡CFB indicates p.Lys565Glu.

DEAP, deficiency of plasma proteins and factor H autoantibody positive. N/a, not applicable. ND, not determined.

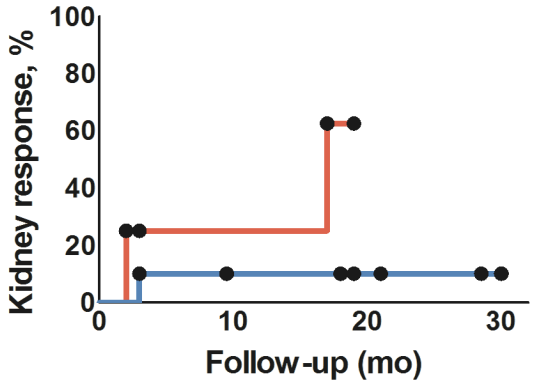
Item S5. Characteristics of the genetic complement defects.

Patient	Gene	Mutated protein(s)	MAF, %	SIFT	PP–2	Defect
M09717	None	N/a	N/a	N/a	N/a	N/a
M99917	*CFI*[1]	p.Pro50Ala	≤0.01	Deleterious	Dam.	Deficiency[63]
	THBD[2]	p.Thr478Ile	0	Tolerated	Benign	Unknown
M01217	None	N/a	N/a	N/a	N/a	N/a
M10216	None	N/a	N/a	N/a	N/a	N/a
M04516	None	N/a	N/a	N/a	N/a	N/a
M01416	None	N/a	N/a	N/a	N/a	N/a
M01016	None	N/a	N/a	N/a	N/a	N/a
M02715	*C3*[1]	p.Arg161Trp	0	Deleterious	Dam.	GOF[66]
M01715	*CFI*[1]	p.Asn151Ser	<0.01	Deleterious	Dam.	Deficiency[63]
M00915	None	N/a	N/a	N/a	N/a	N/a
M04010	*CFH*[1]	p.Cys853Tyr	0	Deleterious	Dam.	LOF[64]
M03307	*C3*[1]	p.Arg161Trp	0	Deleterious	Dam.	GOF[66]
M04306	*CD46*[1]	p.Asp271_Ser272del	0	N/a.	N/a.	Deficiency[43]
	CFH[2]	p.Gln950His	>0.1	Deleterious	Dam.	LOF(?)[65]
M00105	*C3*[1]	p.Arg161Trp	0	Deleterious	Dam.	GOF[66]
M03802	None	N/a	N/a	N/a	N/a	N/a
M05486	*C3*[1]	p.Arg161Trp	0	Deleterious	Dam.	GOF[66]
M06880	None	N/a	N/a	N/a	N/a	N/a

The variants have been classified as [1](likely) pathogenic or [2]uncertain significance.

Dam., damaging. GOF, gain–of–function. LOF, loss–of–function. MAF, minor allele frequency according to Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/) and Exome Aggregation Consortium databases (ExAC, http://exac.broadinstitute.org/). N/a, not applicable. PP–2, Polymorphism Phenotyping V2. SIFT, Sorting Intolerant From Tolerant.

Item S6. Kaplan Meier shows the cumulative incidence of kidney survival and/or recovery in eculizumab treated (red, *n*=5) and treatment naive patients (blue, *n*=12).



Log–rank test, *P*=0.08.

4

Diagnostic and risk factors for complement defects in patients with hypertensive emergency and thrombotic microangiopathy

Sjoerd A.M.E.G. Timmermans,[1, 2] Alexis Wérion,[3] Jan G.M.C. Damoiseaux,[4] Johann Morelle,[3, 5] Chris P. Reutelingsperger,[2] and Pieter van Paassen.[1, 2]

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Summary. Hypertensive emergency can cause thrombotic microangiopathy (TMA) in the kidneys with high rates of end–stage kidney disease (ESKD) and vice versa. The conundrum of hypertension as the cause of TMA or consequence of TMA on the background of complement dysregulation remains difficult. Patients with hypertensive emergency and TMA on kidney biopsy were tested for *ex vivo* C5b9 formation on the endothelium and rare variants in complement genes to identify complement–mediated (C–)TMA. We identified factors associated with complement dysregulation and poor kidney outcomes. Massive *ex vivo* C5b9 formation was found on resting endothelial cells in 18 (69%) out of 25 cases at presentation, including the 9 patients who carried at least 1 rare genetic variant. Thirteen (72%, *N*=18) and 3 (38%, *N*=8) patients with massive and normal *ex vivo* complement activation, respectively, progressed to ESKD (*P*=0.03). In contrast to blood pressure (BP) control, inhibition of C5 activation prevented ESKD to occur in 5 (83%, *N*=6) patients with massive *ex vivo* complement activation. TMA–related graft failure occurred in 7 (47%, *N*=15) donor kidneys and was linked to genetic variants. The assessment of both *ex vivo* C5b9 formation and screening for rare variants in complement genes may categorize patients with hypertensive emergency and TMA into different groups with potential therapeutic and prognostic implications. We propose an algorithm to recognize patients at the highest risk for complement dysregulation.

Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Division of Nephrology, Cliniques universitaires Saint–Luc, BE.
[4]Central Diagnostic Laboratory, Maastricht UMC, NLD.
[5]Institute de Recherche Expérimentale et Clinique, UCLouvain, BE.

Mild–to–moderate hypertension may cause a slow decline in kidney function, while acute ESKD can occur in patients presenting with hypertensive emergency. ESKD is attributed to hypertension in up to 25% adult cases in Europe and the United States[84] with, unfortunately, no confirmative proof, assuming that the kidneys are the victim rather than culprit of disease. Parenchymal renal disease, including TMA, can therefore be missed.[85] TMA represents tissue responses to endothelial damage caused by distinct mechanisms, such as mechanical stress to the endothelium in hypertensive emergency[72] and defects in complement regulation in primary atypical hemolytic uremic syndrome (HUS).[8,9]

We recently demonstrated that identical defects in complement regulation can be linked to ESKD in patients presenting with hypertensive emergency and TMA on kidney biopsy, resembling primary atypical HUS.[73] The defects in complement regulation are often caused by rare variants in genes encoding proteins that either regulate or activate complement and/or autoantibodies that inhibit complement regulation, lowering the threshold for unrestrained C5 activation and TMA to occur.[30] The generalized endothelial damage in primary atypical HUS can induce thrombosis, consumptive thrombocytopenia, and mechanical hemolysis, contributing to target organ damage, mostly affecting the kidneys. In clinical practice it remains challenging to identify primary atypical HUS among patients with hypertensive emergency because many of such patients do not present with profound hematologic abnormalities;[50,73] we therefore prefer the term C–TMA. The correct identification of C–TMA, however, is critical given the potential therapeutic benefit of complement inhibition.[13-15] Reliable diagnostic methods are lacking.[86] KDIGO therefore questioned whether a functional *ex vivo* complement test, clinical manifestations, and/or pathologic features on kidney biopsy can aid the differential diagnosis in patients with hypertensive emergency.[11]

In the present study, patients with hypertensive emergency and TMA on kidney biopsy were screened for massive *ex vivo* C5b9 formation, a specific test to detect unrestrained C5 activation on the endothelium,[51] and rare variants in complement genes linked to defects in complement regulation. In addition, we analyzed clinical manifestations and kidney biopsies for specific features to distinguish C–TMA from hypertensive emergency as the sole cause of TMA.[11] Thus, our objectives were to assess factors associated with C–TMA and to propose a clinical algorithm to recognize C–TMA among patients presenting with hypertensive emergency. Reliable factors may guide the use of therapeutic complement inhibition.

MATERIAL AND METHODS

Patient population. Patients with hypertensive emergency and TMA with typical pathologic features of severe hypertension (i.e., mucoid intimal edema) on kidney biopsy were recruited from the Limburg Renal Registry, Maastricht, The

Netherlands,[54] and the Cliniques universitaires Saint–Luc, Brussels, Belgium, respectively. Miscellaneous causes of TMA other than hypertensive emergency were ruled out;[10,11] the enzymatic activity of ADAMTS13, i.e., von Willebrand factor cleaving protease, was assessed by using the "FRETS–VWF73" assay in selected cases.[87] Hypertensive emergency was defined as a systolic and/or diastolic BP of >180/120 mmHg and evidence of impending or progressive extrarenal target organ damage secondary to hypertension.[88] Patients were screened for neurologic and cardiac disease as considered appropriate. Neurologic disease was defined as acute onset of severe headache, seizures, coma, cerebral infarction or bleeding on imaging; cardiac disease was defined as ventricular dysfunction or acute ischemia on electrocardiogram and/or ultrasound.

At the time of presentation and during follow–up, serum samples were obtained, processed, and immediately stored at –80 degrees Celsius to prevent *in vitro* complement activation.[59] The study was approved by the appropriate ethics committees and is in accordance with the Declaration of Helsinki.

Kidney tissue specimens. Kidney tissue sections were processed as described.[54] The sections were scored as glomerular TMA or isolated intimal edema, the latter of which reflects mucoid intimal edema and clear absence of acute glomerular lesions (e.g., thrombosis, endothelial cell swelling, and mesangiolysis). Tubular atrophy and interstitial fibrosis were scored as mild (<25%), moderate (25–50%), or severe (>50%).

Also, sections from snap frozen kidney specimens were stained with rabbit anti–human C5b9 pAb (1:100; Calbiochem, San Diego, CA) followed by Alexa488 labeled goat anti–rabbit Ab (1:100; Life Technologies, Carlsbad, CA).

Complement work–up. *Ex vivo* C5b9 formation on microvascular endothelial cells of dermal origin (ATCC, Manassas, VA) was assessed to identify patients with complement dysregulation as described.[86] Briefly, endothelial cells were plated on glass culture slides and used when >80% confluent, incubated with serum diluted in test medium for 3 hours at 37 degrees Celsius, fixed in 3% formaldehyde, and blocked with 2% BSA for 1 hour. In selected experiments, endothelial cells were preincubated with 10 μM adenosine diphosphate for 10 minutes to mimic a perturbed endothelium.[51] Rabbit anti–human C5b9 pAb (Calbiochem) and Alexa488 labeled goat anti–rabbit Ab (Life Technologies) were used. The results were compared with pooled normal human serum (NHS) run in parallel.

Patients were screened for rare variants, i.e., variants with a minor allele frequency <1%, and single nucleotide polymorphisms in coding regions of *CFH*, *CFI*, *CD46*, *CFB*, *C3*, *CFHR1–5*, *THBD*, and *DGKE* using DNA sequencing. The classification of variants was based on international standards.[89] Pathogenic

variants were defined as those with functional studies supporting a defect in complement regulation, including null variants in genes linked to complement regulation and/or variants that cluster in patients with primary atypical HUS as demonstrated by Osborne and colleagues.[23] Likely pathogenic variants were defined as those with functional studies supporting a defect in complement regulation that have been located in a mutational hotspot and/or critical functional domain. Rare variants not fulfilling these criteria have been classified as uncertain significance. The *CFH–CFHR1–5* genomic region was analyzed for rearrangements by multiplex ligation probe amplification. Factor H autoantibodies were assessed by ELISA in selected cases.[58]

C–TMA was defined as massive *ex vivo* C5b9 formation on resting endothelial cells at presentation and/or the presence of (likely) pathogenic variants in complement genes.

Statistical analysis. Continuous variables were presented as mean (±SD) or median (interquartile range [IQR]) as appropriate. Differences in continuous and categorical variables were checked using the unpaired t or Mann–Whitney U test and the chi–square or Fisher's exact test, respectively. The *ex vivo* formation of C5b9 on the endothelium was compared with NHS run in parallel by the paired sample *t* test or Wilcoxon signed rank test as appropriate. Survival was assessed using the Kaplan–Meier method and log–rank test.

RESULTS

Patient population. Twenty six patients (European, *n*=22; African, *n*=4) with hypertensive emergency and TMA on kidney biopsy were included (Table 1). Patients invariably presented with severe kidney failure (median serum creatinine 723 µmol/L, IQR 423–1,071) and proteinuria, 17 (65%) of whom initially required dialysis. Kidney biopsies were needed to detect the TMA in 19 (73%) cases because profound systemic hemolysis was not present. The enzymatic activity of ADAMTS13 appeared normal in the 17 patients who have been tested for; the other patients presented with platelet counts of over 95,000 per µL, making thrombotic thrombo–cytopenic purpura highly unlikely.[76] Drug use, infection, autoimmune disease, and pregnancy as causes of TMA were ruled out. Familial disease was not noted. Extrarenal manifestations included severe hypertensive retinopathy (i.e., grade III and/or IV; *n*/*N*=22/25, 88%), cardiac disease (*n*/*N*=18/22, 82%), and/or neurologic disease (*n*/*N*=6/26, 23%).

Kidney biopsy findings. Mucoid intimal edema was invariably present either with glomerular TMA (*n*=13) or not (*n*=13), i.e., those with isolated intimal edema. Mucoid intimal edema, however, was not found in C–TMA without hypertensive emergency

Table 1. The patients' characteristics at the time of presentation.

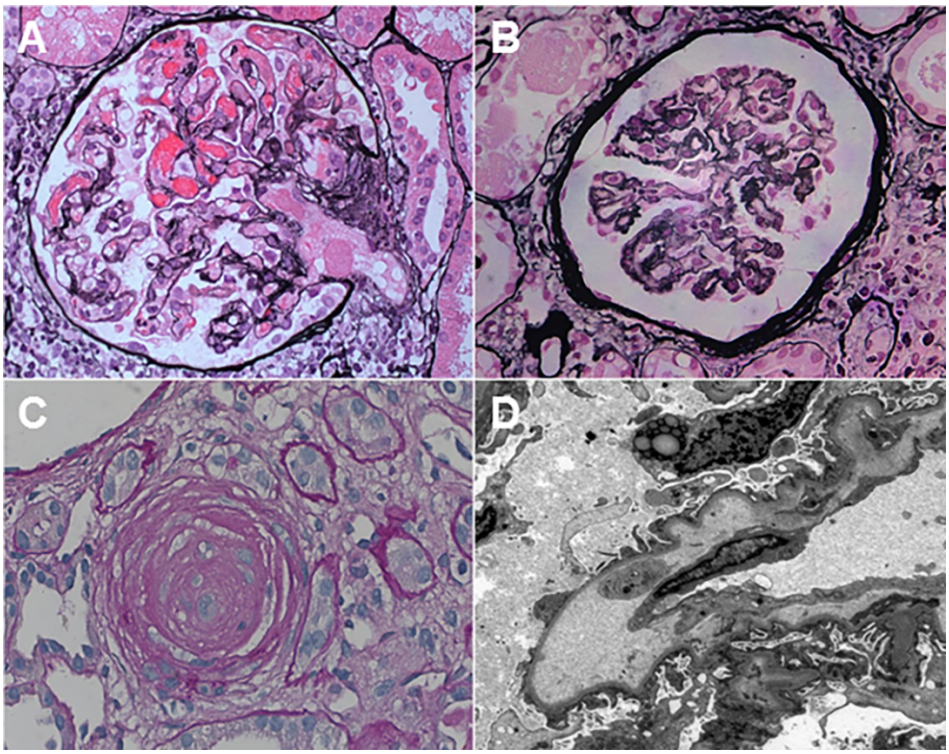
	Total	C–TMA	Normal C regulation	*P* value
N	26	18	8	
M/F	15/11	9/9	6/2	0.39
European, %	22, 85	18, 100	4, 50	<0.01
Age, years	39 (±11)	40 (±9)	40 (±8)	0.81
SBP, mmHg	217 (±32)	210 (±29)	236 (±35)	0.08
DBP, mmHg	130 (120–148)	120 (120–140)	146 (140–154)	0.02
LDH, U/L	731 (335–1,168)	638 (303–1,200)	762 (612–1,128)	0.33
Platelets, $\times 10^9$/L	168 (±79)	184 (±78)	113 (±55)	0.04
Creatinine, µmol/L	723 (423–1,071)	820 (579–1,152)	605 (410–971)	0.19
Dialysis, %	17, 65	14, 78	3, 38	0.08
Glomerular thrombosis	13	12	1	0.03
Extrarenal manifestations				
Neurologic disease, *n*/*N*	6/26	5/18	1/8	0.63
Retinopathy, *n*/*N*	22/25	14/17	8/8	0.53
Cardiac disease, *n*/*N*	18/22	11/14	7/8	1.00
Systemic hemolysis, %	7, 27	4, 22	3, 38	0.64
Complement				
Low C4, *n*/*N*	0/22	0/15	0/7	
Low C3, *n*/*N*	7/24	6/17	1/7	0.62
Rare variant(s), %	9, 35	9, 50	0, 0	0.02
Outcome				
Renal response, %	10, 38	5, 28	5, 63	0.19
ESKD at three months, %	16, 62	13, 72	3, 38	0.03
Donor kidneys	15	12	3	
Relapse	7	7	0	0.20

Systemic hemolysis was defined as microangiopathic hemolytic anemia (i.e., hemoglobin <10 g/dL, lactate dehydrogenase >500 U/L, schistocytes on peripheral blood smear) and platelets <150,000/µL.

ESKD, end–stage kidney disease. C, complement. DBP, diastolic blood pressure. F, female. M, male. SBP, systemic blood pressure.

(data not shown). The typical features on kidney biopsy have been depicted in Figure 1. On average, 16 (±7) glomeruli were present. Most tissue specimens classified as glomerular TMA showed acute lesions, that is, glomerular thrombosis, endothelial cell swelling, endocapillary hypercellularity, and/or mesangiolysis; 2 (15%) out of 13 specimens classified as glomerular TMA showed chronic rather than acute lesions, i.e., membranoproliferative glomerulonephritis. The glomeruli from those classified as isolated intimal edema showed wrinkling of the glomerular basement membrane (GBM) either with ischemic collapse of the capillary tuft or not; one of these specimens (analyzed, *n*/*N*=6/13) showed abnormalities linked to TMA on electron microscopy, that is, widening of the GBM with electron lucent material. Mild tubular atrophy and interstitial fibrosis was present in 11, moderate in 8, and severe in 7 cases. Frozen kidney tissue specimens of 20 patients were available for immunofluorescence microscopy. Focal granular C3c and C5b9 deposits were found at the vascular pole and/or along segments of the GBM in 9 (53%, *N*=17) and 7 (47%, *N*=15) cases, respectively; co–localization of C3c and C5b9 was found in 6 of these cases. Focal granular IgM deposits were found along segments of the GBM in 7 (44%, *N*=16) and sclerotic areas in 3 (19%, *N*=16) cases, while immune complex

Figure 1. Morphologic features on kidney biopsy of 2 patients with a pathogenic variant in *C3* (i.e., c.418C>T; p.Arg161Trp).



Mucoid intimal edema was present (C; periodic acid–Shiff stain, 400×) either with glomerular TMA (A; Jones methenamine silver, 400×) or not (B; Jones methenamine silver, 630×). Electron microscopy revealed widening of the GBM with electron lucent material (D, 1900×).

deposits were not appreciated on electron microscopy (analyzed, *n*/*N*=9/10), indicating unspecific entrapment of IgM.

Complement work–up. At presentation, serum samples were available to test for *ex vivo* C5b9 formation from all but 1 patient (i.e., the patient with combined variants in *CFI* and *THBD* as described below). Massive *ex vivo* C5b9 formation on the resting endothelium was found in 17 (68%) out of 25 cases tested for. Of note, *ex vivo* C5b9 formation normalized on resting but not on perturbed endothelial cells at the time of quiescent disease (analyzed, *n*/*N*=7/17), underscoring the key role of a second hit for unrestrained C5 activation to occur. The 8 samples with normal test results at presentation also showed normal *ex vivo* C5b9 on the perturbed endothelium, indicating normal complement regulation.[51,86] It is noteworthy that the *ex vivo* test's specificity is 97% (data not shown).

DNA samples were tested and rare variants in complement genes were found in

9 (35%) out of 26 patients. The characteristics of the variants, including *C3* (*n*=2), *CFH* (*n*=2), *CFI* (*n*=2), *CD46* (*n*=1), and/or THBD (*n*=1) have been depicted in Table 2; 6 out of 8 variants were considered (likely) pathogenic. Two patients presented with combined variants: *CFI* with *THBD* and *CD46* with *CFH*. The at–risk *CFH*–H3 and MCP_{GGAAC} haplotypes were found in 8 (32%) and 2 (8%) cases, respectively; the homozygous genomic deletion of *CFHR1* and *CFHR3* was identified in 2 patients, while factor H autoantibodies were not found.

Thus, C–TMA was diagnosed in 18 (69%) out of 26 patients. Details about the complement work–up are provided in Table 2 and Table 3.

Patient characteristics and outcome depending on complement defects. None of the 4 patients from African descent had C–TMA (*P*=0.004). The diastolic BP appeared to be slightly lower, whereas platelet counts were higher in patients with C–TMA as compared to those with no complement defects (Table 1). Extrarenal manifestations did not differ between both groups. Most of the patients with C–TMA presented with glomerular TMA on kidney biopsy (*n*/*N*=12/13 versus *n*/*N*=6/13 with isolated intimal edema, *P*=0.03), while the prevalence of rare variants in complement genes did not differ between both pathologic groups (*n*/*N*=6/13 versus *n*/*N*=3/13, *P*=0.4). No specific staining for C–TMA was found on immunofluorescence microscopy (data not shown).

Patients were followed for a median of 2.6 (IQR, 0.8 – 10.8) years. BP was controlled by intravenous administration of antihypertensive agents. Thirteen (72%) of the 18 patients with C–TMA and 3 (38%) of the 8 patients with no complement defects had ESKD at 3 months follow–up (*P*=0.03), while the other 5 patients in both groups had chronic kidney disease (CKD). Except for 1 case, none of the patients with C–TMA achieved a renal response (i.e., recovery of kidney function or >25% decrease in serum creatinine) upon BP control alone. Five patients with no complement defects who had CKD at three months follow–up achieved a renal response after BP control; the 3 non–responders, however, had a serum creatinine of >700 µmol/L at presentation. From 2015 onwards, the anti–C5 mAb eculizumab was started in 7 cases (C–TMA, *n*=6) because the lack of a renal response. Five (83%) out of 6 patients with C–TMA who received eculizumab recovered and/or improved kidney function (Figure 2A); no response, however, was achieved in the patient with no complement defects. Follow–up serum samples from 6 treated patients (C–TMA, *n*=5), indeed, confirmed adequate inhibition of C5 (Table 3).

Fifteen donor kidneys were transplanted in 7 recipients with C–TMA (carriers of pathogenic variants, *n*=6) and 2 recipients with no complement defects (Figure 2B). TMA manifested in 7 donor kidneys of 4 recipients with pathogenic variants in complement genes (i.e., *C3*, *n*=3 and *CD46* combined with *CFH*, *n*=1) but not in those with no variants identified (*P*=0.1). Recurrence of TMA after kidney transplantation was linked to graft failure in all cases.

Table 2. Complement work–up in 26 patients with hypertensive emergency and TMA.

							C5b9 formation on HMEC–1, % control	
Patient no.	**Variant(s)**	**MAF, %**	***In vitro* defect**	***CFH*-H3/ *MCP*$_{GGAAC}$**	**Δ*CFHR1 –3***	**FHAA**	**resting**	**ADP–activated**
M99918	None			N/N	N	ND	121%	121%
M09018	*C3* c.463A>C[2]	0.2–0.4	GOF(?)	Y/Y	N	ND	326%**	403%***
M06018	None			N/Y	Y	None	297%***	ND
M09717	None			N/N	Y	None	223%**	190%**
M99917	*CFI* c.148C>G[1]	<0.02	LOF	Y/N	Y	ND	ND	ND
	THBD c.1433C>T[2]	<0.01	Unknown					
M01217	None			Y/N	Y	None	160%	117%
M10216	None			N/N	Y	None	353%**	205%**
M04516	None			N/N	N	None	198%***	204%*
M01416	None			N/N	N	None	245%***	340%***
M01016	None			Y/N	N	None	224%*	ND
M02715	*C3* c.481C>T[1]	<0.01	GOF	N/N	Y†	None	373%***	246%**
M01715	*CFI* c.452A>G[1]	<0.01	LOF	N/N	N	ND	395%***	252%*
M04010	*CFH* c.2558G>A[1]	0	LOF	Y/N	N	ND	339%**	253%**
M03307	*C3* c.481C>T[1]	<0.01	GOF	Y/N	N	ND	463%***	404%***
M04306	*CD46* c.811_816delGACAT[1]	0	LOF	Y/N	Y	None	284%***	272%***
	CFH c.2850G>T[2]	0.1–0.4	LOF(?)					
M00105	*C3* c.481C>T[1]	<0.01	GOF	N/N	N	None	310%***	325%**
M03802	None			N/N	N	None	140%	92%
M05595	None			N/N	N	ND	ND	95%
M05486	*C3* c481C>T[1]	<0.01	GOF	Y/N	N	ND	336%***	283%***
M06880	None			Y/N	N	ND	306%***	255%**
B47	None			N/N	N	None	99%	121%
B30	None			N/N	N	None	162%	125%
B17	None			N/N	N	None	87%	117%
B04	None			N/N	N	None	394%**	ND
B03	None			N/N	Y§	None	159%	85%
B02	None			N/N	N	None	197%*	ND

$^{*}P$ value <0.05, $^{**}P$ value <0.01, and $^{***}P$ value <0.001. †Δ*CFHR1-CFHR3* came in homozygosity. The variants have been classified as [1](likely) pathogenic or [2]uncertain significance

FHAA, factor H autoantibodies. GOF, gain–of–function. LOF, loss–of–function. MAF, minor allele frequency according to Exome Variant Server (EVS) and Genome Aggregation Database (gnomAD). N, not found. ND, not determined. Y, found.

◀**Table 2.** Continued.
The *in vitro* defects have been studied elsewhere: *CFH* c.2558G>A (Ref.[64]), *CFH* c.2850G>T (Ref.[65]), *CFI* c.148C>G (Ref.[63]), *CFI* c.452A>G (Ref.[63]),*CD46* c.811_816delGACAT (Ref.[43]), *C3* c.463A>C (Ref.[90]), and *C3* c.481C>T (Ref.[66]).

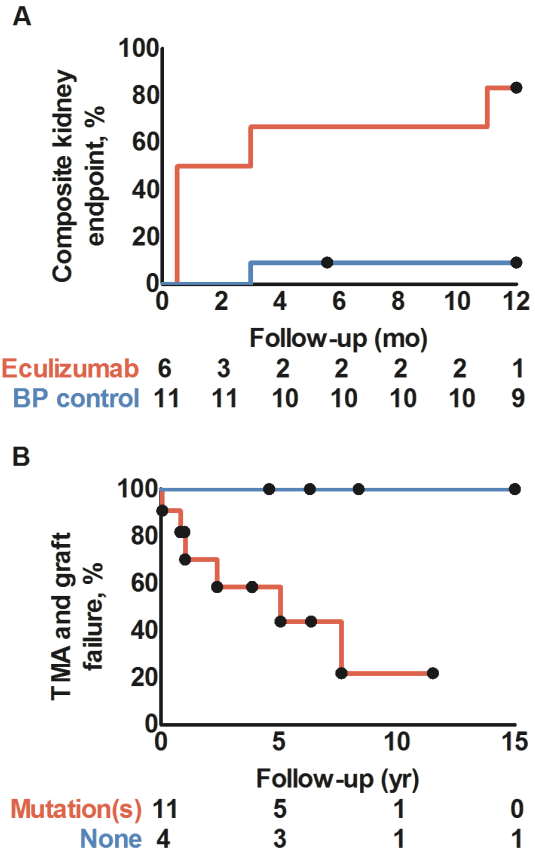
Table 3. *Ex vivo* C5b9 formation during follow–up in patients treated with eculizumab or not.

Patient no.	Variant(s)	Interval of eculizumab	Hb, mmol/L	LDH, U/L	Platelets, ×10^9/L	SCr, µmol/L	C4, g/L	C3, g/L	CPFA, %	*Ex vivo* C5b9, % of the control	
										resting	ADP–activated
TMA, active disease											
M03307[†]	*C3*[1]	N/a	6.1	383	210	426	0.27	0.90	65	366***	307***
TMA, quiescent disease											
M04516	None	N/a[‡]	7.3	85	ND	ESKD	ND	ND	ND	136	ND
M01416	None	N/a[‡]	7.2	192	301	187	ND	ND	121	74	251*
M02715	*C3*[1]	N/a	7.0	239	333	ESKD	0.34	1.14	114	160	231*
M01715	*CFI*[1]	N/a[‡]	6.6	142	399	278	0.33	1.41	>125	141	256**
M04010[§]	*CFH*[1]	N/a	6.8	197	174	205	0.20	0.47	107	163	293**
M03307[§]	*C3*[1]	N/a	6.1	181	232	220	0.28	0.97	77	87	170**
M00105[§]	*C3*[1]	N/a	7.5	ND	198	98	ND	ND	ND	116	248***
TMA, eculizumab treatment											
M06018	None	3 wk	7.7	134	236	399	0.22	1.10	3	ND	14**
M99917	*CFI,*[1] *THBD*[2]	2 wk	8.9	277	211	227	ND	1.09	2	ND	3***
		3 wk	8.6	165	210	190	ND	ND	21	25**	45*
M01217	None	1 wk	4.9	241	309	ESKD	ND	ND	4	0	0
M04516	None	2 wk	5.5	143	492	ESKD	0.34	1.36	6	0	0
M01416	None	2 wk	6.3	154	298	234	0.28	1.12	2	17***	14**
M01715	*CFI*[1]	2 wk	5.5	179	291	315	ND	ND	0	0**	24**
		4 wk	7.4	141	393	288	0.29	1.16	102	160	229***
M04010[§]	*CFH*[1]	4 wk	6.8	138	216	132	0.21	0.56	0	9***	29**
M03307[§]	*C3*[1]	2 wk	6.7	264	216	437	ND	ND	2	ND	12***

$^{*}P$ value <0.05, $^{**}P$ value <0.01, and $^{***}P$ value <0.001. [†]Kidney donor recipient. [‡]Eculizumab has been discontinued for over 3 months. Reference values for C4, C3, and CPFA are 0.11–0.35 g/L, 0.75–1.35 g/L, and >75%, respectively. The variants have been classified as [1](likely) pathogenic or [2]uncertain significance.

CPFA, functional activity of the classical pathway. ESKD, end–stage kidney disease. ND, not determined. SCr, serum creatinine.

Figure 2. The clinical outcome prior to and after kidney transplantation.



(A) The cumulative incidence of the composite kidney endpoint, i.e., renal response and/or recovery, was higher in patients with C–TMA who had been treated with eculizumab versus those who had not been treated; the composite renal endpoint occurred after a median of 1.8 months (P=0.001). (B) TMA and subsequent graft failure was common in kidney donor recipients with pathogenic variants; TMA recurred after a median of 5.0 years (P=0.05).

DISCUSSION

Here, we demonstrated that massive *ex vivo* C5b9 formation on the endothelium predicted a poor response upon BP control in patients with hypertensive emergency and TMA on kidney biopsy, while therapeutic complement inhibition appeared effective in most of the treated cases. Half the patients with abnormal test results carried rare variants in complement genes, confirming the genetic predisposition for C–TMA to develop. Neither clinical manifestations nor pathologic features on kidney biopsy appeared specific for C–TMA. Thus, our findings indicate that massive *ex*

vivo C5b9 formation holds promise for the recognition of C–TMA and may be suitable to assess the effectiveness of therapeutic complement inhibition in patients with hypertensive emergency. Moreover, pathogenic variants in complement genes predicted TMA and subsequent graft failure after kidney transplantation.

Patients with hypertensive emergency may present with acute kidney failure and TMA in over 50% and up to 25% of cases, respectively.[79] TMA may be under–recognized as most patients with hypertensive emergency and TMA on kidney biopsy lack systemic hemolysis. Thus, a kidney biopsy is imperative to detect the TMA; obviously, control of BP and other clinical routines is mandatory to lower the risk of bleeding.[91] Patients with TMA should be screened for severe ADAMTS13 deficiency and other causes.[10,11] The conundrum of hypertensive emergency as a cause or consequence of TMA, however, remains difficult.

DNA testing can confirm the genetic predisposition for C–TMA in half the patients,[11] but does not reflect the dynamic process of complement activation. Levels of circulating complement proteins and markers of complement activation also lack sensitivity and specificity as complement in C–TMA is activated on the endothelium (i.e., the solid phase) rather than the fluid phase.[51,86] *Ex vivo* C5b9 formation reflects solid phase but not fluid phase complement activation.[51,86] Here, we demonstrated that massive *ex vivo* C5b9 formation reflects unrestrained C5 activation on the endothelium regardless of the genetic predisposition. *Ex vivo* C5b9 formation, indeed, normalized on resting but not on perturbed endothelial cells at the time of quiescent disease. The clinical course of patients with massive *ex vivo* C5b9 formation was poor, with early ESKD occurring in most patients. BP control was ineffective in all but 1 case, while eculizumab blocked *ex vivo* C5b9 formation and improved kidney function among patients classified as C–TMA. In contrast, BP control was effective in over half the patients with normal *ex vivo* test results at presentation, resembling the natural course of so–called malignant nephro–sclerosis.[74] Thus, our observations indicate that massive *ex vivo* C5b9 formation differentiates C–TMA from TMA caused by hypertensive emergency alone.[86]

C–TMA was associated with a poor prognosis, indicating C5 as a therapeutic target for the treatment of TMA in patients with hypertensive emergency. Eculizumab's dosing schedule and treatment duration, however, remain to be established. In line with Galbusera and colleagues,[92] a prolonged interdose interval of 3 to 4 weeks retained normal *ex vivo* C5b9 formation on the perturbed endothelium despite a residual activity of the classical pathway above the recommended goal of <10% during therapeutic complement inhibition.[11] None of the reported patients to date developed a relapse of disease, suggesting that trough levels below the recommended target concentration[93] can prevent unrestrained C5 activation, potentiating lower costs of treatment.

Knowledge of the genetic predisposition is instrumental to make informed

decisions regarding transplantation in potential recipients with TMA in their native kidneys.[11] Patients with (likely) pathogenic variants in *CFH*, *CFB*, *C3*, and those with combined mutations, are at high–risk of disease recurrence.[44] In our cohort, TMA recurrence occurred in high–risk recipients who carried pathogenic variants in complement genes,[11] underscoring the importance of genetic testing prior to kidney transplantation. Eculizumab can prevent TMA to manifest and its sequelae.[94] Living kidney donation and adequate BP control may postpone the initiation of eculizumab[95] and, in particular, because TMA developed as a late complication after kidney transplantation. However, one should keep in mind that the allograft's capacity to recover is limited as compared with native kidneys.[96]

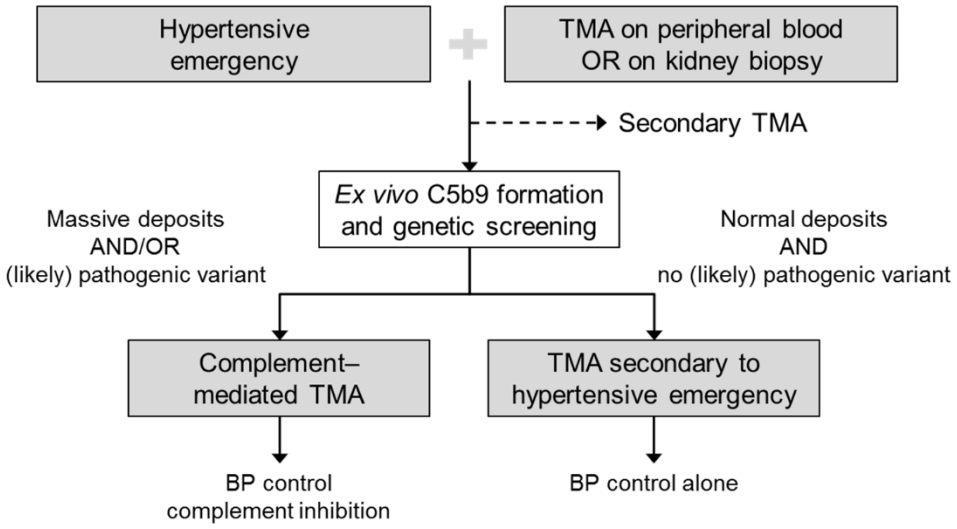
Of note, in our experience, several patients with C–TMA in their native kidneys have been misdiagnosed as hypertensive ESKD. Neither clinical manifestations nor pathologic features on kidney biopsy appeared specific for C–TMA in patients with hypertensive emergency. Thus, impending hypertensive organ damage outside the kidneys, including severe retinopathy, should not be used to exclude primary atypical HUS as such manifestations are common in patients with primary atypical HUS who present with hypertensive emergency.[86,97] Larsen and colleagues suggested that the presence of isolated intimal edema on kidney biopsy can exclude C–TMA as none of their patients carried rare variants in complement genes,[98] contrasting our data. Further conclusions from their study, however, were limited by incomplete clinical data. Most of Larsen's *et al*. patients are from African descent, a population with a high burden of hypertensive emergency and ESKD as compared to those from European descent.[99] No complement defects were identified in our patients from African descent and all showed an excellent response upon BP control, pointing towards hypertension as the sole cause of disease in this particular group of patients. Larsen's *et al*. cohort might represent a mixture of distinct causes, some of which may be related to well–known secondary etiologies not linked to complement.[100,101] Potential recipients classified as hypertensive ESKD with proof or a high suspicion of TMA should therefore be screened for rare variants in complement genes.

Based on our studies,[73,86] we propose an algorithm for the evaluation of TMA in patients presenting with hypertensive emergency (Figure 3). Patients with hypertensive emergency and severe kidney failure who lack systemic hemolysis should be biopsied to assess whether TMA is present or not. Patients should be screened for other causes of TMA and, if no cause can be identified, for *ex vivo* C5b9 formation. Abnormal results are indicative of a poor response to BP control and thus, C–TMA. Furthermore, patients and, in particular, potential kidney donor recipients, should be screened for rare variants in complement genes to adopt suitable measures prior to kidney transplantation. Prospective studies, however, are needed to test the hypothesis that therapeutic complement inhibition, either with

eculizumab or other therapies under development, will improve the outcome of patients with massive *ex vivo* C5b9 formation. The predictive role of pathologic features, such as chronic vascular and tubulointerstitial damage,[83,102] and clinical phenotype regarding the response to treatment should also be addressed.

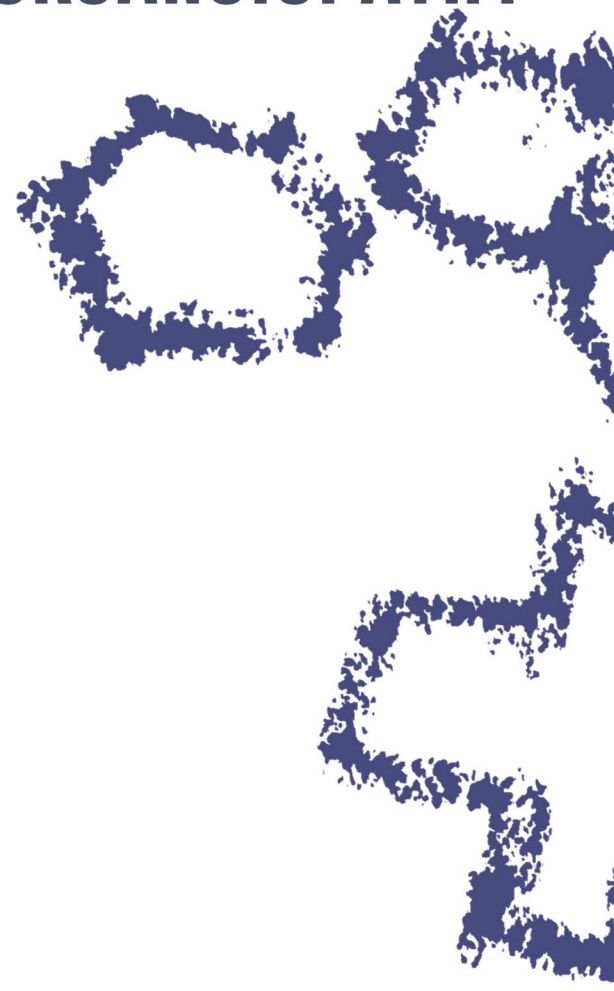
In conclusion, these observations show that assessment of both *ex vivo* C5b9 formation and screening for rare variants in complement genes may categorize the TMA in patients with hypertensive emergency into different groups, with potential therapeutic and prognostic implications. Furthermore, *ex vivo* C5b9 formation may guide the dosage of treatment. These findings should now be confirmed in independent prospective cohort studies.

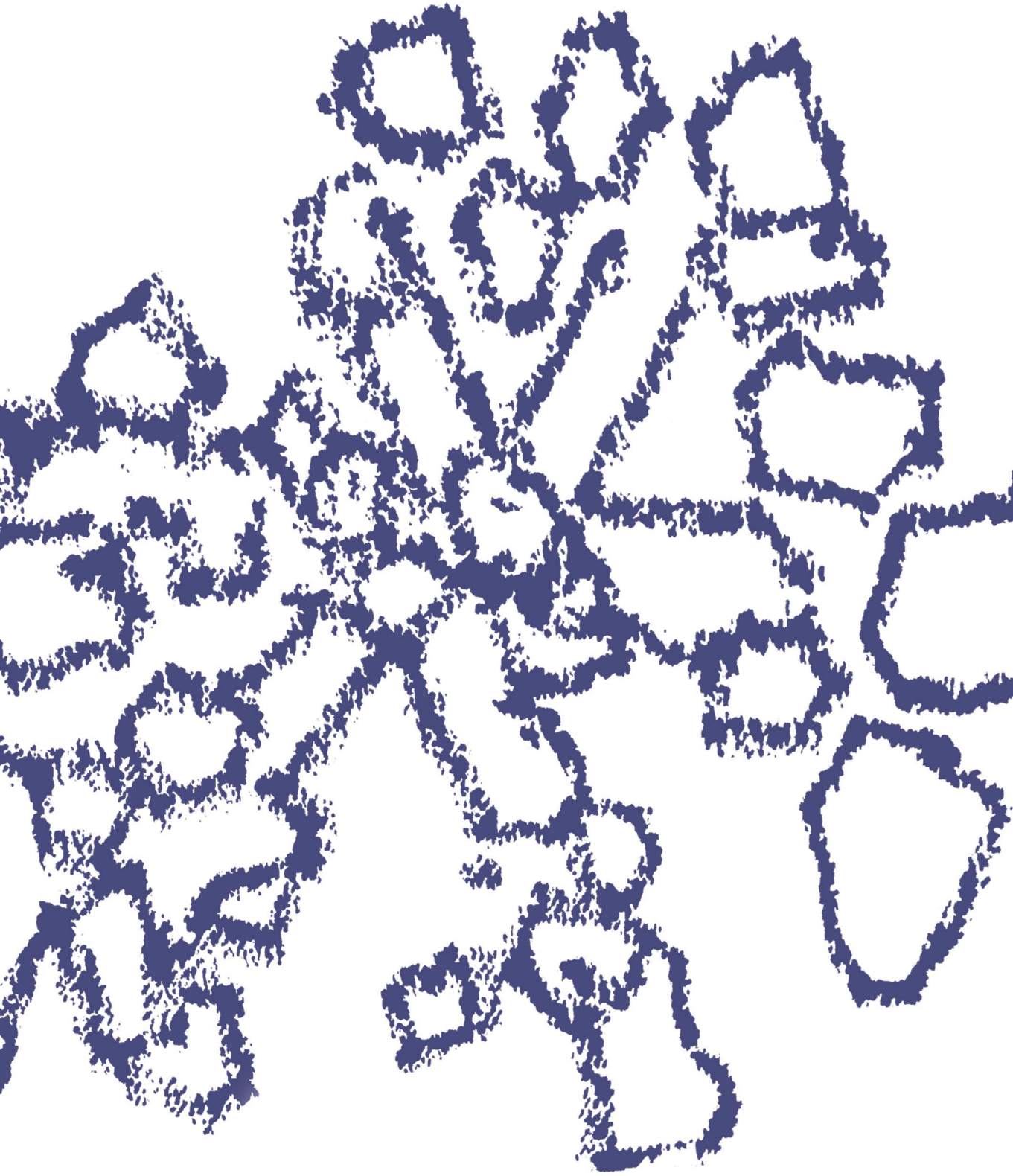
Figure 3. Clinical algorithm for the evaluation of TMA in patients with hypertensive emergency.



BP, blood pressure.

PART II
THE RECOGNITION OF COMPLEMENT-MEDIATED THROMBOTIC MICROANGIOPATHY





“*A man should look for what is, and not for what he thinks should be.*”

Albert Einstein

5

Functional and genetic landscape of complement dysregulation along the spectrum of thrombotic microangiopathy and its potential implications on clinical outcomes

Sjoerd A.M.E.G. Timmermans,[1, 2] Jan G.M.C. Damoiseaux,[3] Alexis Wérion,[4] Johann Morelle,[4, 5] Chris P. Reutelingsperger,[2] and Pieter van Paassen.[1, 2]

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Introduction. The syndromes of thrombotic microangiopathy (TMA) are diverse and represent severe endothelial damage caused by various mechanism. The complement system plays a major role in a subset of patients with TMA and its recognition is of clinical importance as it guides choice and duration of treatment.
Methods. We studied a well–defined cohort of patients with TMA and hypothesized that assessment of serum–induced *ex vivo* C5b9 formation on the endothelium and screening for rare variants in complement genes can better categorize the TMA.
Results. Massive *ex vivo* C5b9 formation was found in all patients with primary atypical hemolytic uremic syndrome (HUS; *n*/*N*=11/11) and in 59% patients with TMA and coexisting conditions (*n*/*N*=30/51). Massive *ex vivo* C5b9 formation was associated with rare genetic variants (41% [*n*/*N*=17/41] versus 0% [*n*/*N*=0/21] patients with normal *ex vivo* C5b9 formation; *P*<0.001). Massive *ex vivo* C5b9 formation was associated with a favorable renal response to therapeutic com–plement inhibition in patients with TMA and coexisting conditions (86% [*n*/*N*=12/14] versus 31% [*n*/*N*=5/16] of untreated patients; *P* <0.001), indicating complement–mediated (C–)TMA rather than secondary disease. Among treated patients, the odds ratio for 1–year kidney survival was 12.0 (95% confidence interval, 1.2–115.4). TMA recurrence was linked to rare genetic variants in all cases. Patients with normal *ex vivo* C5b9 formation had an acute, non–relapsing form of TMA.
Conclusions. *Ex vivo* C5b9 formation and genetic testing appears to categorize TMAs into different groups as it identifies complement as a driving factor of disease, with potential therapeutic and prognostic implications.

Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Central Diagnostic Laboratory, Maastricht UMC, NLD.
[4]Division of Nephrology, Cliniques universitaires Saint–Luc, BE.
[5]Institute de Recherche Expérimentale et Clinique, UCLouvain, BE.

The syndromes of TMA are diverse and represent tissue responses to severe endothelial damage caused by various mechanisms.[2] TMAs translate into microvascular thrombosis, thrombocytopenia, microangiopathic hemolysis, and ischemic organ damage, often affecting the kidneys. Complement dysregulation related to rare variants in complement genes and/or autoantibodies that interfere with complement regulation is a major risk factor for TMA in a subset of patients,[8,9] referred to as C–TMA. Ever since the approval of therapeutic complement inhibition,[13-15] the approach of TMAs has transformed, focusing on the recognition of complement dysregulation in the earliest possible stage of disease.[10,11]

C–TMA should be considered after exclusion of other well–established causes of TMA that are unrelated to complement dysregulation, for example, thrombotic thrombocytopenic purpura and Shiga toxin–producing E. *coli* infection.[103] The diagnosis of C–TMA is challenging as reliable tests are lacking and, according to the current nomenclature, should be reserved for patients not presenting with coexisting conditions (i.e., primary atypical HUS).[10,11] Many of such patients, however, require a coexisting condition, such as hypertension, to lower the threshold for C–TMA.[12,104] Recently, we demonstrated that massive serum–induced *ex vivo* C5b9 formation on the endothelium indicates C–TMA in patients with coexisting hypertensive emergency, pointing to complement dysregulation rather than hypertension as the cause of TMA.[86,105] C–TMA, in particular, was common in patients not responding to standard of care (i.e., blood pressure control), with high rates of progression to end-stage kidney disease (ESKD).[105]

We hypothesized that the prevalence of C–TMA is underappreciated in patients presenting with coexisting conditions beyond hypertensive emergency and that the assessment of both *ex vivo* C5b9 formation and screening for rare variants in complement genes better categorizes patients along the spectrum of TMA into different groups, with potential therapeutic and prognostic implications. We tested this premise in a well–defined cohort of 65 patients with TMA, either with coexisting conditions or not, and severe kidney involvement, often confirmed on kidney biopsy. Furthermore, the dynamics of *ex vivo* C5b9 formation in patients treated with therapeutic complement inhibition and a prolonged interdose interval were studied.

METHODS

***Patient population and definitions*.** Patients with TMA were recruited from the Limburg Renal Registry, Maastricht, The Netherlands,[54] and the Cliniques universitaires Saint–Luc, Brussels, Belgium. TMA was defined as typical morphologic features of TMA on kidney biopsy and/or the triad of microangiopathic hemolytic anemia (hematocrit <30%, hemoglobin <10 g/L, lactate dehydrogenase >500 U/L, and schistocytes on peripheral blood smear), platelets <150 $\times 10^9$/L, and acute kidney injury. Patients presenting with coexisting conditions were classified as

secondary atypical HUS according to HUS International's nomenclature (Item S1).[10,11] Patients with thrombotic thrombocytopenic purpura, defined as an enzymatic activity of von Willebrand factor cleaving protease <10% or the combination of platelets <30 $\times 10^9$/L and serum creatinine ≤200 μmol/L,[76] and those with a Shiga toxin–producing E. *coli* infection were excluded.

Clinical and laboratory data were documented at the time of presentation and during follow–up. The information was specified in the Limburg Renal Registry and the patient's medical records. Complete renal remission (CR) was defined as the restoration of an estimated glomerular filtration >60 mL/min/1.73m^2; partial renal remission (PR) was defined as the recovery of kidney function after dialysis or >25% decrease in serum creatinine. The stage of chronic kidney disease (CKD) was based on international consensus;[106] ESKD was defined as the need for chronic kidney replacement therapy.

At the time of presentation and during follow-up, serum samples were obtained, processed, and immediately stored at –80 degrees Celsius to prevent *in vitro* complement activation.[59] The study was approved by the appropriate ethics committees and is in accordance with the Declaration of Helsinki.

Routine complement measures. C4 and C3 serum levels and the functional activity of the classical pathway (Svar Life Sciences, Malmo, Sweden) were assessed.

Ex vivo C5b9 formation on the endothelium. *Ex vivo* C5b9 formation on microvascular endothelial cells of dermal origin (i.e., HMEC–1; ATCC, Manassas, VA) was assessed as described.[86] Briefly, HMEC–1 were plated on glass culture slides and used when >80% confluent, incubated with serum diluted in test medium for 3 hours at 37 degrees Celsius, fixed in 3% formaldehyde, and blocked with 2% BSA for 1 hour. The results of 26 patients have been published.[105] In selected experiments, HMEC–1 were preincubated with 10 μM ADP for 10 minutes to mimic a perturbed endothelium.[51] Rabbit anti–C5b9 pAb (Calbiochem, San Diego, CA) and Alexa488 labeled goat anti–rabbit Ab (Life Technologies, Carlsbad, CA) were used. Fluorescent staining on HMEC–1 was acquired in 15 fields and the staining area was evaluated using ImageJ (National Institutes of Health, Bethesda, MD). The samples were compared with pooled serum from 10 healthy controls run in parallel; *ex vivo* C5b9 formation on the resting and perturbed endothelium did not differ between individuals (data not shown).

Rare variants in complement genes and FHAA. Patients were screened for rare variants, i.e., variants with a minor allele frequency <0.1%, and single nucleotide polymorphisms in coding regions of *CFH*, *CFI*, *CD46*, *CFB*, *C3*, *CFHR1*, *CFHR2*,

CFHR3, *CFHR4*, *CFHR*5, *THBD*, and *DGKE* using DNA sequencing. The results of 49 patients have been published.[101] The classification of variants was based on international standards.[89] Pathogenic variants were defined as those with functional studies supporting a defect in complement regulation, including null variants in genes linked to complement regulation, variants located in a mutational hotspot, variants located in a functional domain, and/or variants that cluster in patients with primary atypical HUS as demonstrated by Osborne and colleagues.[23] Rare variants not fulfilling these criteria have been classified as uncertain significance.

Rearrangements in the *CFH–CFHR1–5* genomic region were analyzed by multiplex-ligation probe amplification. In selected cases, the presence of factor H autoantibodies (FHAA) was assessed by ELISA.[58]

***Statistics*.** Continuous variables were presented as mean (±SD) or median (interquartile range [IQR]) as appropriate. Differences in continuous and categorical variables were checked using the unpaired *t* or Mann–Whitney U test and the chi–square or Fisher's exact test, respectively. *Ex vivo* C5b9 formation on the endothelium was compared with normal human serum run in parallel by the paired sample *t* test or Wilcoxon signed rank test as appropriate. Logistic regression was used to compute an odds ratio with 2–sided 95% confidence interval. Survival was assessed using the Kaplan–Meier methods and log–rank test.

Massive *ex vivo* C5b9 formation and/or pathogenic variants in complement genes defined C–TMA.

RESULTS

***Patient population*.** Ninety–three patients with TMA were recruited (Item S2). Fifteen patients with acquired thrombotic thrombocytopenic purpura and 13 patients with antiphospholipid syndrome–related TMA, described previously,[107] were excluded; serum samples obtained at the time of presentation from 14 of these patients were used as disease controls for *ex vivo* C5b9 formation. Thus, 65 patients with TMA were included (Table 1); 59 patients were from European descent, 4 patients from African descent, 1 patient from Latin American descent, and 1 patient from Asian descent. Patients invariably presented with severe kidney involvement, including 38 (58%) patients who initially needed dialysis. Microangiopathic hemolysis, low platelets, or both were present in 10 (15%), 16 (25%), and 26 (40%) cases. TMA was confirmed on kidney biopsy in 47 (72%) cases, including 39 patients not presenting with systemic hemolysis. Low levels of C4 and C3 measured at the time of presentation, were found in 5 (*N*=57, 9%) and 19 (*N*=59, 32%) patients, respectively. Fifty–two (80%) patients presented with coexisting conditions and should have been classified as secondary atypical HUS according to HUS International's nomenclature.

Table 1. Main clinical data of 65 patients with TMA.

	C–TMA	Normal C regulation	*P* value
HUS International's nomenclature, *N*[10,11]	44	21	
Primary atypical HUS (%)	13 (30)	0 (0)	0.006
Secondary atypical HUS (%)	31 (70)	21 (100)	0.006
Hypertensive emergency	18	12	
Pregnancy	8	0	
TMA after kidney transplantation	2	3	
Postsurgical TMA	2	1	
Streptococcal HUS	1	0	
HELLP	0	3	
Drug–induced TMA	0	2	
Features at presentation, *N*	44	21	
M/F	19/25	12/9	0.4
European (%)	43 (98)	16 (76)	0.01
Age, years	36±18	42±13	0.1
Creatinine, µmol/L	492 (314–804)	485 (231–778)	0.5
Dialysis (%)	27 (61)	11 (52)	0.6
Hemolysis (%)	25 (57)	11 (52)	0.8
Systemic hemolysis (%)	18 (41)	8 (38)	1.0
Platelets, $\times 10^9$/L	101 (44–228)	95 (52–178)	0.8
LDH, U/L	842 (398–2,103)	762 (465–1,222)	0.6
ADAMTS13's activity >10%, *n*/*N*	31/31	17/17	
Low C4, *n*/*N*	5/39	0/18	0.2
Low C3, *n*/*N*	18/41	1/18	0.005
Massive *ex vivo* C5b9 formation, *n*/*N*	41/41	0/21	<0.001
Rare variant(s)/FHAA (%)	20 (45)	0 (0)	<0.001
Pathogenic (%)	17 (37)	0 (0)	0.006
Combined variants	2	0	1.0
MCP_{GGAAC}, *n*/*N*	16/31	12/19	0.6
Treatment, *N*	44	21	
Plasma therapy (%)	31 (70)	7 (33)	0.007
Immunosuppression (%)	12 (27)	2 (10)	0.1
Eculizumab (%)	19 (43)	5 (24)	0.2
Days after diagnosis, median	6 (range, 0–100)	4 (range, 2–37)	0.8
Doses, median	13 (range, 2–70)	4 (range, 1–10)	0.009
Ongoing, *n*/*N*	3/19	0/5	0.6
Clinical outcome, *n*/*N*	43/44	20/21	
Follow–up, years	2.0 (0.6–3.8)	0.5 (0.3–2.4)	0.002
Renal response (%)	24 (56)	9 (45)	0.6
Complete remission	15	4	0.4
Partial remission	9	5	0.8
ESKD at 3 months (%)	17 (40)	7 (35)	0.8
ESKD at last follow–up (%)	19 (44)	8 (45)	0.8
Patients with TMA recurrence (%)	11 (26)	0 (0)	0.01
Deceased at 3 months (%)	1 (2)	1 (5)	0.5
Deceased at last follow–up (%)	3 (7)	2 (10)	0.6

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 C, complement. ESKD, end–stage kidney disease. FHAA, factor H autoantibodies.

Patients with *de novo* TMA after kidney transplantation presented with ESKD related to glomerular disease (anti–neutrophil cytoplasmic antibody–associated glomerulonephritis, *n*=1; IgA nephropathy, *n*=1; focal segmental glomerulosclerosis, *n*=1), renovascular disease (*n*=1), and reflux nephropathy (*n*=1) in their native kidneys.

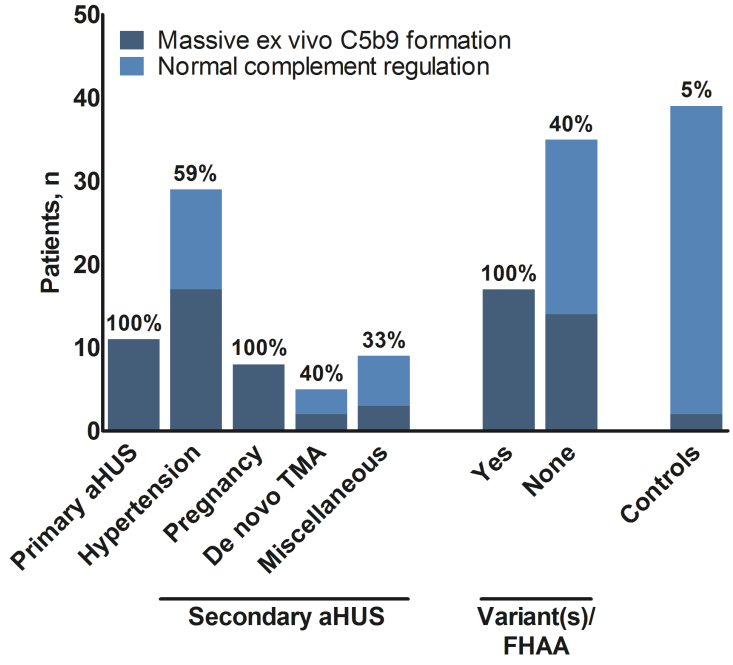
Complement workup. Patients were screened for complement dysregulation using functional (Figure 1) and genetic tests (Table 2); treatment naive serum samples from 62 (95%) out of 65 patients with TMA (coexisting conditions, *n*=51; primary atypical HUS, *n*=11) were tested for *ex vivo* C5b9 formation at the time of presentation. No baseline serum sample was available from 3 patients with pathogenic variants in complement genes.

C-TMA. Massive *ex vivo* C5b9 formation on the resting endothelium was found in 41 (66%) out of 62 patients tested for, including 30 (59%) out of 51 patients with coexisting conditions. This was particularly the case in patients with coexisting hypertensive emergency (*n*/*N*=17/29, 59%), pregnancy (*n*/*N*=8/8, 100%), and *de novo* TMA after kidney transplantation (*n*/*N*=2/5, 40%). At the time of quiescent disease, *ex vivo* C5b9 formation normalized on the resting (*n*/*N*=12/12, 100%) but not perturbed endothelium (*n*/*N*=7/9, 78%) when using samples from patients not treated with therapeutic complement inhibition who had massive *ex vivo* C5b9 formation at the time of acute TMA. Thus, massive *ex vivo* C5b9 formation is not a secondary phenomenon triggered by acute TMA (e.g., hemolysis[108] and fibrinolysis[36]).

Ten rare variants in complement genes were identified in 17 (41%) out of 41 patients with massive *ex vivo* C5b9 formation (Table 2, Item S4); 7 variants were considered pathogenic and 3 as of uncertain significance. The genomic deletion of *CFHR1* and *CFHR3* was found in homozygosity in 3 patients and associated with FHAA in 1 case, i.e., deficiency of CFHR plasma proteins and autoantibody positive (DEAP)–HUS. In addition, 3 patients not tested for *ex vivo* C5b9 formation had pathogenic variants identified (i.e., M99917, M00004, B03), 1 of whom also carried 1 variant of uncertain significance. Altogether, 44 patients had C–TMA. Two (5%) out of 44 patients presented with combined variants.

Normal complement regulation. Normal *ex vivo* C5b9 formation on both the resting and perturbed endothelium was found in 21 patients with coexisting conditions, indicating true secondary atypical HUS with normal complement regulation.[51] The specificity of normal *ex vivo* C5b9 formation on the perturbed endothelium for normal complement regulation is 95% as based on 39 control samples (Figure 1, Item S3). Low C3 levels were found in 1 out of 18 patients tested for, contrasting patients with C-TMA (*n*/*N*=18/41, *P*=0.005). None of the patients with normal complement regulation carried rare variants in complement genes. FHAA were not found in the patient with a loss of CFHR1 and CFHR3. Thus, none of the patients with normal *ex vivo* C5b9 formation had identified genetic or acquired abnormalities associated with complement dysregulation.

Figure 1. Massive *ex vivo* C5b9 formation along the spectrum of TMA on resting endothelial cells.



Controls have been tested on perturbed endothelial cells (**Item S3**).

***The clinical course of TMA*.** Main clinical data of 65 patients with TMA classified according to HUS International's nomenclature have been depicted in Item S4.

C–TMA and coexisting conditions. Thirty–one (61%) out of 51 patients with coexisting conditions had C–TMA rather than secondary atypical HUS. Patients presented with coexisting hypertensive emergency (*n*=18), pregnancy (*n*=8), *de novo* TMA after kidney transplantation (*n*=2), postsurgical TMA (*n*=2), or streptococcal HUS (*n*=1); rare variants in complement genes confirmed the genetic predisposition in 11 patients (Table 2).

Of the patients with C–TMA and coexisting conditions, 30 had follow–up data available, with a median follow-up of 2.3 (IQR, 0.7–6.5) years (Table 1). Twenty–one (70%) out of 30 patients were treated with plasma therapy. Fourteen patients not responding to standard of care, including plasma therapy in 12 patients, were treated with eculizumab (Item S5); treatment was initiated after a median of 7 (range, 1–100) days and a median of 14 (range, 4–70) doses were administered. Patients treated with eculizumab received meningococcal vaccines and/or antibiotics. In addition, 11 patients were treated with immunosuppressive drugs, including 2 kidney donor recipients (i.e., prophylactic treatment for rejection; Item S6).

Table 2. The phenotype of patients carrying rare variants (minor allele frequency <0.1%) in complement genes or FHAA.

Patient no.	Sex/ age	SCr, µmol/L	MAHA	Platelets, $\times 10^9$/L	Treatment	Outcome	Gene(s)/ FHAA	Variant	Protein	MAF, %	*In vitro*
C-TMA and no coexisting conditions (i.e., primary atypical HUS)											
M00018	F/3	92	+	12	PEX, Ecu	CR, R	*C3*[1]	c.481C>T	R161W	<0.01	GOF
							CFI[1]	c.392T>G	L131R	<0.01	LOF
M11317	M/65	372	+	44	PEX	CR	*CFHR5*[2]	c.1412G>A	G471E	<0.07	Unknown
M00016	M/4	311	+	28	PEX, CS	CR	FHAA	c.CFHR1/3 del	ΔCFHR1/3	N/a	N/a
M01609	M/20	287	+	345	PEX	CR	*C3*[1]	c.481C>T	R161W	<0.01	GOF
M03103	F/12	339	+	264	PEX	ESKD, R	*C3*[1]	c.481C>T	R161W	<0.01	GOF
M00004	F/49	800	+	<150	PEX	ESKD, R	*CFI*[1]	c.1420C>T	R474*	<0.01	LOF
B12	F/31	359	–	23	PEX, Ecu	CR	*CFH*[1]	c.3486delA	K1162Nfs*7	0	Unknown
B27	M/53	441	+	53	PEX, Ecu	CKD G3b	*CD46*[2]	c.478G>T	V160F	0	Unknown
B39	M/25	469	+	7	PEX	CR	*C3*[2]	c.3125G>A	R1042Q	0	Unknown
C–TMA and coexisting hypertensive emergency											
M99917	M/47	1,980	–	272	Ecu	CKD G3b	*CFI*[1]	c.148C>G	P50A	<0.02	LOF
							THBD[2]	c.1433C>T	T478I	<0.01	Unknown
M02715	F/28	1,065	–	228	PEX	ESKD	*C3*[1]	c.481C>T	R161W	<0.01	GOF
M01715	F/41	334	–	291	PEX, Ecu	CKD G4	*CFI*[1]	c.452A>G	N151S	<0.01	LOF
M04010	F/32	1,138	+	142	PEX	ESKD, R	*CFH*[1]	c.2558G>A	C853Y	0	LOF
M03307	M/37	586	+	100	PEX	ESKD, R	*C3*[1]	c.481C>T	R161W	<0.01	GOF
M04306	M/40	1,195	+	158	PEX	ESKD, R	*CD46*[1]	c.811_816delGACAT	ΔD271/S272	0	LOF
M00105	F/38	1,730	+	228	–	ESKD, R	*C3*[1]	c.481C>T	R161W	<0.01	GOF
M05486	M/39	1,089	–	101	–	ESKD, R	*C3*[1]	c.481C>T	R161W	<0.01	GOF
C–TMA and coexisting pregnancy (i.e., pregnancy–associated atypical HUS)											
M00503	F/32	1,388	+	212	PEX, CS	ESKD, R	*C3*[1]	c.481C>T	R161W	<0.01	GOF
B46	F/31	557	+	77	PEX, Ecu	ESKD	*CFI*[1]	c.772G>A	A258T	<0.03	LOF
C–TMA and coexisting kidney transplantation (i.e., *de novo* TMA after kidney transplantation)											
B33	M/24	309	–	252	PEX, Ecu	CKD G4/T	*CFI*[1]	c.148C>G	P50A	<0.02	LOF

The variants have been classified as [1](likely) pathogenic or [2]uncertain significance.

CKD, chronic kidney disease. CR, complete renal remission. CS, corticosteroids. Ecu, eculizumab. ESKD, end–stage kidney disease. FHAA, factor H autoantibodies GOF, gain–of–function. LOF, loss–of–function. MAF, minor allele frequency in the European American population according to the Exome Variant Server (EVS) and Genome Aggregation Database (gnomAD). MAHA, microangiopathic hemolytic anemia. PEX, plasma therapy. R, recurrence. SCr, serum creatinine.

◀**Table 2.** Continued.

The *in vitro* defects have been studied elsewhere: *CFI* c.148C>G (Ref.[63]), *CFI* c.392T>G (Ref.[109]), *CFI* c.452A>G (Ref.[63]), *CFI* c.772G>A (Ref.[109]), *CFI* c.1420C>T (Ref.[110]), *CFH* c.2558G>A (Ref.[64]), *CD46* c.811_816delGACAT (Ref.[43]), and *C3* c.481C>T (Ref.[66]).

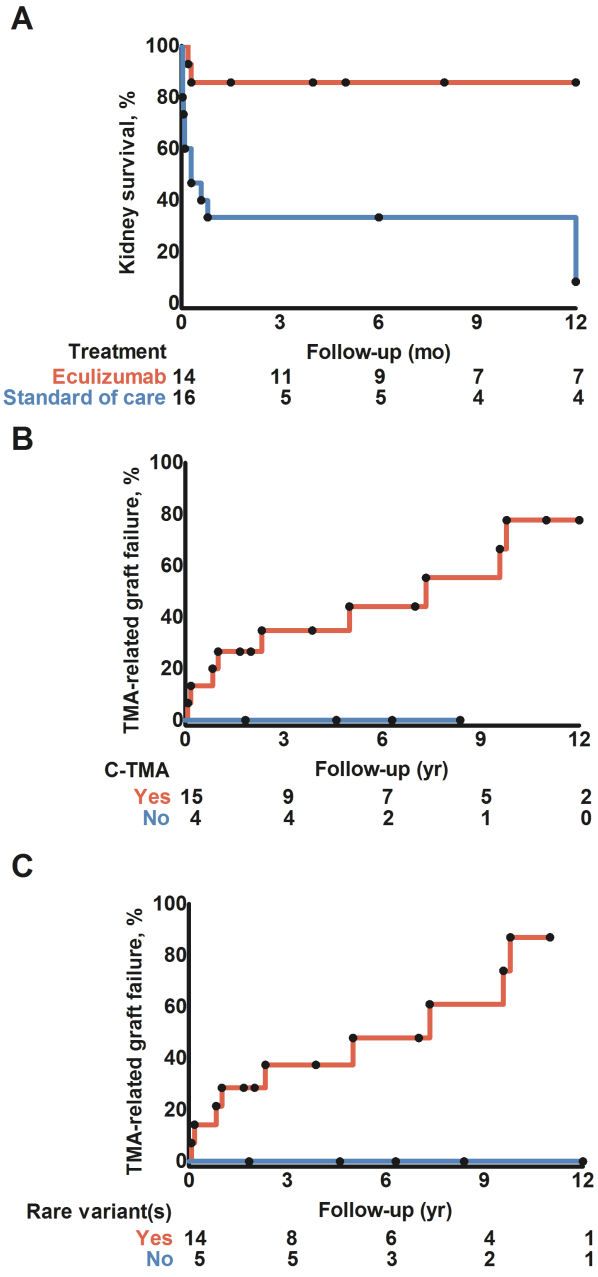
In 14 (47%) out of 30 patients, a renal response was achieved, either a CR (*n*=6) or PR (*n*=8). The cumulative incidence of renal response did not differ between patients with rare variants in complement genes and those with no variants identified (3 out of 11 versus 11 out of 19 patients, *P*=0.1). PR was associated with CKD stage G3–G4 in 7 patients and G5 in 1 patient at 3 months; the patient with CKD stage G5 improved to G4 at 1 year. Renal response rates were higher in patients treated with eculizumab as compared to untreated patients (Figure 2A), as is discussed later. Of note, 13 (93%) out of 14 remitted patients (i.e., those patients who achieved a renal response) had a sustained clinical remission during follow–up. The patient with no sustained clinical remission presented with streptococcal HUS recurrence in the native kidneys, similar to the first event, that is, linked to massive *ex vivo* C5b9 formation and a CR upon a 3–month course of eculizumab.

The cumulative incidence of ESKD was 16 (53%), including 15 patients who progressed to ESKD within 3 months. ESKD did not differ between patients with rare variants in complement genes and those with no variants identified (8 out of 11 versus 8 out of 19 patients, *P*=0.1). Eculizumab was associated with better 1–year kidney survival as 12 (86%) out of 14 treated patients achieved a renal response, whereas 11 (69%) out of 16 untreated patients progressed to ESKD within 3 months (Figure 2A); baseline characteristics have been depicted in Item S5. The odds ratio for 1–year kidney survival was 12.0 (95% confidence interval, 1.2–115.4) when treated with eculizumab. Of note, the non–responding patients presented either with anuric or oliguric kidney disease, serum creatinine >550 μmol/L, and severe interstitial fibrosis/tubular atrophy on kidney biopsy (i.e., >50%).

Thirteen donor kidneys were transplanted in 8 recipients, including 7 patients with pathogenic variants in complement genes. Most kidneys were transplanted before the approval of eculizumab by the European Medicines Agency's in 2011. Preemptive eculizumab was initiated in 1 patient who carried a pathogenic variant in *CFH* and prevented graft failure for at least 7 years. Ten episodes of TMA recurrence were documented in 9 donor kidneys from 7 recipients, all but 1 patient carried pathogenic variants in complement genes. TMA recurrence resulted in graft loss in all but 1 case (Figure 2B–C). Of note, 1 recipient with advanced graft failure (serum creatinine, 486 μmol/L) on the background of chronic TMA lost his donor kidney after 12 months despite eculizumab.[111,112]

Three patients died during follow-up; the cause of death was cardiac disease (*n*=2) and cancer (*n*=1).

Figure 2. Kaplan–Meier curves.



(A) ESKD was less prevalent in patients with C–TMA and coexisting conditions who had been treated with eculizumab as compared to untreated patients (log–rank test, $P<0.001$). TMA recurrence after kidney transplantation was common in patients with C–TMA and linked to rare variants in complement genes (B and C, respectively; log-rank test, $P<0.05$).

C-TMA and no coexisting conditions (i.e., primary atypical HUS). Most patients with C–TMA classified as primary atypical HUS presented with profound systemic hemolysis and less severe kidney disease as compared to those with C–TMA and coexisting conditions (Item S4). The prevalence of rare variants in complement genes did not differ from patients with C–TMA and coexisting conditions.

Follow–up data were available for all patients, with a median follow–up of 2.1 (IQR, 1.0–9.1) years. Ten (77%) out of 13 patients were treated with plasma therapy. Eculizumab was initiated in 5 patients. The patient with DEAP–HUS was treated with immunosuppressive drugs.

Ten (77%) out of 13 patients achieved a renal response, either a CR (*n*=9) or PR (*n*=1). The cumulative incidence of renal response did not differ from patients with C–TMA and coexisting conditions, whereas ESKD at 3 months appeared more common in the latter. Three donor kidneys were transplanted in 2 recipients with pathogenic variants; TMA recurrence and subsequent graft failure were documented in each case (Figure 2B–C).

Patients with normal complement regulation. Twenty–one patients presented with normal complement regulation and coexisting hypertensive emergency (*n*=12), kidney transplantation (*n*=3), HELLP (hemolysis, elevated liver enzymes, low platelets; *n*=3), drug–induced TMA (*n*=2), and postsurgical TMA (*n*=1).

Of these patients, 20 had follow–up data, with a median follow–up of 0.5 (IQR, 0.3–2.4) years (Table 1). Seven (35%) out of 20 patients were treated with plasma therapy; eculizumab was initiated in 5 patients not responding to standard of care (Item S7). Two kidney donor recipients were treated with immunosuppressive drugs (i.e., prophylactic treatment for rejection; Item S6).

Nine (45%) out of 20 patients achieved a renal response, either a CR (*n*=5) or PR (*n*=4). Eight remitted patients had been treated with standard of care only, contrasting patients with C–TMA and coexisting conditions (8 out of 9 versus 2 out of 14, $P<0.001$). ESKD developed in 9 patients, including 3 (60%) out of 5 patients who had been treated with eculizumab. Of note, 1 patient who had received 1 dose of eculizumab had achieved a PR prior to drug administration. In contrast to patients with C–TMA, none of the patients experienced TMA recurrence (Item S4), including 4 kidney donor recipients (Figure 2B–C).

Two patients died during follow-up; the cause of death was duodenal perforation (*n*=1) and unknown (*n*=1).

Ex vivo C5b9 formation, eculizumab, and recurrent TMA. Follow–up samples from 14 patients with C–TMA treated with eculizumab (coexisting conditions, *n*=11; primary atypical HUS, *n*=3), including 6 patients who carried rare variants in

complement genes, were available to test for *ex vivo* C5b9 formation. C5b9 formation on the perturbed endothelium was attenuated when incubated with samples from patients on eculizumab, confirming the assay's specificity. We prolonged the interdose interval beyond 2 weeks and measured the CPFA and *ex vivo* C5b9 formation on the perturbed endothelium in 7 cases (Figure 3, Table 3). Persistent inhibition of *ex vivo* C5b9 formation was achieved in 6 patients, including 2 patients with a CPFA >10%. None of the patients experienced a relapse despite a prolonged interdose interval.

Four patients with TMA recurrence not treated with therapeutic complement inhibition were tested for *ex vivo* C5b9 formation. Patients invariably presented with massive *ex vivo* C5b9 formation on the resting endothelium, whereas samples from these patients obtained at the time of quiescent disease showed normal *ex vivo* C5b9 formation on the resting endothelium.

DISCUSSION

The recognition of complement dysregulation as the cause of TMA is important to select patients for therapeutic complement inhibition. Here, we demonstrate that complement dysregulation, defined by massive *ex vivo* C5b9 formation and/or pathogenic variants in complement genes, is prevalent in patients with TMA presenting with coexisting conditions and that these features are linked to poor kidney outcomes. Massive *ex vivo* C5b9 formation was associated with rare variants in complement genes and favorable renal response to therapeutic complement inhibition, confirming that these patients fall within the spectrum of C–TMA. Normal *ex vivo* C5b9 formation indicated an acute non–relapsing form of TMA.

Over the last decade, the approach of the TMAs has transformed and focused on the recognition of complement dysregulation in the earliest possible stage of disease.[10,11] It is important to stress that profound systemic hemolysis can be lacking in up to 60% patients with C–TMA and a kidney biopsy may therefore be needed to detect the TMA. Low C3 levels may suggest C–TMA at an early stage of disease, although routine complement measures are not specific for C-TMA.[67,86] DNA sequencing is considered of high specificity but time–consuming and thus, cannot be used to select patients for treatment. Here, we demonstrate that *ex vivo* C5b9 formation can categorize patients along the spectrum of TMA, including those patients presenting with coexisting conditions. Most patients with massive *ex vivo* C5b9 formation who had been treated with eculizumab achieved a favorable renal response, whereas the prognosis of untreated patients was dire. Massive *ex vivo* C5b9 formation can therefore contribute to a rapid diagnosis of C–TMA and moreover, may guide treatment decisions.

At the time of quiescent disease, *ex vivo* C5b9 formation normalized on the resting but not the perturbed endothelium, indicating that the endothelium's capacity

Table 3. Clinical parameters and complement measures in patients treated with eculizumab and a prolonged interdose interval.

Patient no.	Coexisting condition	Variant(s)	Interdose interval, wk	Hb, mmol/L	LDH, U/L	Platelets, ×10⁹/L	SCr, µmol/L	C4, g/L	C3, g/L	CPFA, %	*Ex vivo* C5b9, % control*
M00018	None	*C3*, *CFI*	4	7.7	294	340	32	ND	ND	1	12
			6	8.2	237	299	38	0.19	1.12	2	8
M06518	Surgery	None	3	6.1	166	156	103	ND	ND	116	30
M06018	Hypertension	None	3	7.7	134	236	399	0.22	1.10	3	14
M99917	Hypertension	*CFI*, *THBD*	3	8.6	165	210	190	ND	ND	21	45
M08316	Pregnancy		4	8.7	139	204	106	0.22	0.82	1	11
			6	8.2	132	174	103	0.24	0.85	2	11
M01715	Hypertension	*CFI*	4	7.4	141	393	288	0.29	1.16	102	229
M04010	Hypertension	*CFH*	4	6.8	138	216	132	0.21	0.56	0	29

Ex vivo C5b9 formation on the perturbed endothelium.
C3; reference range, 0.75–1.35 g/L. C4; reference range, 0.11–0.35 g/L. CPFA, functional activity of the classical pathway; reference range, >75%.

Figure 3. Prolonged interdose interval and *ex vivo* C5b9 formation on the perturbed endothelium.

Ex vivo C5b9 formation after incubation of perturbed endothelial cells with serum from patients treated with eculizumab using various interdose intervals, i.e., 1 week (*n*=3; dose, 900 mg), 2 weeks (*n*=9; dose, 1200 mg), 3 weeks (*n*=3; dose, 1200 mg), 4 weeks (*n*=4; dose, 1200 mg), and/or 6 weeks (*n*=2; dose, 1200 mg). Two patients with a prolonged interdose interval of 3 weeks and attenuated *ex vivo* C5b9 formation on the perturbed endothelium had a classical pathway functional activity (CPFA) above the recommended cut–off of 10% (Table 3). Also, serum from 4 patients not treated with eculizumab for at least 12 weeks obtained at the time of quiescent disease were tested (pathogenic variant in *CFI*, *n*=1; pathogenic variant in *CFH*, *n*=1; no genetic variants, *n*=2); these samples induced massive *ex vivo* C5b9 formation on the perturbed endothelium, confirming the risk for unrestrained complement activation. Each patient has been denoted by a distinct symbol and color; dots tag patients with rare variants in complement genes. Normal range, *ex vivo* C5b9 formation of 78,78% to 178.62% as compared to normal human serum (NHS).

to regulate complement normalized. In line with Galbusera and colleagues,[92] TMA recurrence was associated with massive *ex vivo* C5b9 formation, similar to the first presentation, underscoring that the *ex vivo* test reflects the dynamic process of endothelium–restricted complement activation. In contrast, the so–called modified Ham test using human endothelial hybrid cells that lack membrane–bound complement regulators (i.e., CD55 and CD59) cannot differentiate acute TMA from quiescent disease.[113] Moreover, serum samples from a subset of patients with HELLP show similar results as compared to those from patients with C–TMA when using the modified Ham test.[114] Indeed, secondary complement activation occurs in HELLP.[115] The occurrence of HELLP in pregnant women treated with eculizumab,[116] absence of "true" pathogenic variants in complement genes,[114] and favorable kidney survival, pleads against complement dysregulation.[117] Thus, our test appears to be more specific than the modified Ham test for the detection of complement dysregulation. We advocate that our *ex vivo* test, when prospectively validated, can be implemented in routine clinical practice, although a specialized laboratory is needed to execute the test.

Rare variants in complement genes and/or FHAA confirmed the predisposition in about half the patients with massive *ex vivo* C5b9 formation on the resting endothelium, resembling primary atypical HUS.[9] Our observation that patients with neither genetic variants nor FHAA may present with massive *ex vivo* C5b9 formation points to a circulating factor that affects complement regulation,[51,118] either related to common variants in complement genes (i.e., minor allele frequency ≥0.1%) with *in vitro* studies showing functional consequences or a yet unidentified factor. TMA recurrence was common in patients with pathogenic variants, corroborating previous studies.[11,44] Patients should therefore be screened for rare variants in complement genes to inform the long–term prognosis and thus, guide treatment decisions during follow–up.

The nomenclature on TMAs, considered to indicate targets for treatment, states that C–TMA should be reserved for patients not presenting with coexisting conditions.[10,11] Three–quarters of our patients with C–TMA, however, presented with coexisting conditions. Many of such patients presented with typical features of TMA on kidney biopsy and coexisting conditions recently linked to a high prevalence of rare variants in complement genes, that is, hypertensive emergency,[73,97,119] pregnancy,[120] and *de novo* TMA after kidney transplantation,[39] whereas profound systemic hemolysis appeared uncommon. The prevalence of rare variants in complement genes is higher as compared to controls (i.e., ~5%).[100] In contrast, rare variants in complement genes were not prevalent (i.e., ~5% patients) in a French cohort of patients with TMA and coexisting drug use, autoimmunity, infection, or cancer among other causes.[100] Pathogenic variants, identified in 2% French patients, were associated with severe kidney involvement and/or TMA recurrence,

similar to C–TMA. Thus, the TMAs in the French cohort likely represent a mixture of distinct causes, only some of which may be linked to complement dysregulation.[101] The phenotype of their cohort resembled our patients with normal *ex vivo* C5b9 formation, that is, an acute non–relapsing form of TMA.

Observational studies showed conflicting results on the efficacy of eculizumab for the treatment of TMA presenting with coexisting conditions.[100,121] Most responding patients presented with drug–induced TMA and mild–to–moderate kidney involvement.[121] Yet, no studies have linked complement dysregulation to drug–induced TMA. The offending drug was stopped in all and the clinical response may therefore reflect the natural course of drug–induced TMA.[122] Here, we demonstrate that most patients with C–TMA and coexisting conditions treated with therapeutic complement inhibition achieved a renal response, contrasting untreated patients. We advocate the use of therapeutic complement inhibition, either eculizumab or therapies under development, in selected patients presenting with coexisting conditions and, in particular, patients with massive *ex vivo* C5b9 formation. Non–responding patients with massive *ex vivo* C5b9 formation presented with severe kidney disease and advanced chronicity scores on kidney biopsy. Future prospective trials, however, are needed to assess the efficacy of therapeutic complement inhibition in patients with TMA and coexisting conditions. Also, the prognostic value of vascular damage, glomerulosclerosis, and interstitial fibrosis on kidney biopsy should be studied.[83]

The optimal treatment regimen, that is, dosage and duration of eculizumab for the treatment of C–TMA, remains to be established. Eculizumab biweekly from the fifth week of treatment onwards has been shown to block C5 activation as indicated by a classical pathway functional activity <10%. Eculizumab, however, can exceed the recommended target by up to 15–fold using the standard regimen.[93] In selected cases, we demonstrate that *ex vivo* C5b9 formation can be attenuated when using a prolonged interdose interval. A prolonged interdose interval was associated with a classical pathway functional activity above the recommended cut–off in some patients, corroborating previous observations.[92] None of the patients experienced a relapse, suggesting that trough levels below the recommended target may prevent TMA recurrence and potentiate lower costs of treatment.

In conclusion, patients with TMA and coexisting conditions may present with C–TMA and should therefore be screened for *ex vivo* C5b9 formation and rare variants in complement genes to categorize the TMA. The risk of ESKD appeared lower in patients with massive *ex vivo* C5b9 formation at presentation and treated with eculizumab. We therefore advocate that *ex vivo* C5b9 formation may be used to select patients for therapeutic complement inhibition, whilst rare variants in complement genes inform the long-term prognosis. Prospective studies are needed to test the hypothesis that therapeutic complement inhibition can improve the outcome of such patients.

SUPPLEMENTAL DATA

Item S1. Patients with TMA were classified according to the current nomenclature.[10,11]

The following definitions have been used:

***Antiphospholipid syndrome–related TMA*.** Patients with TMA and persistent phospholipid serum reactivity (i.e., lupus anticoagulant, anti–β2 glycoprotein I IgG, and/or anti–cardiolipin IgM/IgG).[123]

***De novo TMA after kidney transplantation*.** Donor kidney recipients with TMA unrelated to calcineurin inhibitor nephrotoxicity, infection, and antibody–mediated rejection (i.e., neither donor specific alloantibodies nor C4d deposits along the peritubular capillaries on allograft biopsy) who presented with end–stage kidney disease not related to TMA in their native kidneys.

***Drug–induced TMA*.** TMA related to drugs reported in the Oklahoma Registry and Blood Center of Wisconsin to have a definite causal association with TMA (the data can be found on: https://www.ouhsc.edu/platelets/DITMA.htm).[124]

***HELLP (hemolysis, elevated liver enzymes, and low platelets)*.** Patients with the onset of TMA during pregnancy, aspartate and/or alanine aminotransferase at least 2 times the upper limit of normal, and clinical improvement after delivery.

***Hypertensive emergency*.** Patients with TMA presenting with typical pathologic features of severe hypertension (i.e., myxoid intimal changes, hypertrophy of the arterial vessel walls, and/or fibrinoid necrosis of arterioles) on kidney biopsy, severe hypertension (i.e., blood pressure levels of at least 180 mmHg systolic and/or 120 mmHg diastolic), and evidence of impending or progressive target organ damage outside the kidneys.[125]

***Postsurgical TMA*.** Patients with the onset of TMA within 30 days after surgery.[126]

***Pregnancy–associated atypical hemolytic uremic syndrome*.** Patients with the onset of TMA during pregnancy or within the first 12 weeks postpartum.[120]

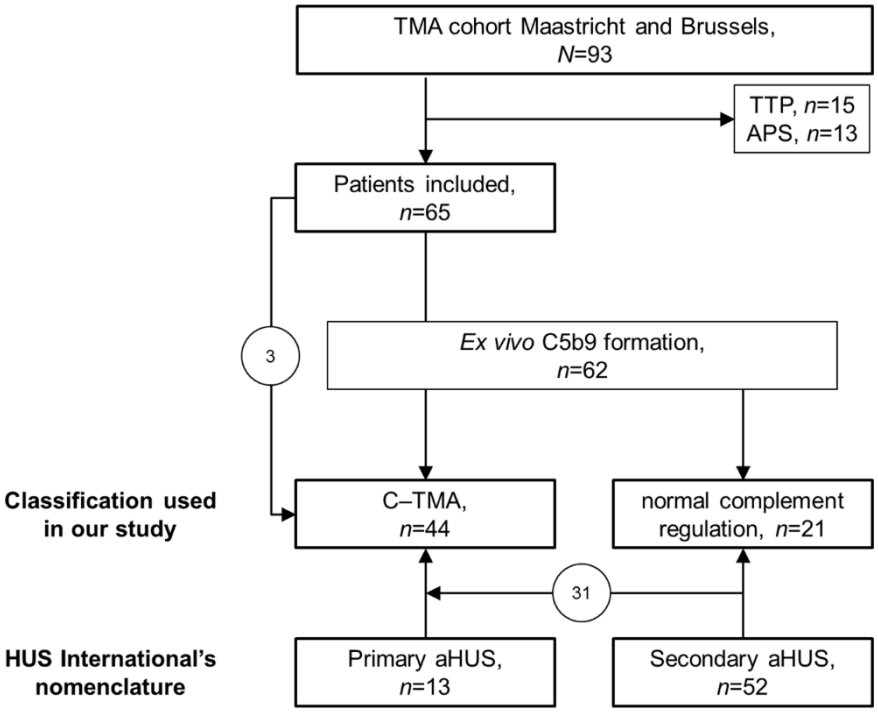
***Primary atypical hemolytic uremic syndrome*.** Patients with TMA not presenting with coexisting conditions.

***Shiga toxin–producing E. coli–associated hemolytic uremic syndrome*.** TMA related to Shiga toxin–producing E. *coli* infection.

***Streptococcal hemolytic uremic syndrome*.** TMA associated with S. *pneumoniae* infection.

***Thrombotic thrombocytopenic purpura*.** Patients with TMA presenting with an enzymatic activity of von Willebrand factor cleaving protease <10% as based on FRETS–VWF73 assay and/or platelets <30 $\times 10^9$/L and serum creatinine ≤200 μmol/L.[76]

Item S2. Flowchart.



Three patients with pathogenic variants in complement genes were classified as C–TMA, although no baseline serum sample was available to test for *ex vivo* C5b9 formation on the endothelium.

APS, antiphospholipid syndrome. TTP, thrombotic thrombocytopenic purpura.

Item S3. Baseline characteristics according to the atypical (a)HUS type classification.

	Primary aHUS	Secondary aHUS	
C-TMA	**Yes**	**Yes**	**No**
HUS International's nomenclature[10,11]			
Coexisting condition(s), *n*/*N*	0/13	31/31[b]	21/21[b]
Hypertensive emergency	0	18[b]	12[b]
Pregnancy	0	8	0[c]
TMA after kidney transplantation	0	2	3
Postsurgical TMA	0	2	1
Streptococcal HUS	0	1	0
HELLP	0	0	3
Drug–induced TMA	0	0	2
Features at presentation			
M/F	5/8	14/17	12/9
European (%)	12 (92)	31 (100)	16 (76)
Age, years	30±25	38±13	42±13
Creatinine, µmol/L	321 (193–407)	561 (356–1,065)[b]	485 (231–778)
Dialysis (%)	5 (38)	22 (71)	11 (52)
Hemolysis (%)	12 (92)	13 (42)[b]	11 (52)[a]
Systemic hemolysis (%)	9 (69)	9 (29)[a]	8 (38)
Platelets, $\times 10^9$/L	36 (12–200)	133 (75–228)[a]	95 (52–178)
LDH, U/L	1,251 (711–2,390)	680 (305–1,486)[a]	762 (465–1,222)[a]
ADAMTS13's activity >10%, *n*/*N*	10/10	21/21	17/17
Low C4, *n*/*N*	0/10	5/29	0/18
Low C3, *n*/*N*	6/11	12/30	1/18[b, c]
Massive *ex vivo* C5b9 formation, *n*/*N*	11/11	30/30	0/21[b, d]
Rare variant(s)/FHAA (%)	9 (70)	11 (35)	0 (0)[b, d]
Pathogenic (%)	6 (46)	11 (35)	0 (0)[b, d]
Combined variants	1	1	0
MCP_{GGAAC}, *n*/*N*	7/11	9/20	12/19
Treatment			
Plasma therapy (%)	10 (77)	21 (68)	7 (33)[b, c]
Immunosuppression (%)	1 (8)	11 (35)	2 (10)
Eculizumab (%)	5 (38)	14 (45)	5 (26)
Days after diagnosis, median	4 (range, 1–19)	7 (range, 1–100)	4 (range, 2–37)
Doses, median	10 (range, 2–21)	14 (range, 4–70)	4 (range, 1–10)
Ongoing, *n*/*N*	1/5	2/14	0/5
Clinical outcome			
Patients, *n*/*N*	13/13	30/31	20/21
Follow–up, years	2.1 (1.0–9.1)	2.3 (0.7–6.5)	0.5 (0.3–2.4)[a, d]
Renal response (%)	10 (77)	14 (47)	9 (45)
Complete remission	9	6	4
Partial remission	1	8	5
ESKD at 3 months (%)	2 (15)	15 (50)[a]	7 (35)
ESKD at last follow–up (%)	3 (23)	16 (53)	8 (45)
Patients with TMA recurrence (%)	3 (23)	8 (27)	0 (0)[a, c]
Deceased at 3 months (%)	0 (0)	1 (3)	1 (5)
Deceased at last follow–up (%)	0 (0)	3 (10)	2 (10)

[a]$P \leq 0.05$ and [b]$P < 0.01$ versus primary aHUS. [c]$P \leq 0.05$ and [d]$P < 0.01$ versus C–TMA and coexisting conditions.

ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13. ESKD, end–stage kidney disease. FHAA, factor H autoantibodies. HELLP, hemolysis, elevated liver enzymes, low platelets.

Item S4. *Ex vivo* C5b9 formation on the perturbed endothelium when using serum samples from disease controls showed a specificity of 95%. Patients' samples have been obtained at the time of presentation prior to treatment.

	N	Massive *ex vivo* C5b9 formation
TMAs		
TTP	7	0
APS–related	7	1
STEC–HUS	1	0
Glomerulopathies		
C3G	10	1
APS nephropathy*	3	0
AGN	2	0
Hypertension		
Arterionephrosclerosis	5	0
Hypertensive emergency†	4	0

*Focal cortical necrosis without morphologic features of TMA on kidney biopsy. †Patients presenting with hypertensive emergency and an estimated glomerular filtration rate >45 mL/min/1.73m^2.

AGN, anti–neutrophil cytoplasmic antibody–associated glomerulonephritis. C3G, C3 glomerulopathy. TTP, thrombotic thrombo–cytopenic purpura.

Item S5. Baseline characteristics of patients with C–TMA and coexisting conditions who had been treated with eculizumab or not.

	Eculizumab	Untreated	*P* value
Patients	14	16	
Coexisting conditions			
Hypertensive emergency	6	11	0.3
Pregnancy	4	4	1.0
De novo after kidney transplantation	1	1	1.0
Miscellaneous	3	0	0.09
Features at presentation			
M/F	6/8	7/9	1.0
Caucasian (%)	14 (100)	16 (100)	1.0
Age, years	39 (29–56)	33 (28–38)	0.2
Creatinine, µmol/L	492 (345–583)	854 (566–1,181)	0.01
Dialysis (%)	8 (57)	14 (88)	0.1
Hemolysis (%)	6 (43)	6 (38)	1.0
Platelets, $\times 10^9$/L	90 (46–277)	138 (95–204)	0.8
LDH, U/L	620 (304–1,098)	867 (312–2,044)	0.6
Low C4, *n*/*N*	4/13	1/15	0.2
Low C3, *n*/*N*	5/13	7/16	1.0
Rare variant(s)/FHAA (%)	5 (36)	8 (50)	0.5
Pathogenic (%)	3 (21)	8 (50)	0.1
Combined variants	2	1	0.6
MCP_{GGAAC}, *n*/*N*	5/11	4/8	1.0
Treatment			
Plasma therapy (%)	12 (86)	9 (56)	0.1
Immunosuppression (%)	6 (42)	5 (31)	0.7
Eculizumab (%)	14 (100)	–	
Days after diagnosis, median	6 (range, 0–100)	–	
Doses, median	14 (9–24)	–	
Ongoing, *n*/*N*	2/14	–	

Item S6. Indications for immunosuppressive agents.

Patient no.	Age/ sex	Coexisting condition	SCr, μmol/L	Drug(s)	Ecu	Indication	Outcome
C–TMA							
M00016	4/M	–	311	CS	–	DEAP–HUS	CR
M06018	26/M	HE	805	CS	+	–	PR
M01416	72/F	HE	356	CS, MMF	+	TIN	PR
M02715	28/F	HE	1,065	CS, MMF	–	–	ESKD
M01715	41/F	HE	334	CS, MMF	+	–	PR
M04010	32/F	HE	1,138	CS	–	–	ESKD
M03307	37/M	HE	586	CS	–	–	ESKD
M00503	32/F	Pregnancy	1,388	CS	–	–	ESKD
M06019	30/F	Pregnancy	411	CS	+	–	CR
M06518	74/F	Surgery	220	CS	+	–	PR
B07	35/M	KTX	519	TAC, MMF, CS	–	KTX*	ESKD
B33	24/M	KTX	309	TAC, MMF, CS	+	KTX*	PR
Normal complement regulation							
M00018	54/F	KTX	242	CS	–	KTX*	PR
B10	52/M	KTX	795	TAC, CS	–	KTX*	ESKD

*Prophylactic treatment for rejection or graft versus host disease.

CR, complete renal remission. CS, corticosteroids. DEAP–HUS, deficiency of CFHR and autoanti–body positive HUS. Ecu, eculizumab. ESKD, end–stage kidney disease. HE, hypertensive emergency. KTX, kidney transplantation. MMF, mycophenolate mofetil. PR, partial renal remission. SCr, serum creatinine. TAC, tacrolimus. TIN, acute tubulointerstitial nephritis.

Table S7. Clinical characteristics of patients with TMA and normal complement regulation treated with eculizumab. (All patients had been treated with plasma exchange.)

							Eculizumab		
Patient no.	**Sex/ age**	**Coexisting condition**	**SCr, μmol/L**	**GS**	**IF/ TA**	**KRT**	**Start (d)**	**Doses**	**Outcome**
M13519	M/38	HE	984	3/13	20%	+	4	4	ESKD
M09419	M/63	HE	546	1/7	40%	+	2	2	ESKD
M11818	M/37	Surgery	626	0/14	<5%	+	3	10	CR
M01217	M/38	HE	726	5/21	40%	+	7	5	ESKD*
B03	M/41	HE	1,017	3/14	15%	+	37	1	PR†

*Patient died. †PR was achieved prior to administration of eculizumab.

CR, complete renal remission. ESKD, end–stage kidney disease. GS, glomerulosclerosis. HE, hypertensive emergency. IF/TA, interstitial fibrosis/tubular atrophy. KRT, kidney replacement therapy. PR, partial renal remission.

6

More about complement in the antiphospholipid syndrome

Sjoerd A.M.E.G. Timmermans,[1, 2] Jan G.M.C. Damoiseaux,[3] Chris P. Reutelingsperger,[2] and Pieter van Paassen;[1, 2] for the Limburg Renal Registry.

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Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Central Diagnostic Laboratory, Maastricht UMC, NLD.

The thrombotic spectrum of antiphospholipid syndrome (APS) is heterogeneous and ranges from mild thrombosis in isolation to a catastrophic and multisystem disease, with thrombotic microangiopathy (TMA) and organ failure. The exact mechanism of APS related thrombosis remains to be elucidated and may differ between subsets of patients. Murine data linked thrombosis, at least in part, to complement activation.[127,128] Chaturvedi and colleagues in their interesting *Blood* paper (Jan. 23 issue) reported that patients' serum induced C5b9 formation on human endothelial hybrid cells and corresponding complement–dependent cell killing (i.e., modified Ham test).[129] The authors argue that with increasing severity of disease, the role of unrestrained complement activation via the alternative pathway becomes more dominant as corroborated by the high prevalence of complement gene variants in catastrophic APS, quite alike primary atypical hemolytic uremic syndrome (HUS). Their data may suggest that such patients should be screened for genetic variants and treated accordingly.[130]

MATERIAL AND METHODS

Patient population. We evaluated Chaturvedi's *et al*. premise in an intrinsically different subset of patients with APS[123] presenting with APS nephropathy on kidney biopsy.[131] Serum samples were obtained at the time of kidney biopsy, processed, and immediately stored at –80°C. The study was approved by the regional ethical committee and was performed in accordance with the declaration of Helsinki.

Phospholipid serum reactivity. The presence of lupus anticoagulant was defined by a prolonged dilute Russell's viper venom time and/or activated partial thromboplastin time which was not effected by addition of normal plasma and normalized upon addition of phospholipids.[123] The presence of anti–β2 glycoprotein I IgG (Thermo Fisher, Uppsala, Sweden) and anti–cardiolipin IgM/IgG (Thermo Fisher) were determined by fluorescent enzyme immunoassay using a positive cut–off of >10 U per mL and >40 MPL/GPL per mL, respectively.[123]

Routine complement assays. C4 and C3 levels were determined using nephelometry. Also, the functional activity of the classical pathway was assessed (Eurodiagnostica, Malmö, Sweden).[60]

Ex vivo complement activation. We used human microvascular endothelial cells (ATCC, Manassas, VA).[86] C5b9 formation on these endothelial cells reflects the dynamics of complement and disease activity in patients with primary atypical HUS.[51,105] Briefly, perturbed endothelial cells were plated on glass culture slides, incubated with serum diluted in medium, and stained with rabbit anti–human C5b9 pAb (1:100; Calbiochem, San Diego, CA) followed by Alexa488 labeled anti–rabbit

Ab (1:100; Life Technologies, Carlsbad, CA); pooled normal human serum and serum from patients with primary atypical HUS were run in parallel. In selected experiments, the endothelium was stained with FITC labeled anti–human C3c (1:20; Dako, Heverlee, Belgium) or anti–human IgG subclasses (1:100; Sigma–Aldrich, St. Louis, MO).

In vivo complement activation. Snap frozen kidney tissue sections were stained with FITC labeled anti–human C1q (in–house) or anti–human C3c (1:20; Dako); mouse anti–human C4d mAb (1:200; Quidel, Alkmaar, The Netherlands) followed by rabbit anti–mouse Ab (1:60; Dako) or rabbit anti–human C5b9 pAb (1:100; Calbiochem) followed by Alexa488 labeled anti–rabbit Ab (1:100; Life Tech–nologies).

Statistical analysis. Continuous variables were presented as median (interquartile range [IQR]) as appropriate. Comparisons were made for each patient comparing serum–induced complement deposits for the patient and normal human serum run in parallel by using the paired sample t test or Wilcoxon signed rank test as appropriate. Between group differences were analyzed by ANOVA. P <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In total, 17 consecutive patients with APS nephropathy were included (Table 1). Female–to–male ratio was 0.9 and the median age at diagnosis was 45 (IQR, 27–55) years. Patients invariably presented with proteinuric kidney disease (nephrotic–range proteinuria, n=4), either with (n=6) or without microscopic hematuria. Kidney tissue sections showed both acute and chronic morphologic features of TMA in 14 cases; subendothelial electron lucent material confirmed the TMA on electron microscopy (n/N=7/8). Three patients (i.e., no. M04984, M00585, and M04386) had focal cortical necrosis without glomerular lesions, reflecting arteriolar thrombosis. Four (24%) patients were triple positive, 8 (47%) were double positive, and 5 (29%) were single positive for lupus anticoagulant, anti–β2 glycoprotein I antibodies, and/or anti–cardiolipin antibodies confirmed on 2 occasions at least 12 weeks apart. Of note, thrombocytopenia but not hemolytic anemia was found in 8 (47%) patients. C4 and C3 levels were low in 2 (N=14, 14%) and 4 (N=14, 29%) patients, respectively. The functional activity of the classical pathway was decreased in 6 (N=13, 46%) patients. None of the patients had systemic lupus erythematosus.

At the time of presentation, 14 (93%) out of 15 patients with APS nephropathy showed normal *ex vivo* C5b9 formation on the perturbed endothelium (Figure 1A), while massive *ex vivo* C5b9 formation was found in 5 patients with primary atypical HUS and a pathogenic gain–of–function variant in *C3*, that is, c.481C>T

Table 1. Baseline characteristics of 17 patients with APS and APS nephropathy on kidney biopsy.

		APS diagnostic criteria										*Ex vivo* C5b9	
Patient no.	**Kidney biopsy**	**Laboratory**	**Clinical manifestations**	**Sex/ age**	**SCr, µmol/L**	**uH**	**uP, g/d**	**Plts, ×10⁹/L**	**C4, g/L***	**C3, g/L***	**CPFA, %***	**% control**	***P* value**
M04984	1984	aCL	–	M/48	80	–	1.90	WNR	0.30	1.36	>125	Normal	NS
M00585	1985	aCL	–	M/45	217	–	1.94	WNR	0.26	1.10	>125	60	NS
M01585	1985	LAC, β2GPI, aCL	CAPS† (1986)	M/50	223	–	0.78	93	ND	ND	ND	ND	
M04386	1986	β2GPI, aCL	AT (2001), pregnancy	F/28	75	–	1.11	WNR	0.14	0.78	ND	109	NS
M01988	1988	aCL	AT (2004)	M/38	214	–	1.02	314	0.20	1.30	3	63	NS
M03188	1988	LAC, aCL	AT (1990)	F/58	168	+	0.38	108	0.07	0.55	58	117	NS
M00799	1999	β2GPI, aCL	VT (1995)	F/26	657	+	1.85	83	ND	ND	ND	ND	
M06999	1999	LAC, β2GPI, aCL	VT (1995)	M/53	155	–	4.80	93	0.18	1.04	47	127	NS
M07200	2000	LAC, β2GPI, aCL	–	M/26	119	–	0.06	152	0.15	0.93	ND	161	NS
M07800	2000	LAC, aCL	Pregnancy	F/45	197	–	1.20	101	0.09	0.60	61	189	0.01
M06502	2002	aCL	–	M/62	216	+	3.64	333	0.11	0.72	57	103	NS
M00205	2005	β2GPI, aCL	–	F/73	284	+	1.02	97	0.13	0.73	103	101	NS
M00707	2007	aCL	–	M/67	276	+	13.06	WNR	0.37	1.18	122	96	NS
M02907	2007	β2GPI, aCL	VT (2002)	F/27	69	+	0.48	314	0.16	1.09	71	71	NS
M03310	2010	LAC, aCL	–	F/19	103	–	0.21	WNR	0.11	1.02	109	160	NS
M09512	2012	β2GPI, aCL	–	M/47	188	–	3.95	101	0.12	1.03	33	123	NS
M01314	2014	LAC, β2GPI, aCL	VT (1998), AT† (2014)	F/39	266	–	2.72	51	ND	ND	>125	78	NS

*Lower limit of normal: C4 0.11 g/L, C3 0.75 g/L, CPFA 75%. †Within 12 months after kidney biopsy.

aCL, anti–cardiolipin antibodies. AT, arterial thrombosis. B2GPI, anti–beta2 glycoprotein–I antibodies. CAPS, catastrophic APS. CPFA, functional activity of the classical pathway. LAC, lupus anticoagulant. ND, not determined. NS, not significant. Plts, platelets. SCr, serum creatinine. uH, hematuria. uP, proteinuria. VT, venous thrombosis. WNR, within normal range.

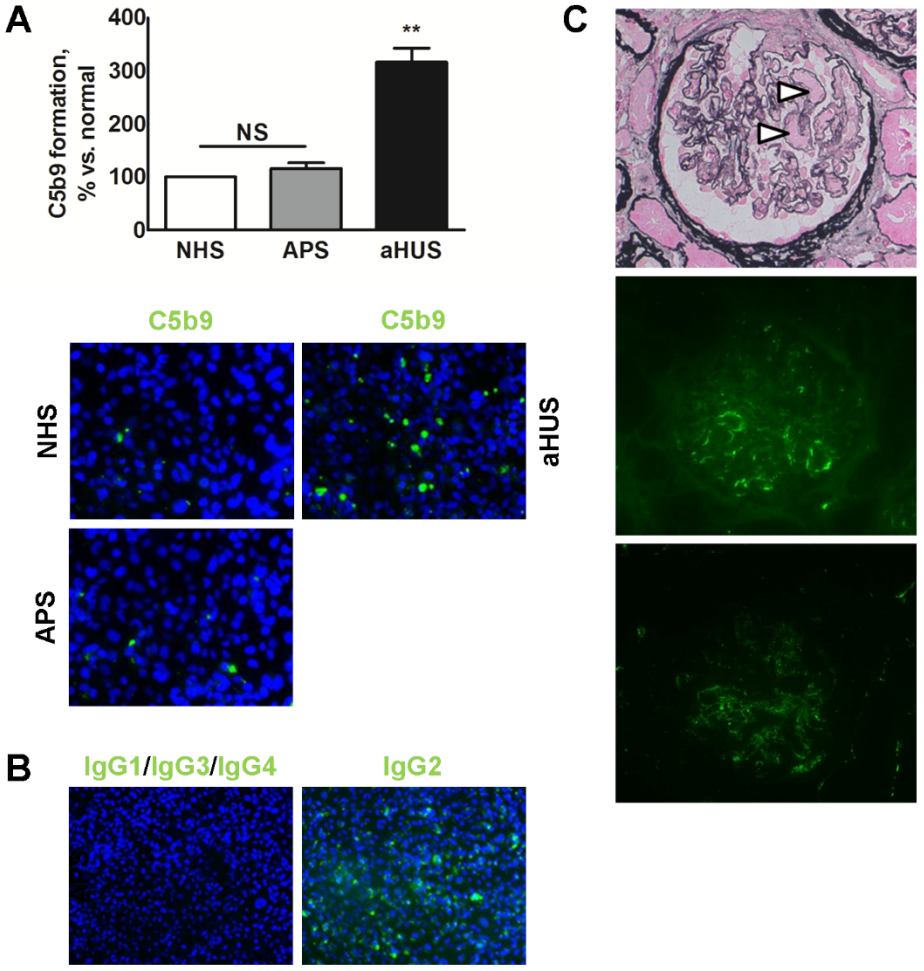
(p.Arg161Trp)[66] run in parallel. To date, none of our patients with primary atypical HUS and at least 1 genetic variant linked to complement dysregulation of the alternative pathway had normal *ex vivo* test results.[101] *Ex vivo* C3c deposits appeared normal in the setting of APS nephropathy (*n*/*N*=4/4), excluding complement activation upstream of C5.

We consider the kidney tissue sections from our patients, with the presence of abundant endothelial cells, to be an ideal *in vivo* counterpart to study complement activation, either with coinciding immunoglobulin deposition or not, in relation to local thrombotic vascular changes. Seven (41%) out of 17 samples revealed scant C3c deposits along segments of the glomerular capillary wall; C3c co–located with C5b9 in 2 cases (Figure 1C). Neither C1q nor C4d were found, making activation of the classical and lectin pathway highly unlikely. No electron dense deposits were found on electron microscopy, underscoring the lack of complement deposits.

The clinical course and kidney survival of our patients did also differ from the dire prognosis of primary atypical HUS not treated with therapeutic complement inhibition.[8,9] Patients were followed for a median of 5.4 (IQR, 1.0–16.8) years; 2 patients were lost to follow–up. The follow–up has been depicted in Table 2. At presentation, anticoagulation and immunosuppression were started in 10 (67%) and 7 (47%) out of 15 patients, respectively. None of the patients received therapeutic complement inhibition. Kidney function stabilized and/or improved in 13 (87%) out of 15 patients. Both patients who presented with end–stage kidney disease did not recover kidney function (no. M00585, M00799). Five patients had recurrent thrombosis, including the 4 patients not on anticoagulation; 1 of the latter patients died because of catastrophic APS (no. M01585).

Altogether, our experimental and clinical findings suggest that a mechanism other than unrestrained complement activation is key for renal thrombosis to occur in APS. We studied the dynamics of complement activation on the endothelium during active arteriolar and/or microvascular thrombosis, whereas the time of sampling in Chaturvedi's *et al*. cohort did not concur with the (macrovascular) thrombotic event.[129] In addition, the modified Ham test uses endothelial hybrid cells that lack the complement regulatory proteins CD55 and CD59, with a lower threshold for complement activation as compared to our *ex vivo* test. Based on our observations, we decided not to test for variants in complement genes.[86,101] Also, it remains to be established whether or not the reported variants in complement genes[129] are causal for (macrovascular) thrombosis. First, the minor allele frequency of some variants exceeds 0.1%; for example, deletion of *CFHR1* and *CFHR3* has been identified in up to 8% of the European population,[25] indicating a non–pathogenic change in the absence of factor H autoantibodies. Second, loss of function variants in *CFB* and *CFHR5* are of no significance as the transcribed proteins are unlikely to overactivate complement.[23] Third, the effects of variants in *CFHR4*, *THBD*, and *DGKE* on

Figure 1. Patients with APS and microvascular thrombosis on kidney biopsy have normal *ex vivo* com–plement activation and lack significant *in vivo* complement deposits.



(A) *Ex vivo* C5b9 formation on the perturbed endothelium did not differ between patients with APS (*n*=15) and normal human serum (NHS), while serum from patients with primary atypical (a)HUS and pathogenic gain–of–function variant in *C3* induced unrestrained complement activation (*n*=5); original magnification, 400×. (B) IgG2 but not the other subclasses bound to the endothelium when incubated with serum from triple positive patients (*n*/*N*=2/2); original magnification, 200×. (C) Microvascular thrombosis on kidney biopsy (arrowheads); scant deposits of C3c (*n*/*N*=7/17) and/or C5b9 (*n*/*N*=2/17) along segments of the glomerular capillary wall were uncommon; original magnification, 400×.

Table 2. Patients' treatment and clinical follow-up.

Patient no.	Laboratory	Clinical manifestations	Extra-criteria manifestations	Treatment	Follow-up, yr.	SCr, µmol/L	CKD	Relapse	Died
M04984	aCL	–	Dystonia	–	Lost	N/a	N/a	N/a	N/a
M00585	aCL	–	–	–	<1	ESKD	G5	–	Yes
M01585	LAC, β2GPI, aCL	CAPS† (1986)	Low platelets, epilepsy	–	1	200	G3b	CAPS (1986)	Yes
M04386	β2GPI, aCL	AT (2001), pregnancy	–	VKA (2001)	24	77	–	AT (2001)	–
M01988	aCL	AT (2004)	Neuritis, CMP	VKA (2004)	27	121	G3a	AT (2004)	–
M03188	LAC, aCL	AT (1990)	Low platelets, VHD	CS, VKA (1990)	12	147	G3a	AT (1990)	Yes
M00799	β2GPI, aCL	VT (1995)	Low platelets, CMP	VKA	1	ESKD	G5	–	–
M06999	LAC, β2GPI, aCL	VT (1995)	Low platelets, livedo	VKA	4	136	G3a	–	–
M07200	LAC, β2GPI, aCL	–	VHD	VKA, ASA	18	82	–	–	–
M07800	LAC, aCL	Pregnancy	Low platelets, VHD	CS/MMF, VKA	17	93	G3a	–	–
M06502	aCL	–	–	CS/CYC, VKA	14	119	G3a	–	–
M00205	β2GPI, aCL	–	Low platelets	VKA	5	145	G3b	–	Yes
M00707	aCL	–	–	VKA	Lost	N/a	N/a	N/a	N/a
M02907	β2GPI, aCL	VT (2002)	–	CS/CYC, VKA	11	87	–	–	–
M03310	LAC, aCL	–	–	CS/MMF, VKA	5	80	–	–	–
M09512	β2GPI, aCL	–	Low platelets, VHD	CS/MMF, VKA	6	144	G3a	–	–
M01314	LAC, β2GPI, aCL	VT (1998), AT† (2014)	Low platelets	MMF, VKA	1	134	G3a	AT (2014)	–

ASA, aspirin. AT, arterial thrombosis. CAPS, catastrophic APS. CKD, chronic kidney disease. CMP, cardiomyopathy. CS, corticosteroids. CYC, cyclophosphamide. ESKD, end–stage kidney disease. MMF, mycophenolate mofetil. N/a, not applicable. SCr, serum creatinine. VHD, valvular heart disease. VKA, vitamin K antagonist. VT, venous thrombosis.

complement regulation are still controversial.

What could be the mechanism for APS nephropathy to occur? Experimental data in mice showed that non–complement–dependent activation of tissue factor is key for renal thrombosis to occur.[128] This fits our observation that non–complement–fixing IgG2 was found on the endothelium after serum incubation, while complement–fixing IgG1 and IgG3 were not found (Figure 1B; *n*/*N*=2/2). APS related thrombosis, indeed, has been linked to anti–β2 glycoprotein I and anti–cardiolipin IgG2 but not to other IgG subclasses.[132,133] We therefore assume that the antiphospholipid antibodies may cause renal thrombosis via a direct effect on the endothelium.[134] In addition, annexin A5 resistance may play a role in a subset of patients, including those with anti–β2 glycoprotein I antibodies.[135]

In conclusion, the suggestion that unrestrained complement activation on the endothelium correlates with thrombosis in APS cannot be extrapolated to patients with APS nephropathy. Future studies on the role of complement in various subsets of patients with APS are needed.

7

Chronic thrombotic microangiopathy in patients with a C3 gain–of–function protein

Sjoerd A.M.E.G. Timmermans,[1, 2] Myrurgia A. Abdul–Hamid,[3] and Pieter van Paassen;[1, 2] for the Limburg Renal Registry.

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Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Dept. Pathology, Maastricht UMC, NLD.

The syndromes of thrombotic microangiopathy (TMA) are rare and occur in patients with severe endothelial damage caused by various mechanisms.[2] The TMAs converge to a final common pathway, inducing microvascular thrombosis with platelet consumption, hemolysis, and ischemic damage, often affecting the kidneys. Endothelial damage can occur on the background of complement dysregulation as demonstrated in primary atypical hemolytic uremic syndrome (HUS).[13-15] Most cases of primary atypical HUS present with acute TMA, while a small subset of patients present with chronic disease. Half the patients have rare variants in complement genes, encoding proteins that either regulate or activate complement, and/or autoantibodies that inhibit complement regulation identified.[2] The genotype–phenotype correlation has clinical significance.[11]

The etiology and disease course of patients with chronic TMA remain poorly understood.[11] Smith–Jackson and colleagues demonstrated that a gain–of–function change in C3 (i.e., p.Asp1115Asn) drives murine TMA with heavy proteinuria and chronic rather than acute TMA on kidney biopsy.[32] The arginine to tryptophan substitution at amino acid 161 (i.e., p.Arg161Trp) in C3 has been identified in 8 (29%) out of 28 patients with primary atypical HUS in the Limburg Renal Registry (Figure 1) but not in 3 asymptomatic relatives; the variant's minor allele frequency is <0.004% according the Genome Aggregation Database and Exome Variant Server. C3 p.Arg161Trp results in a gain–of–function protein and has been linked to nephrotic–range proteinuria in more than half the patients,[66] suggesting chronic damage to podocytes. *In vivo* and clinical observations therefore suggest that C3 gain–of–function proteins may cause chronic TMA. Human morphological data, however, are not available.

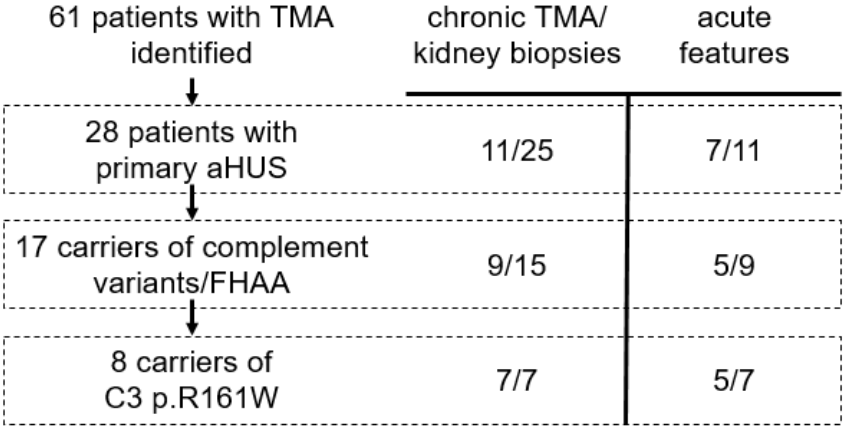
MATERIAL AND METHODS

Herein, we evaluated morphologic features in 7 patients with primary atypical HUS and C3 p.Arg161Trp included in the Limburg Renal Registry; 1 patient was excluded because no kidney tissue sections were available.[54,86] No rare variants in *CFH*, *CFI*, *CD46*, *CFB*, *THBD*, and *DGKE* were found using DNA sequencing. *CFH*–H3 but not MCP_{GGAAC} was found in 3 (43%) patients. The homozygous deletion of *CFHR1–CFHR3* was identified in 1 patient with no factor H autoantibodies. We therefore analyzed C3 p.Arg161Trp's effect in isolation. Our observations add to the understanding of the etiology and disease course of patients with chronic TMA.

RESULTS

Baseline characteristics and outcome data have been depicted in Table 1, corroborating previous observations from the French cohort.[66] Patients presented with proteinuric kidney failure (mean serum creatinine, 1,008 ± 477 µmol/L), either with nephrotic range proteinuria (*n*=4) or not. Normal platelet counts were found in

Figure 1. Patients with primary atypical (a)HUS included in the Limburg Renal Registry.



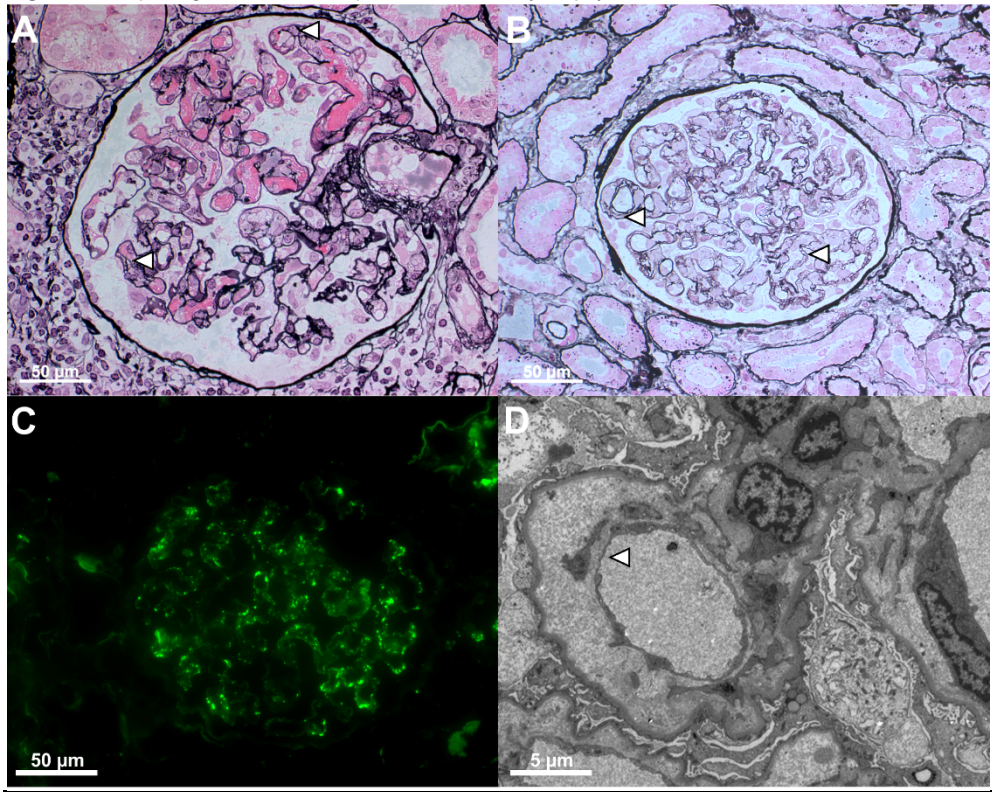
Patients with chronic features of TMA on kidney biopsy not related to C3 p.Arg161Trp had a variant in *CFI* (p.Asn151Ser; *n*=1), *CD46* (p.Asp271_Ser272del; *n*=1), or no variant (*n*=2) identified.
FHAA, factor H autoantibodies.

5 (71%) patients. C3 but not C4 levels were low in 6 (86%) patients. Kidney tissue sections showed double contour formation of the glomerular basement membrane, mesangiolysis, and global foot process effacement, either with fibrin thrombi (*n*=5) or not (Figure 2). Moderate–to–severe interstitial fibrosis and tubular atrophy was present. These morphologic features therefore suggest a smoldering rather than acute onset of disease. C3c but not immune complex deposits were found along segments of the glomerular basement membrane on immunofluorescence micro–scopy (*n*/*N*=5/7; Figure 2). Electron microscopy showed subendothelial electron lucent material but not electron dense deposits (*n*/*N*=4/4; Figure 2), excluding C3 glomerulopathy;[136] global foot process effacement was found in 3 cases. Two patients presented with extrarenal manifestations. Patient no. M05486 had seizures and left ventricular hypertrophy and no. M03307 had dilated cardiomyopathy at presentation.

Plasma exchange was started in 5 patients. Plasma exchange was associated with a complete clinical remission for at least 3 years (i.e., chronic kidney disease stage G1) in patient no. M01609. The other patients (*n*=6, 86%) required dialysis and did not recover kidney function.

C3 p.Arg161Trp has been linked to a high–risk of recurrent primary atypical HUS.[11] Six patients received a total of 10 kidney donors. Eight out of 10 kidney donors were transplanted before eculizumab's approval by the European Medicines Agency. Both other recipients (no. M05486 and M02715) were transplanted with a transplantation protocol not using eculizumab prophylaxis.[95] Five (83%) out of 6 recipients developed TMA on allograft biopsy (episodes, *n*=8), with normal platelet

Figure 2. Morphologic features on representative kidney biopsy.



Double contour formation of the glomerular basement membrane (arrowheads) and mesangiolysis were appreciated, either with thrombosis (A) or not (B); Jones methenamine silver stain; original magnification, 400×. C3c (C) but not immune complex deposits were found along the glomerular basement membrane; fluorescein isothiocyanate labeled anti–C3c (Dako, Heverlee, Belgium), original magnification 400×. Electron lucent material was found in the subendothelial space (D), while electron dense deposits were lacking on electron microscopy; original magnification 1,400×.

counts in 5 (63%) episodes (Figure 3, Table 2). Four episodes presented early, that is, <12 months after kidney transplantation, with microangiopathic hemolysis (*n*=4), low platelet counts (*n*=2), and acute TMA on donor kidney biopsy. Remarkably, 4 episodes presented "late" with nephrotic–range proteinuria, normal platelet counts, and morphologic features consistent with chronic TMA and C3c deposits (*n*/*N*=3/4), identical to native kidney biopsies; thrombosis was not found. Neither donor specific alloantibodies nor C4d deposits were present along the peritubular capillaries and thus, chronic transplant glomerulopathy was considered unlikely.

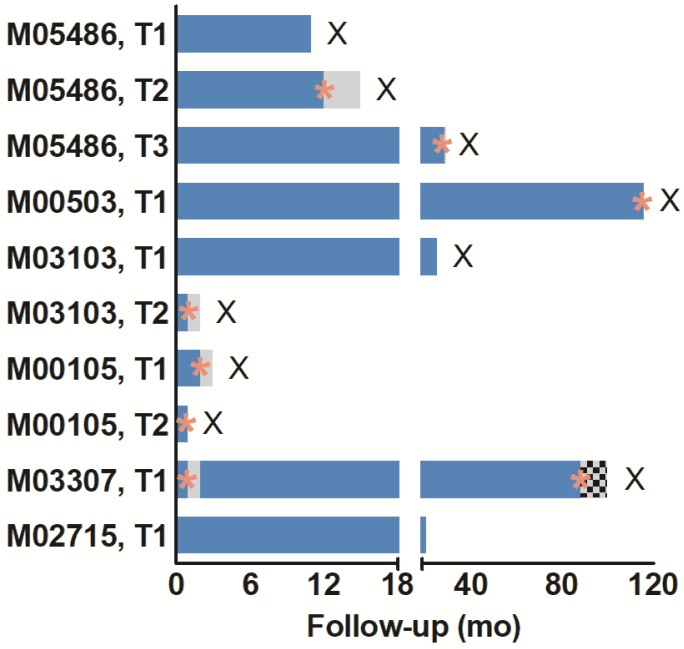
In all cases, including patient no. M03307 who had been treated with C5 inhibition, that is, eculizumab, graft loss developed. Patient no. M03307, however, presented with a creatinine of 486 µmol/L.

Table 1. Patient's characteristics and clinical outcome.

Patient no.	M05486	M00503	M03103	M00105	M03307	M01609	M02715
Year of presentation	1986	2003	2003	2005	2007	2009	2015
Sex/age, yr.	M/39	F/32	F/18	F/38	M/37	M/20	F/28
Precipitant(s)	HE	Pregnancy	None	HE	HE	None	HE
Platelets, $\times 10^9$/L	179	212	20	228	100	345	228
MAHA	–	+	+	+	+	+	–
LDH, U/L	680	4,106	1,000	1,800	2,125	1,251	298
Creatinine, µmol/L	1,089	1,388	884	1,730	586	283	1,065
Proteinuria, g/d	Oliguria	>3.5	0.4	Anuria	6.4	4.5	5.5
C3, g/L (Ref., >0.75)	0.74	0.69	0.72	0.69	0.88	0.72	0.72
CFH-H3/*MCP*$_{GGAAC}$	+/–	–/–	+/–	–/–	+/–	–/–	–/–
Kidney biopsy							
Pattern on light microscopy	CresGN, ischemia	DC, thrombi	DC, thrombi	DC, thrombi	DC, Mes., thrombi	DC, Mes.	DC, Mes., thrombi
Electron lucent material	+	ND	ND	ND	+	+	+
FPE, %	80	ND	ND	ND	>50	80	40
Neurologic disease	+	–	–	–	–	–	–
Cardiac disease	+	–	–	–	+	–	–
Plasma exchange	–	+	+	+	+	+	+
Follow-up, yr.	30.6	15.4	16.0	11.4	11.8	3.0	4.6
Outcome	ESKD, deceased	ESKD	ESKD	ESKD	ESKD	CKD G1	ESKD
Donor kidney(s)	3	1	2	2	1	0	1
Graft failure (TMA)	3 (2)	1 (1)	2 (1)	2 (2)	1 (1)	N/a.	0

CKD, chronic kidney disease. CresGN, crescentic glomerulonephritis. DC, double contour formation of the glomerular basement membrane. ESKD, end–stage kidney disease. FPE, foot process effacement. HE, hypertensive emergency. MAHA, microangiopathic hemolytic anemia. Mes., mesangiolysis. N/a., not applicable. ND, not determined. TMA, recurrent disease.

Figure 3. Disease course after kidney transplantation.



Asterisk, recurrent primary atypical HUS. Blue bars, allograft survival. Checkered bar, eculizumab treatment. Grey bars, treatment for recurrent disease, including immunosuppression (e.g., steroid and/or rituximab). X, graft loss.

T1, donor kidney 1. T2, donor kidney 2. T3, donor kidney 3.

Table 2. Patients' characteristics at the time of recurrent primary atypical HUS.

Patient/ TX	Donor type	Platelets, $\times 10^9$/L	MAHA	LDH, U/L	SCr, µmol/L	uP, g/d	Precipitant(s)
M05486/T2	DCD	334	–	366	751	5.1	TAC
M05486/T3	DCD	158	–	560	460	3.7	TAC
M00503/T1	DCD	205	–	159	557	3.0	TAC
M03103/T2	LR	253	–	200	769	Anuria	TAC
M00105/T1	DCD	142	+	1,274	495	1.4	TAC
M00105/T2	LUR	116	+	793	399	3.3	TAC
M03307/T1(1)	LR	127	–	367	203	0.5	TAC, CMV
M03307/T1(2)	LR	237	–	255	486	4.1	TAC

CMV, cytomegalovirus reactivation. DCD, donation after cardiac death. LR, living–related donation. LUR, living–unrelated donation. MAHA, microangiopathic hemolytic anemia. SCr, serum creatinine. TAC, tacrolimus. uP, proteinuria

DISCUSSION

In the current study, we demonstrated that primary atypical HUS linked to C3 p.Arg161Trp can present with chronic morphologic features of TMA, characterized by nephrotic–range proteinuria, normal platelet counts, and a poor prognosis. The clinical and pathological observations on kidney biopsy are consistent with the murine data.[32] C3 p.Arg161Trp's affinity for CD46 (i.e., membrane cofactor protein) is decreased,[66,137] identical to C3 p.Asp1115Asn.[138] This might explain the chronic morphologic features of TMA, identical to some patients with pathogenic variants in *CD46*.[112] Also, cardiac and neurologic manifestations appeared prevalent in patients carrying C3 p.Arg161Trp.[66]

C5 inhibition rescued affected mice despite the presence of chronic TMA.[32] In clinical practice, the use of therapeutic complement inhibition for the treatment of chronic TMA is debatable.[11] Patient no. M03307 who relapsed "late" after kidney transplantation progressed to graft loss despite eculizumab. The donor kidney's capacity to recover is limited as compared to native kidneys.[96] Early recognition is therefore of utmost importance. Proteinuria >1 g/d, although aspecific, may be a marker of smoldering disease after kidney transplantation.

In conclusion, C3 p.Arg161Trp and probably other C3 gain–of–function proteins, may present with proteinuria related to chronic TMA, often with normal platelet counts. Recognition of these patients at an early stage of disease may improve the prognosis and, in particular, kidney survival in the era of therapeutic complement inhibition.

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The syndromes of thrombotic microangiopathy: a critical appraisal on complement

Sjoerd A.M.E.G. Timmermans[1, 2] and Pieter van Paassen;[1, 2] for the Limburg Renal Registry.

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Summary. Thrombotic microangiopathy (TMA) is a rare and potentially life–threatening condition that can be caused by a heterogeneous group of diseases, often affecting the brain and kidneys. TMAs should be classified according to etiology to indicate targets for treatment. Complement dysregulation is an important cause of TMA that defines cases not related to coexisting conditions, that is, primary atypical hemolytic uremic syndrome (HUS). Ever since the approval of therapeutic complement inhibition, the approach of TMA has focused on the recognition of primary atypical HUS. Recent advances, however, demonstrated the pivotal role of complement dysregulation in specific subtypes of patients considered to have secondary atypical HUS. This particularly is the case in patients presenting with coexisting hypertensive emergency, pregnancy, and kidney transplantation, shifting the paradigm of disease. In contrast, complement dysregulation is uncommon in patients with other coexisting conditions, such as bacterial infection, drug use, cancer, and hematopoietic stem cell transplantation. In this review, we performed a critical appraisal on complement dysregulation and the use of therapeutic complement inhibition in TMAs associated with coexisting conditions and outline a pragmatic approach to diagnosis and treatment. For future studies, we advocate the term complement–mediated (C–)TMA as opposed to the traditional atypical HUS type classification.

Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.

TMA is a rare, potentially life–threatening condition that reflects tissue responses to severe endothelial damage caused by distinct disorders, including thrombotic thrombocytopenic purpura and HUS. Despite heterogeneity, TMAs typically manifest with consumptive thrombocytopenia, microangiopathic hemolytic anemia, and ischemic organ damage, often affecting the brain and kidneys. TMAs should be classified according to etiology to indicate targets for treatment (Figure 1).[10,11] For example, thrombotic thrombocytopenic purpura is caused by a severe deficiency of von Willebrand factor cleaving protease (also known as a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 [ADAMTS13])[5] and thus, treatment should restore ADAMTS13's function. The term HUS, either atypical or not, has been used to define any TMA with a normal functional activity of ADAMTS13.

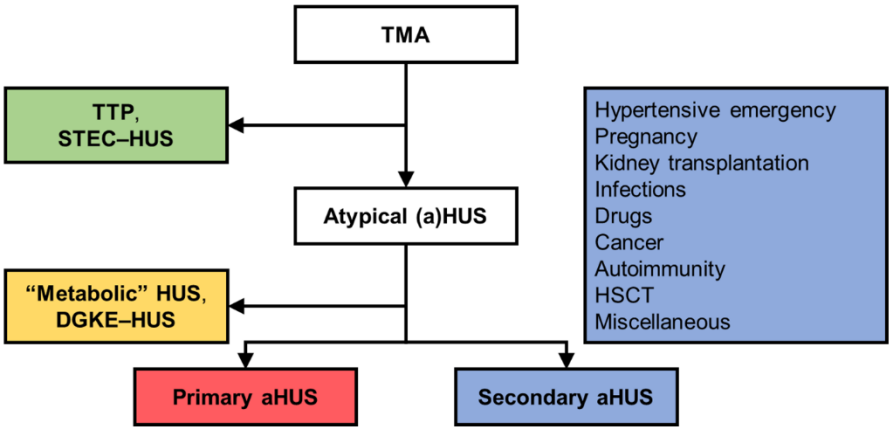
HUS occurring on the background of complement dysregulation defines primary atypical HUS, indicating a diagnosis of exclusion.[10] Many of such patients present with rare variants in complement genes and/or autoantibodies that inhibit complement regulatory proteins.[8,9] Primary atypical HUS is considered an orphan disease, with an incidence <1 per million population per year.[45] Most patients with HUS (i.e., ~90%) present with coexisting conditions, assumed to be the etiologic factor of disease, and have been termed secondary atypical HUS (Figure 1).[139] Known coexisting conditions linked to secondary atypical HUS are hypertensive emergency, pregnancy, kidney transplantation, (bacterial) infection, drug use, cancer, autoimmunity, and hematologic stem cell transplantation (HSCT) among others. Recent advances, however, linked complement dysregulation to specific subtypes of so–called secondary atypical HUS and poor kidney outcomes.[39,101,120,140] Thus, the traditional atypical HUS type classification is not absolute because complement dysregulation can be present along the spectrum of HUS. In the era of therapeutic complement inhibition,[13-15] the challenge is to recognize patients with complement dysregulation in the earliest possible stage to prevent end–stage kidney disease (ESKD).

In this review, we performed a critical appraisal on complement dysregulation and therapeutic complement inhibition in HUS presenting with coexisting conditions and outline a pragmatic approach to diagnosis and treatment. We advocate to use the term C–TMA to define cases related to complement dysregulation.

TMA, COEXISTING CONDITIONS, AND COMPLEMENT DYSREGULATION

Recent studies demonstrated that complement dysregulation is prevalent in specific subtypes of "secondary" atypical HUS and linked to poor kidney outcomes, resembling primary atypical HUS (hereafter referred to as C–TMA).[140]

Figure 1. The atypical (a)HUS type classification.[10]



DGKE, diacylglycerol kinase epsilon. HSCT, hematopoietic stem cell transplantation. STEC, Shiga toxin–producing E. *coli*. TTP, thrombotic thrombocytopenic purpura.

***Hypertensive emergency*.** Hypertensive emergency has been linked to activation of the renin-angiotensin system.[141] Renin can cause the C3 convertase to form via activation of C3.[142] *In vivo* data showed that further activation of C5 may play a role in the development of hypertension–associated kidney damage.[143,144]

Kidney disease is common (i.e., ~25%) in patients with hypertensive emergency and has been associated with hemolysis.[79] Patients with kidney disease are at risk for ESKD despite blood pressure control.[74] Many of such patients have been classified as "hypertensive" ESKD with no confirmative proof on kidney biopsy, assuming that the kidneys are the victim rather than culprit of disease. Thus, parenchymal kidney disease, including TMA, can be missed. This is particularly the case in patients without profound hematologic abnormalities.[105] We, for the first time, demonstrated the high prevalence of pathogenic variants in complement genes in patients with TMA and coexisting hypertensive emergency, which was associated with ESKD and TMA recurrence.[73] Our observations have been validated in independent cohorts, confirming C–TMA associated with complement gene variants in ~50% of patients with hypertensive emergency and severe kidney disease.[97,119] It remains to be established whether or not complement dysregulation plays a role in patients with mild–to–moderate kidney disease.

The effect of blood pressure control, the cornerstone of treatment, is limited in patients with hypertensive emergency and severe kidney disease.[74] The high prevalence of rare variants in complement genes and/or massive *ex vivo* C5b9 formation on the endothelium pointed to complement as a potential target for treatment.[86] Retrospective studies from France,[97] Spain/Portugal,[119] and our own group[105] included 29 patients with hypertensive emergency and severe kidney

Table 1. The effects of therapeutic complement inhibition in patients with TMA and coexisting hyper–tensive emergency or pregnancy; single case reports have not been included.

		Presentation		Genotyping		Outcome at 12 months		
	Ecu, *n*/*N*	SCr, mg/dL	Dialysis	Variants	Patho–genic	Kidney response	ESKD	Death
Hypertensive emergency								
Ref.[119]	9/19	8 (7–9)	8, 89%	5, 56%	3, 33%	7, 78%	2, 22%	0
Ref.[97]	13/76	?	?	7, 54%	?	9, 69%	4, 31%	0
Ref.[105]*	7/26	7 (4–9)	4, 57%	2, 29%	2, 29%	5, 71%	1, 14%	1, 14%
	29/122	?	?	14, 48%	?	21, 72%	7, 24%†	1, 3%
Pregnancy–associated atypical HUS								
Ref.[120]	4/87	?	?	2, 50%	?	3, 75%	1, 25%	0
Ref.[145]	10/22	4 (3–5)	3, 30%	4, 40%	4, 40%	10, 100%	0	0
Ref.[117]	3/7	5 (4–6)	3, 100%	1, 33%	0	2, 67%	1, 33%	0
	17/116	?	?	7, 41%	?	15, 88%	2, 25%‡	0
***De novo* TMA after kidney transplantation**§								
No data								

*Patients with follow–up <12 months were excluded. †60–75% of patients not treated with eculizumab had ESKD at 12 months. ‡49–55% of patients not treated with eculizumab had ESKD at 12 months. §Neither related to antibody–mediated rejection nor calcineurin inhibition.

Ecu, eculizumab. ESKD, end–stage kidney disease. SCr, serum creatinine presented as median (interquartile range); µmol/L, conversion factor ×88.4.

disease, including patients on dialysis, who had been treated with eculizumab (Table 1). At 12 months, a renal response was achieved in 21 (72%) patients, suggesting a benefit of treatment as compared to historical data.[74] Future prospective trials are warranted to test the hypothesis that therapeutic complement inhibition will improve the outcome of patients presenting with severe kidney disease. Furthermore, the predictive role of functional *ex vivo* complement measures[105] and pathologic features, such as chronic vascular and tubulointerstitial damage,[83] should be addressed.

Pregnancy. TMA can develop in 1 per 25,000 births,[146] the etiology of which varies from thrombotic thrombocytopenic purpura[147] to pregnancy-associated atypical HUS (P–aHUS) in late pregnancy and the postpartum period.[47] In an international cohort, P–aHUS resembled C–TMA based on the high incidence of ESKD and prevalence of complement gene variants, that is, 41% to 56%.[120,145] Pregnancy, indeed, has been linked to the first episode of primary atypical HUS in ~20% of women.[47] Rare variants in complement genes per se, however, cannot predict the risk of P–aHUS in a given pregnancy.[148] P–aHUS often develops in the setting of coexisting conditions, such as preeclampsia and bleeding.[148,149]

Preeclampsia and HELLP, both microangiopathies of late pregnancy, have been linked to complement activation but not to complement dysregulation. Variants in complement genes were found in up to 18% (*n*/*N*=7/40)[150] and 38% (*n*/*N*=9/24),[114,151]

respectively. Most variants, however, should be classified as uncertain or no significance according to current standards and guidelines.[89] Patients typically present with mild kidney disease and are at low risk for ESKD.[139] Moreover, preeclampsia and HELLP can develop in pregnant women treated with eculizumab,[116] suggesting a mechanism not related to C5 activation. Preeclampsia or HELLP, however, may mask P–aHUS when the kidney function does not improve after delivery.

KDIGO advocated that patients with P–aHUS should be treated as C–TMA (Table 1).[11]

Kidney transplantation. Activation of complement has been linked to various stages of kidney transplantation, including but not limited to organ preservation, reperfusion during surgery, and rejection.

TMA after kidney transplantation, both *de novo* and recurrent disease, has been linked to rare variants in complement genes in 29% (*n*/*N*=7/24)[39] and 68% (*n*/*N*=39/57),[44] respectively. The risk of TMA after kidney transplantation is >36 times higher in patients with C–TMA in the native kidney as compared to those with ESKD due to other causes[62] and is associated with the genetic fingerprint.[44] Of note, "hypertensive" ESKD was diagnosed prior to kidney donation in 3 recipients with *de novo* TMA who carried rare variants in complement genes (pathogenic, *n*/*N*=2/3).[39] Thus, C–TMA may be missed in the native kidneys, particularly in patients with a history of "hypertensive" ESKD,[112] as is discussed earlier. Most cases of *de novo* TMA in transplant recipients, however, are related to concurrent medications (e.g., calcineurin inhibition)[152] or antibody–mediated rejection.[153] The clinical course of both conditions is not consistent with complement dysregulation as 1–year graft survival was common.[122,154] The precise prevalence of rare variants in complement genes, however, has not been studied.

No data are available on eculizumab for the treatment of *de novo* TMA in transplant recipients, but eculizumab's efficacy has been proven for C–TMA recurrence.[94] The graft's capacity to recover is limited as compared to the native kidneys,[96] favoring preemptive treatment in selected cases. Prophylaxis prevented C–TMA recurrence and improved graft survival in patients at moderate and high risk but should not be used in patients with a variant in *CD46* alone because the donor kidney does not express mutated CD46. TMA related to antibody–mediated rejection, characterized by C4d deposits in peritubular capillaries and donor specific alloantibodies, often fails to respond to eculizumab.[154] C4d deposits reflect activation via the classical pathway and therefore, C1 inhibition upstream of C5 may be a better target for treatment.

TMAs UNRELATED TO COMPLEMENT DYSREGULATION

The prevalence of rare variants in complement genes in patients with secondary TMA equals controls (i.e., <10%)[100] and standard of care, that is, treatment directed against the underlying cause, has improved the prognosis over recent decades.[139] Thus, clinical observations (i.e., genetics, clinical response to standard of care, and low risk of TMA recurrence) indicate normal complement regulation (Table 2).

Shiga toxin–producing E. coli and other (bacterial) infections. Shiga toxin, the causative factor of Shiga toxin–producing E. *coli* (STEC)–HUS, can be internalized to the renal endothelium's cytosol via globotriaosyl ceramide. After internalization, activation of C3, either via the lectin[184] or alternative pathway,[185] and glomerular thrombosis have been observed in mice.

Most patients with STEC–HUS or those with post-diarrheal HUS not related to STEC present with increased levels of soluble C5b9, while pathogenic variants in complement genes have been found in 2% (*n*/*N*=3/125) and 3% (*n*/*N*=1/33), respectively.[103,155,156] Although >50% patients present with severe kidney disease, the clinical outcome appeared favorable, with rapid improvement in kidney function.[103,157,186] ESKD developed in 1 patients with a pathogenic variant in *CFH*, indicating C–TMA.[187]

The 2011's STEC–HUS outbreak in Germany, the largest to date (HUS developed in 855 out of 3,842 STEC infected patients),[188] provided new data on the clinical course and effects of various treatment strategies, including therapeutic complement inhibition.[157] Eculizumab was used in patients with severe disease but appeared of no short-term clinical benefit as compared to matched controls with similar disease severity treated with plasma exchange. Data of double blind randomized controlled trials (NCT02205541, EudraCT2016–000997–39) focusing on the efficacy of eculizumab for the treatment of STEC–HUS are awaited. The German data, however, corroborated previous observations,[158,186] indicating that STEC–HUS is an acute but self–limiting disease.

Patients with pneumococcal HUS may present with rare variants in complement genes.[160] The clinical outcome appeared rather favorable as compared to C–TMA[161] and confirmative studies are therefore needed.

Drug–induced TMA (DITMA). Many drugs have been reported to cause DITMA, although causative associations are often lacking. The Oklahoma Registry and Blood Center of Wisconsin provided support for causal associations between specific drugs and TMA.[124,189] Quinine, either related to drug–dependent antibodies or not, was the most common cause of definite DITMA. These antibodies react with platelets and, in some instances, the endothelium.[190] Most patients showed a clinical response after discontinuing the drug, confirming a mechanistic link.[100,165] Of note,

Table 2. The prevalence of rare variants in complement genes (i.e., *CFH*, *CFI*, *CD46*, *CFB*, *C3*, *THBD,* and *CFHR1–CFHR5*) and disease course in patients with secondary TMA confirm normal complement regulation; single case reports have not been included.

		Genetics/FHAA					Treatment	
	N	**Rare variants***	**Patho–genic**	**Dialysis**	**ESKD†**	**Relapse**	**SoC**	**Ecu (*n*)**
STEC–HUS								
Ref.[103]	79	6/75	3/75	56% ~6 d	1%	?	+	= (12)
Ref.[155]	25	1/25	0/25	80% ~7 d	?	?	?	?
Ref.[156]	26	2/25	0/25	65% ~16 d	–	–	+	?
Ref.[157]	298	?	?	54% ~10 d	1%	?	+	= (67)
Ref.[158]	770	?	?	57%	7% at discharge	?	+	?
Ref.[159]	491	?	?	57%	4% at discharge	?	+	= (193)
		9/125, 7%	3/125, 2%	>50%	1–7%	–‡	+	Unproven
Post–diarrheal HUS								
Ref.[103]	33	2/23, 9%	1/33, 3%	41% ~9 d	0%	–	+	= (3)
		2/23, 9%	1/33, 3%	41%	0%	–	+	Unproven
Pneumococcal HUS								
Ref.[160]	5	3/5	2/5	80%	– 40% died	–	±	?
Ref.[161]	37	?	?	73% ~15 d	23%	?	±	?
Ref.[162]	14	?	?	57%	15% at discharge	–	+	?
		3/5, 60%	2/5, 40%	>50%	14–23%	–	±	Unproven
Drug–induced TMA								
Ref.[100]	32	2/32	1/32	22%	16%	3%	+	= (13)
Ref.[163]	11	0/2	0/2	45%	?	?	+	?
Ref.[121] PI	15	0/15	0/15	33%	–	?	?	+ (15)
Ref.[164] VEGFi	13	0/13	0/13	–	–	?	+	?
Ref.[165] Quinine	19	?	?	90%	17%	–	+	?
		2/62, 3%	1/62, 2%	Variable	<17%	–‡	+	Unproven
Cancer								
Ref.[100]	11	2/11	1/11	55%	30%	–	+	= (8)
Ref.[166]	154	?	?	17%	?	±	+	?
		2/11, 18%	1/11, 9%	Variable	Competes w/ survival	Cancer–related	+	Unproven
Autoimmunity								
Ref.[100]	26	1/26	0/26	52%	61%	–	–	NR (8/9)
Ref.[121]	8	1/8	0/8	63%	57%	–	?	NR (6/8)
Ref.[129] CAPS	10	1/10	0/10	?	?	?	?	?
Ref.[167] SLE	11	2/10	0/10	73%	27%	?	?	NR (4/11)
		2/34, 6%	0/34, 0%	Variable	~10%,[107] >50%	–	–	Unproven

Table 2. Continued.

	N	Genetics/FHAA		Dialysis	ESKD[†]	Relapse	Treatment	
		Rare variants[*]	Patho–genic				SoC	Ecu (*n*)
HSCT–TMA								
Ref.[168]	34	3/34	0/34	–	?	?	–	?
Ref.[169]	30	4/30	0/30	23%	?	?	?	+ (64)
				23%	CKD in sur–vivors[170,171]	–	83% died[172]	Improved survival[169]
DGKE–HUS								
Ref.[173]	44	3/44[174]	0/44[174]	52%	23%	70%	+	= (3)
Ref.[175]	15	0/15	0/15	50%	13%	53%	+	= (6)
Ref.[176]	13	0/13	0/13	69%	31%	77%	+	= (1)
		3/72, 4%	0/72, 0%	50–69%	"Late"	>50%	+	Unproven
Cobalamin C deficiency								
Ref.[177]	36	2[¥]/15, 13%	0/15, 0%	22%	<15%	–	+	– (5)
		2[¥]/15, 13%	0/15, 0%	22%	<15%	–	+	Unproven
Monoclonal gammopathy								
Ref.[178]	20	?	?	55%	50%	No	±	– (1)
		?	?	55%	50%	No	±	Unproven

[*]Rare variants indicate those with a minor allele frequency of <0.1%; detailed characteristics of the genetic variants can be found in Table 3; number indicates (likely) pathogenic variants and variants of uncertain significance. [†]ESKD often within 1 year; "Late" ESKD indicates >10 year. [‡]TMA recurrence linked to rare variant(s) in complement genes. [¥]FHAA were found in 1 patient with normal copies of CFHR1 and CFHR3 and thus, FHAA may not be relevant. +, effective (>75% response). ±, may be effective. –, not effective (i.e., <50% response). ?, unknown.

CAPS, catastrophic antiphospholipid syndrome. CKD, chronic kidney disease. DGKE, diacylglycerol kinase epsilon. ESKD, end–stage kidney disease. HSCT, hematopoietic stem cell transplantation. NR, no response. PI, proteasome inhibition. SLE, systemic lupus erythematosus. SoC, standard of care. STEC, Shiga toxin–producing E. *coli*. VEGFi, vascular endothelial growth factor inhibition.

Table 3. Classification of rare variants in *CFH*, *CFI*, *CD46*, *CFB*, *C3*, *THBD*, and *CFHR1–5* found in patients with secondary TMA.

	No.	Gene*	cDNA*	Protein*	MAF (%)	PP–2/ SIFT	*In vitro*
STEC–HUS							
Ref.[103]	1	*CFH*†, ‡	c.2850G>T	p.Q950H	>0.1	Dam./Del.	LOF(?)[65]
	2	*THBD*‡	c.1483C>T	p.P495S	<0.09	Benign/Tol.	LOF[28]
	3	*CFH*	c.1145C>A	p.A382E	0	Dam./Tol.	LOF[103]
	4	*CD46*	c.503_504insA	p.N170Kfs*7	0	N/a	LOF[103]
	?	*C3*†, ‡	c.2203C>T	p.R735W	>0.1	Dam./Del.	WT[138]
	?	*C3*†, ‡	c.2203C>T	p.R735W	>0.1	Dam./Del.	WT[138]
	?	*C3*†, ‡	c.2203C>T	p.R735W	>0.1	Dam./Del.	WT[138]
	?	*C3*†	c.4369G>C	p.D1457H	0.1	Dam./Del.	–
	?	*C3*	c.1618G>T	p.A540S	<0.01	Benign/Tol.	–
	?	*CFB*‡	c.978A>C	p.E326D	<0.1	Benign/Tol.	–
	?	*CFI*†, ‡	c.782G>A	p.G261D	0.1	Benign/Tol.	WT[179]
	?	*THBD*	c.829G>T	p.G277W	0	Dam./Del..	–
Ref.[155]	?	*C3*	c.476T>A	p.V159E	0	Dam//Tol.	–
	?	*CFI*†, ‡	c.1657C>T	p.P553S	0.1	Benign/Tol.	–
Ref.[156]	P14	*C3*‡	c.3656G>A	p.R1219H	<0.01	Benign/Tol.	–
	P21	*CFH*‡	c.2867C>T	p.T956M	0.1	Dam./Tol.	WT[64]
	P25	*C3*†, ‡	c.463T>G	p.K155Q	>0.2	Benign/Tol.	GOF(?)[90]
	P29	*CFH*‡	c.172T>G	p.S58A	0.02	Benign/Tol.	LOF[64]
Post–diarrheal HUS							
Ref.[103]	5	*THBD*†, ‡	c.127G>A	p.A43T	>0.2	Benign/Tol.	LOF[28]
	6	*CFH*‡	c.3628C>T	p.R1210C	0.02	Benign/Tol.	LOF[22]
		C3‡	c.4319A>C	p.D1440A	<0.05	Benign/Tol.	–
	?	*CFH*†, ‡	c.2867C>T	p.T956M	0.1	Dam./Tol.	WT[64]
	?	*C3*†, ‡	c.4855A>C	p.S1619R	>0.1	Dam./Tol.	WT[180]
	?	*C3*†, ‡	c.4855A>C	p.S1619R	>0.1	Dam./Tol.	WT[180]
	?	*C3*	c.4177C>T	p.R1393W	<0.01	Dam./Del.	–
Pneumococcal HUS							
Ref.[160]	3	*CFH*	c.3445C>T	p.R1149X	<0.01	N/a	–
	4	*CFI*‡	c.148C>G	p.P50A	<0.02	Dam./Del.	LOF[63]
	5	*THBD*	c.131C>T	p.T44I	<0.01	Benign/Tol.	–
Drug–induced TMA							
Ref.[100]	3	*CFI*	c.11T>A	p.L4H	0.02	Benign/Tol.	–
	6	*CFH*‡	c.3596T>C	p.F1199S	0	Dam./Del.	LOF[181]
Cancer							
Ref.[100]	2	*THBD*	c.91G>A	p.V31I	0	Benign/Tol.	–
	5	*CFH*‡	c.3047A>G	p.Y1016C	0	Dam./Del.	–
Autoimmunity							
Ref.[100]	1	*THBD*	c.707C>G	p.A236G	0.02	Benign/Tol.	–
Ref.[121]	22	*CFHR1*†	c.869T>C	p.L290S	<0.01	Benign/Tol.	–
		CFHR1†	c.887C>T	p.A296V	<0.1	Benign/Tol.	–
Ref.[129]	CAPS3	*THBD*†, ‡	c.1502C>T	p.P501L	0.2	Benign/Del.	LOF[28]
	CAPS6	*CFHR4*	c.860G>A	p.R287H	<0.01	Benign/Tol.	–
Ref.[167]	1	*THBD*‡	c.127G>A	p.A43T	>0.2	Benign/Tol.	LOF[28]
	2	*CFH*‡	c.184G>A	p.V62I	>5	Benign/Tol.	–
		CFH‡	c.1204C>T	p.H402Y	>5	Benign/Tol.	–
	6	*CFB*‡	c.724A>C	p.I242L	<0.2	Benign/Tol.	–
		CFHR5	c.384G>T	p.S128S	0.1	Synonymous	–
		CD46	c.989-78G>A	N/a	35	N/a	–
		CFH‡	c.1204C>T	p.H402Y	>5	Benign/Tol.	–
	8	*THBD*‡	c.1456G>T	p.D486Y	<0.2	Benign/Tol.	LOF[28]
		CFH‡	c.184G>A	p.V62I	>5	Benign/Tol.	–
	9	*CD46*	c.989-78G>A	N/a	35	N/a	–
	11	*CFI*	c.1217G>A	p.R406H	0.1–1	Benign/Tol.	WT[109]

Table 3. Continued.

	No.	Gene*	cDNA*	Protein*	MAF (%)	PP–2/ SIFT	*In vitro*
HSCT–HUS							
Ref.[168]	?	*CD46*†, ‡	c.971C>T	p.P324L	0.2	Dam./Del.	LOF[182]
	?	*CFI*†, ‡	c.1246A>C	p.I416L	0.4	Benign/Tol.	LOF[63]
	?	*CD46*†, ‡	c.796G>A	p.D266N	>0.3	Benign/Tol.	–
	?	*C3*	c.1243C>A	p.P415T	0	Benign/Tol.	–
	?	*THBD*‡	c.1208G>A	p.R403K	<0.03	Benign/Tol.	–
		CFHR5†	c.486_487insA	p.E163Rfs*35	0.2	N/a	–
		CFHR5†	c.622T>C	p.C208R	0.2	Dam./Del.	–
	?	*CFI*†	c.1217G>A	p.R406H	>0.1	Benign/Tol.	WT[109]
	?	*C3*†, ‡	c.463T>G	p.K155Q	>0.2	Benign/Tol.	GOF(?)[90]
	?	*CFI*†, ‡	c.1534+5G>T	N/a	>0.9	N/a	–
	?	*CFI*†, ‡	c.1534+5G>T	N/a	>0.9	N/a	–
		CFI†, ‡	c.1657C>T	p.P553S	0.1	Benign/Tol.	–
	?	*CFI*†, ‡	c.1322A>G	p.K441R	0.1	Benign/Tol.	–
		CFB†, ‡	c.1697A>C	p.E566A	0.7	Benign/Tol.	–
	?	*CFB*‡	c.1729G>A	p.V577I	0.01	Benign/Tol.	–
		CFB‡	c.2005G>C	p.V669L	0.01	Benign/Tol.	–
Ref.[169]	1	*C3*†, ‡	c.2203C>T	p.R735W	>0.2	Dam./Del.	WT[138]
		CFHR3†	c.839_840del	p.I280fs	0.1	N/a	–
	2	*CFB*†	c.95G>A	p.R32Q	>5	Benign/Tol.	–
		CFHR3†	c.786A>T	p.P262P	>5	N/a	–
	3	*CFB*†	c.95G>A	p.R32Q	>5	Benign/Tol.	–
	4	*CFHR5*	c.486_487insAA	p.E163fs	0	N/a.	–
	5	*CFB*†	c.95G>A	p.R32Q	>5	Benign/Tol.	–
		CFH†, ‡	c.2850G>T	p.Q950H	>0.1	Dam./Del.	LOF(?)[65]
	6	*THBD*†, ‡	c.1502C>T	p.P501L	0.2	Benign/Del.	LOF[28]
	7	*CFHR3*†	c.786A>T	p.P262P	>5	N/a	–
		CFHR5	c.1067G>A	p.R356H	>2	Dam./Del.	–
	8	*CFB*†	c.95G>A	p.R32Q	>5	Benign/Tol.	–
	11	*CFB*	c.559G>A	p.V187I	0	Dam./Del.	–
		CD46†, ‡	c.1058C>T	p.A353V	>1	Benign/Tol.	WT[183]
	13	*CFB*†, ‡	c.1697A>C	p.E556A	0.7	Benign/Tol.	–
	14	*CFB*†, ‡	c.1697A>C	p.E556A	0.7	Benign/Tol.	–
		CFHR1	c.310C>T	p.H104Y	<0.03	Benign/Tol.	–
	18	*CFB*†, ‡	c.1697A>C	p.E556A	0.7	Benign/Tol.	–
		CFH	c.3506T>C	p.I1169T	<0.01	Benign/Del.	–
DGKE–HUS							
Ref.[173]	HUS39	*THBD*†, ‡	c.1456G>T	p.D486Y	<0.2	Benign/Tol.	LOF[28]
	HUS40	*THBD*†, ‡	c.1456G>T	p.D486Y	<0.2	Benign/Tol.	LOF[28]
	HUS272	*C3*‡	c.784G>T	p.G262W	0	Dam./Del.	–
Cobalamin C deficiency							
Ref.[177]	27	*CFH*	Unknown	N/a	N/a	?	?

*Red indicates (likely) pathogenic variants; orange indicates variants of uncertain significance; green indicates benign variants. †Variant has been reclassified according to international standards.[23,89] Pathogenic variants were defined as those with functional studies supporting a defect in complement regulation, including null variants in genes linked to complement regulation (not including *CFB* and *C3*) and at least one of the following: located in a mutational hotspot, located in a functional domain, and/or cluster in patients with primary atypical HUS.[23] Benign variants were defined as those with a MAF ≥0.1% and/or functional studies supporting normal complement regulation. Rare variants, that is, those with a minor allele frequency of <0.1%, not fulfilling these criteria have been classified as uncertain significance. ‡Variant has been identified in the database of complement gene variants (http://www.complement-db.org/home.php).

Dam., possibly/probably damaging. Del., deleterious. DGKE, diacylglycerol kinase epsilon. GOF, gain–of–function. HSCT, hematopoietic stem cell transplantation. LOF, loss–of–function. MAF, minor allele frequency according to the Exome Variant Server (EVS) and Genome Aggregation Database (gnomAD). STEC, Shiga toxin–producing E. *coli*. Tol., tolerated. WT, normal and/or similar to wild type.

variants in complement genes were not found in patients with DITMA related to quinine.[165] Similar observations have been documented for proteasome inhibition[163] among other drugs. The observed efficacy of eculizumab in 12 patients with DITMA may therefore reflect the natural course; none of the patients carried pathogenic variants in complement genes.[121]

Monoclonal anti–vascular endothelial growth factor (VEGF) antibodies have been linked to TMA.[191] VEGF plays a role in the homeostasis of the glycocalyx and is therefore important to maintain the integrity of the glomerular's endothelium.[192] VEGF inhibition attenuates factor H function on the endothelium and causes chronic rather than acute TMA, as is seen in C–TMA.[191] Indeed, no variants in *CFH*, *CFI*, and *CD46* were found in patients with TMA following anti–VEGF treatment.[164]

No firm conclusions can be drawn on the role of complement in DITMA as drugs and thus, mechanisms differ. C–TMA, however, is not anticipated in DITMA.

Cancer. TMA is a well–described complication of cancer, mostly linked to treatment (i.e., DITMA). The exact mechanism of cancer–related TMA is unknown, although cancer emboli and mucin produced by adenocarcinomas (e.g., gastric, lung, prostate) can damage the endothelium.[193] Most patients have mild–to–moderate kidney injury, pointing to normal complement regulation.

Autoimmunity. Defects in complement and clearance of apoptotic bodies are key for systemic lupus erythematosus to occur. Activation of complement on the other hand, has been related to major organ involvement and, in particular, lupus nephritis.[194] TMA can be found in up to 20% of patients with lupus nephritis, mostly related to anti–phospholipid antibodies.[195] In contrast to patients with lupus nephritis alone,[196] those with lupus nephritis and TMA, either related to anti–phospholipid antibodies or not, may present with variants of uncertain significance (*n*/*N*=2/10).[167] We found that patients with antiphospholipid syndrome unrelated to lupus nephritis and TMA on kidney biopsy have normal complement regulation; the prognosis is rather favorable as compared to patients with C–TMA.[197]

No data have been published on complement dysregulation in systemic sclerosis.[198]

Hematologic stem cell transplantation (HSCT)–TMA. TMA after HSCT is a serious complication, the incidence of which appeared to be 39% in a recent prospective study at the Cincinnati Children's Hospital Medical Center,[172] although lower rates have been reported. Patients with proteinuria and elevated soluble C5b9 levels had a very poor prognosis, with death rates exceeding 80% at 12 months.[172] Homozygous deletion of *CFHR1* and *CFHR3*, either with factor H autoantibodies or not,[199] have been found in a small subset of patients, while pathogenic variants in

complement genes are uncommon.[168,169] The high incidence of HSCT–TMA as compared to C–TMA in the general population suggests that HSCT–TMA is not a simple Mendelian trait. HSCT–TMA has been associated with other factors, such as conditioning regimens, concurrent drugs, graft versus host disease, and infections. These factors may lower the threshold for TMA to manifest via neutrophils, neutrophil extracellular trap formation, and complement activation.[200,201]

Remarkably, 64 patients with severe HSCT–TMA were treated with eculizumab for a median of 9 weeks, improving 1–year survival to 66% (*n*/*N*=41/64).[169] No relapse occurred ever since discontinuation. Currently, an open label phase II study is enrolling patients to assess eculizumab's efficacy (NCT03518203).

These observations linked (secondary) complement activation rather than dysregulation to the mechanism of HSCT–TMA.

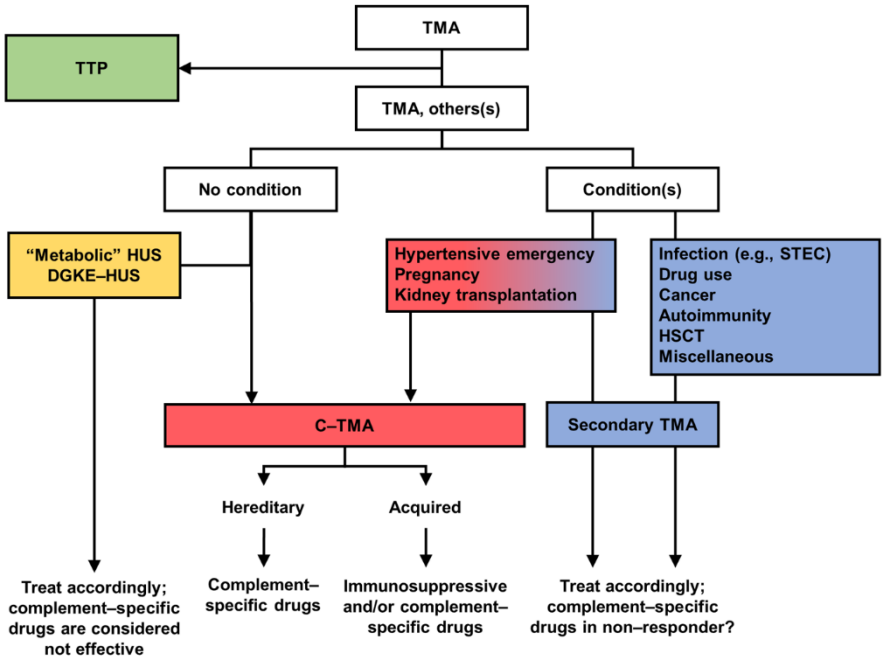
***Miscellaneous conditions*.** TMA can also occur in relation to metabolic disorders (e.g., cobalamin C deficiency),[177] loss of diacylglycerol kinase epsilon (DGKE),[173,176] and, perhaps, monoclonal gammopathies.[178] Metabolic disorders and loss of DGKE typically present in young children. Monoclonal gammopathies may be common in patients with TMA >50 years.[178] Also, TMA can occur in the postoperative period.[126] These TMAs do usually not develop on the background of complement dysregulation.

PROPOSAL FOR A PRAGMATIC APPROACH

With the current state of knowledge and availability of therapeutic complement inhibition, either eculizumab or other therapies under investigation, the central consideration in the management of patients with TMA is the recognition of C–TMA and thus, patients who would likely benefit from such therapies.

We propose that TMAs presenting with a normal ADAMTS13 should be classified according to etiology (Figure 2). Profound systemic hemolysis can be absent[140] and therefore, a tissue (e.g., kidney) biopsy may be needed to detect the TMA. Morphologic features, however, cannot define etiology (Figure 3).[105] Patients should be screened for coexisting conditions and, if absent, complement dysregulation. Patients with coexisting conditions and a severe clinical phenotype, that is, severe kidney disease not responding to standard of care and/or TMA recurrence, should also be screened for complement dysregulation. Many patients with coexisting hypertensive emergency, pregnancy, and, to a lesser extent, *de novo* TMA after kidney transplantation fulfill these criteria and have C–TMA rather than secondary disease.[101,140] In contrast, C–TMA is uncommon in patients with bacterial infection, DITMA, cancer, autoimmunity, HSCT–TMA, and miscellaneous conditions, indicating secondary TMA; rapid improvement in kidney function should be expected in such cases (Table 2). TMA related to metabolic disorders (e.g., cobalamin C

Figure 2. Pragmatic approach to diagnosis and treatment of TMA.



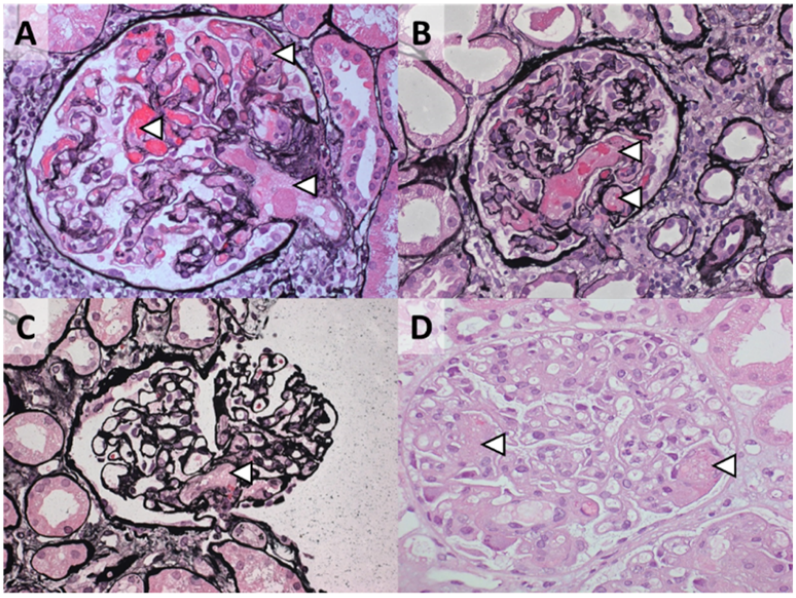
Patients with TMA should be tested for the enzymatic activity of ADAMTS13 (i.e., >10% excludes thrombotic thrombocytopenic purpura [TTP]). Patients with a normal activity of ADAMTS13 should be screened for coexisting conditions. Of note, patients with coexisting hypertensive emergency, pregnancy, or *de novo* TMA after kidney transplantation may have C–TMA rather than secondary TMA. Most patients with no coexisting conditions have C–TMA, although primary TMA related to recessive variants in *DGKE* and metabolic causes should be considered in children.

DGKE, diacylglycerol kinase epsilon. HSCT, hematopoietic stem cell transplantation. STEC, Shiga toxin–producing E. *coli*.

deficiency) or loss of DGKE is common in children and, in particular, infants. The term idiopathic, a subset of primary TMA, should be used judiciously because the etiology can be found in almost every patient.[139]

Tests recommended to screen for complement dysregulation include routine complement measures, genotyping, and autoantibody testing. Routine complement measures, however, are not specific and lack sensitivity.[51,86] Genetics should include sequencing of *CFH*, *CFI*, *CD46*, *CFB*, *C3*, and multiplex–ligation probe amplification to detect hybrid genes and/or the loss of *CFHR1* and *CFHR3*. Factor H serum reactivity should be assessed and, in particular, in children and patients with a homozygous deletion of *CFHR1* and *CFHR3*.[26] Hereditary and/or acquired factors inform the long–term prognosis[11] and should be used for classification (Figure 2) and to adopt suitable prophylactic measures.[40] For example, patients with

Figure 3. Morphologic features of TMA on kidney biopsy cannot define etiology.



Representative cases of TMA presenting with coexisting hypertensive emergency (A, 28–year–old woman with a gain–of–function C3 protein [p.Arg161Trp]; B, 47–year–old man with no rare variants in complement genes identified), after surgery (C, 37–year–old man with no rare variants in complement genes identified), and coexisting pregnancy (D, 28–year–old woman with no rare variants in complement genes identified). The arrowheads indicate glomerular thrombosis, often accompanied by mesangiolysis. Jones methenamine silver (A, B, C) and hematoxylin and eosin (D) staining; original magnification, ×400.

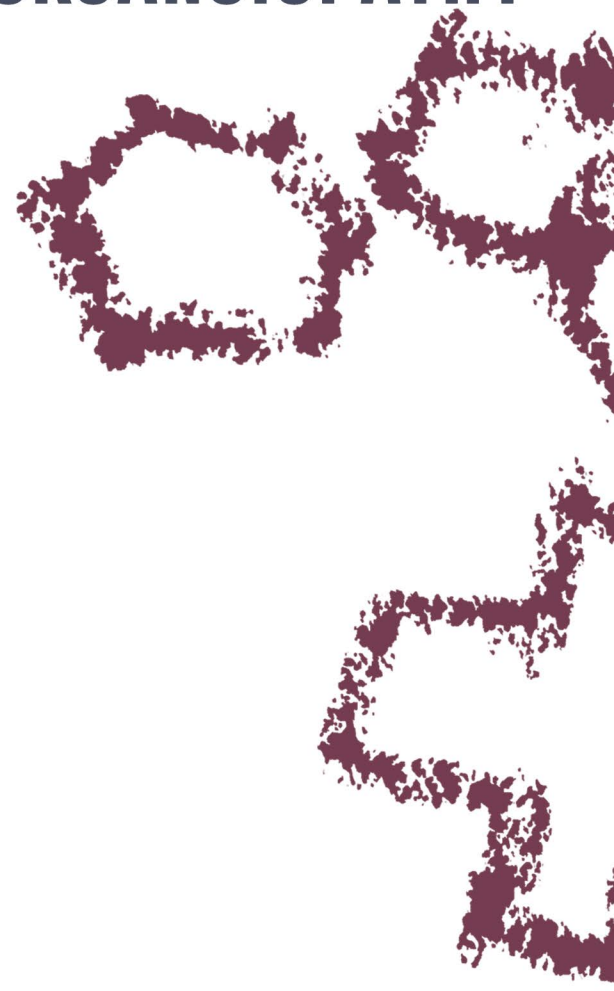
pathogenic variants identified or high levels of factor H autoantibodies are at high risk of TMA recurrence and sequelae, contrasting patients with neither hereditary nor acquired factors. Functional assessment of *ex vivo* complement activation appears a promising method for the detection of unrestrained complement activation on the endothelium and thus, C–TMA, irrespective of rare variants in complement genes.[51,86] Two functional tests have been developed using either microvascular endothelial cells of dermal origin (i.e., HMEC–1)[51,86] or endothelial hybrid cells that lack membrane–bound CD55 and CD59 (i.e., modified Ham test).[113] The HMEC–1 test reflects the dynamics of complement activation on the endothelium with massive *ex vivo* C5b9 formation on resting endothelial cells at the time of active but not quiescent disease. The modified Ham test does not differentiate active from quiescent disease and lack specificity.[113,114] Prospective studies are needed to test the hypothesis that functional tests can guide diagnosis and treatment.

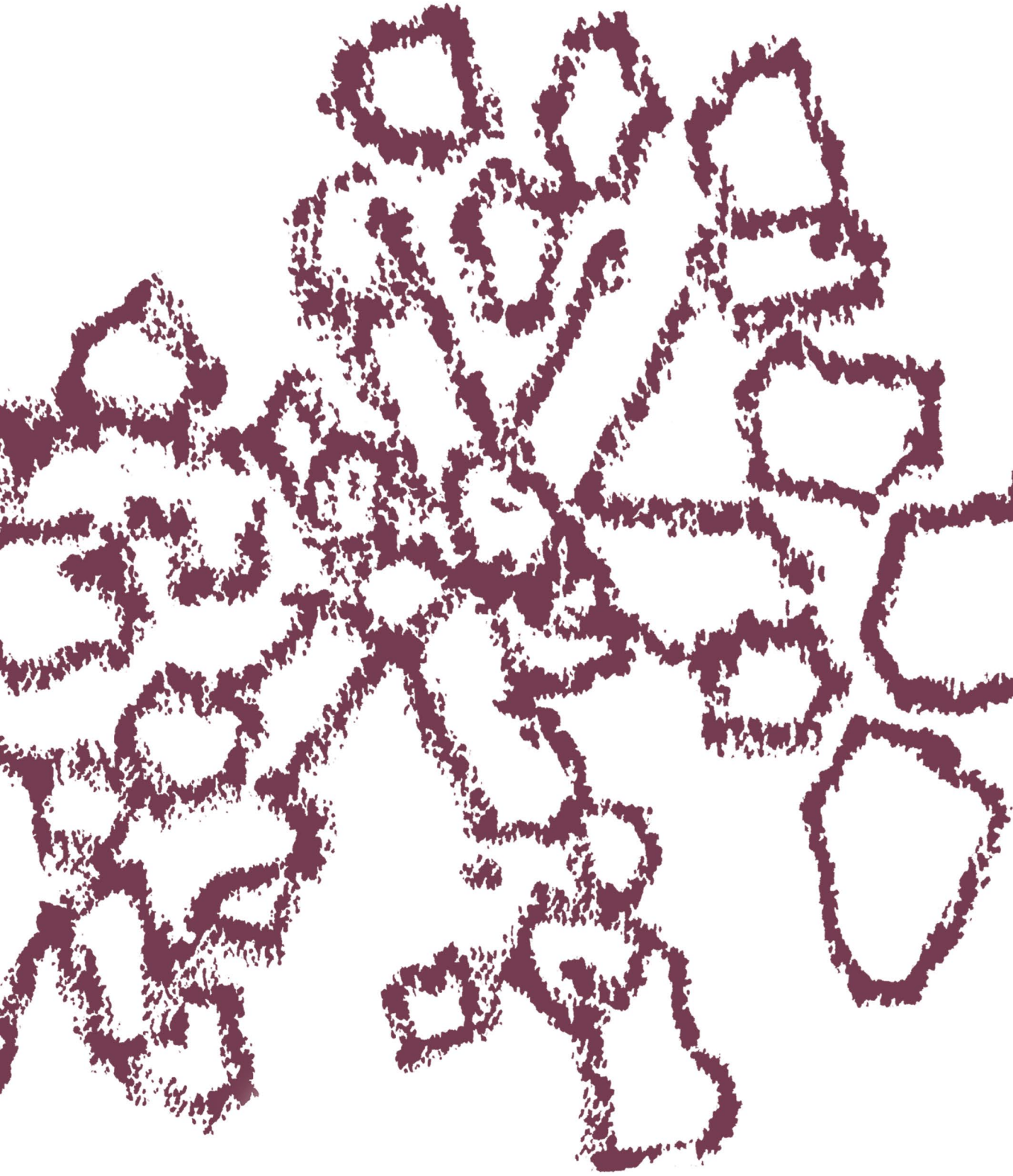
Rapid initiation of therapeutic complement inhibition is warranted in C–TMA, including patients with coexisting conditions. It remains unknown whether or not therapeutic complement inhibition should be used to treat specific subtypes of

secondary TMA.[100,121] The efficacy of ravulizumab, a long–acting monoclonal antibody that blocks C5 activation, for the treatment of secondary TMA is being studied (NCT04743804). The results of this long–awaited randomized controlled trial will aid the debate of therapeutic complement inhibition in secondary TMA.

In conclusion, recent advances have clearly changed the landscape of TMAs. Knowledge on complement dysregulation has enabled breakthroughs in the diagnosis and treatment of C–TMA. The proposed approach will increase diagnostic and prognostic accuracy and thus, may optimize the efficacy of treatment.

PART III
Improving patient care beyond the thrombotic microangiopathy





9

The natural course of pregnancies in women with primary atypical hemolytic uremic syndrome and asymptomatic relatives

Sjoerd A.M.E.G. Timmermans,[1, 2] Alexis Wérion,[3] Marc E.A. Spaanderman,[4] Chris P. Reutelingsperger,[2] Jan G.M.C. Damoiseaux,[5] Johann Morelle,[3, 6] and Pieter van Paassen.[1, 2]

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Summary. Pregnancy has been linked to various microangiopathies, including primary atypical hemolytic uremic syndrome (HUS). Complement dysregulation, often linked to rare variants in complement genes, is key for primary atypical HUS to manifest and may play a role in pregnancy complications of the mother and fetus. The burden of such complications is unknown, making counseling of women with primary atypical HUS and asymptomatic relatives difficult. We analyzed the maternal and fetal outcomes of 39 pregnancies from 17 women with primary atypical HUS and 2 asymptomatic relatives. Seven out of 39 pregnancies were complicated by pregnancy–associated atypical HUS (P–aHUS). Five out of 32 pregnancies not linked to P–aHUS were complicated by preeclampsia or HELLP (i.e., hemolysis, elevated liver enzymes, low platelets). Rare genetic variants were identified in 10 women (asymptomatic relatives, *n*=2) who had a total of 14 pregnancies, including 10 uncomplicated pregnancies. Thirty–five out of 39 pregnancies resulted in live birth. Eight out of 19 women had progressed to end–stage kidney disease (ESKD), with an incidence of 2.95 (95% confidence interval, 1.37–5.61) per 100 person–years after the first pregnancy. Thus, we emphasized the frequency of successful pregnancies in women with primary atypical HUS and asymptomatic relatives. Pregnancies should be monitored closely. Rare genetic variants cannot predict the risk of a given pregnancy.

Affiliations.
[1]Dept. Nephrology and Clinical Immunology, Maastricht UMC, NLD.
[2]Dept. Biochemistry, Cardiovascular Research Institute Maastricht, NLD.
[3]Division of Nephrology, Cliniques universitaires Saint–Luc, BE.
[4]Dept. Obstetrics and Gynecology, Maastricht UMC, NLD.
[5]Central Diagnostic Laboratory, Maastricht UMC, NLD.
[6]Institute de Recherche Expérimentale et Clinique, UCLouvain, BE.

Linked article.
Commentary (DOI: 10.1111/bjh.16694).

Healthy pregnancy has been linked to significant hemodynamic and immunologic shifts for maternal adaptation, placentation, and fetal tolerance. Defects in these processes can lead to a spectrum of microangiopathies, having great impact on maternal and fetal morbidity and mortality. Microangiopathic disorders of pregnancy range from preeclampsia to HELLP and, although rare, the syndromes of thrombotic microangiopathy (TMA), including thrombotic thrombocytopenic purpura and primary atypical HUS. Most of these microangiopathies occur late in pregnancy, suggesting a common denominator. In the last decade, complement has been linked to the mechanism of primary atypical HUS, either related to pregnancy[47] or not,[8,9] and, to a lesser extent, preeclampsia[150] and HELLP.[114,151]

The complement cascade is part of innate immunity and an effector system involved in host homeostasis and the defense against pathogens, which can be activated via the classic, lectin, and alternative pathway (AP). The latter is a continuously active surveillance system operating in the circulation and on cell surfaces. Host cells, including those from the placenta, are protected from the harmful effects of complement by regulatory proteins. Of note, tight complement regulation at the fetomaternal surface is crucial for pregnancy to succeed.[48] Rare variants in genes encoding proteins that either regulate or activate complement and/or autoantibodies that affect AP regulation can cause complement dysregulation and are prevalent in primary atypical HUS.[8,9] These abnormalities per se are not sufficient for TMA to occur. Pregnancy, however, may precipitate the onset or subsequent relapses of life–threatening episodes of primary atypical HUS.[120] Furthermore, the incidence of preeclampsia and HELLP may be higher in pregnant women with primary atypical HUS.[120]

The risk for complications in pregnant women prone for complement dysregulation, that is, patients with primary atypical HUS and asymptomatic relatives carrying rare variants in complement genes, is therefore considered high. In clinical practice, however, it is difficult to counsel such women as robust clinical data are lacking.[202] Moreover, the impact of pregnancies on fetuses and the role of prophylactic measures remains to be established.[149] The current study focused on maternal and fetal outcomes of 39 pregnancies in a well–defined cohort of women with primary atypical HUS and asymptomatic relatives. Furthermore, we report the long–term follow–up.

MATERIAL AND METHODS

***Patient population*.** Female patients with TMA and at least one reported pregnancy were recruited from the Limburg Renal Registry, Maastricht, The Netherlands[54] and the Cliniques universitaires Saint–Luc, Brussels, Belgium. TMA was defined as typical morphologic features of TMA on kidney biopsy or microangiopathic hemolytic anemia (hematocrit <30%, hemoglobin <10 g/L, lactate dehydrogenase >500 U/L,

and schistocytes on peripheral blood smear), platelets <150 $\times 10^9$/L, and acute kidney injury in patients with no pathologic proof of TMA. Patients with primary atypical HUS, defined as TMA, ADAMTS13's (i.e., von Willebrand factor cleaving protease) enzymatic activity of at least 10%, and proven complement defects as detailed below,[11] were included. Patients with the onset of TMA during pregnancy or within the first 12 weeks postpartum were classified as P–aHUS.[120] Also, asymptomatic female relatives carrying rare variants in complement genes were included; relatives from patients with primary atypical HUS, either related to pregnancy or not, were screened at the discretion of the physician. Disease definitions for preeclampsia,[203] HELLP,[203] and chronic kidney disease (CKD),[106] were based on standard international criteria. ESKD was defined as the need for kidney replacement therapy. Normal birth weight was defined as a birth weight between 10th and 90th percentile corrected for gender and gestational age; small for gestational age was defined as a birth weight <10th percentile corrected for gender and gestational age.[204]

The clinical data were obtained from the Limburg Renal Registry and/or the patients' medical records. The study was approved by the appropriate ethics committees and is in accordance with the Declaration of Helsinki.

***Complement analysis*.** DNA was tested for rare variants, that is, variants with a minor allele frequency <1%, and single nucleotide polymorphisms in coding regions of *CFH*, *CFI*, *CD46*, *CFB*, *C3*, *CFHR1–5*, *THBD*, and *DGKE* using sequencing.[73] Rare variants were classified according to international standards.[89] Pathogenic variants were defined as those with functional studies supporting a defect in complement regulation, including null variants in genes linked to complement regulation and variants that cluster in patients with primary atypical HUS as demonstrated by Osborne and colleagues.[23] Likely pathogenic variants were defined as those with functional studies supporting a defect in complement regulation that have been located in a mutational hotspot and critical functional domain. The *CFH–CFHR1–5* genomic region was analyzed for rearrangements by multiplex ligation probe amplification.[57] Factor H autoantibodies were assessed by enzyme–linked immunosorbent assay in selected cases.[58]

Patients with no variants identified were screened for unrestrained *ex vivo* C5b9 formation on microvascular endothelial cells of dermal origin (HMEC–1; ATCC, Manassas, VA) as described.[51,86] Briefly, HMEC–1 were used when >80% confluent, incubated with serum diluted in test medium for 3 hours at 37 degrees Celsius, fixed in 3% formaldehyde, and blocked with 2% BSA for 1 hour. Rabbit anti–human C5b9 pAb (Calbiochem, San Diego, CA) and Alexa488–labeled anti–rabbit Ab (Life Technologies, Carlsbad, CA) were used. The results were compared with pooled normal human serum run in parallel.

Table 1. Maternal outcomes of the 39 pregnancies.

Patient no.	Variant(s)	CKD	Compli–cated P	Adverse event(s)	Treat–ment	Seque–lae	Follow –up,* yr	Age ,yr	CKD at last visit
Primary atypical HUS prior to first pregnancy									
B39	*C3* c.3125G>A[2]	–	0/2	–	N/a	–	4	28	–
P–aHUS									
M00503	*C3* c.481C>T[1]	–	3/3	HT, P–aHUS (+60 d)	PEX	ESKD	18	47	G5+/T
M04813	–	–	6/7	PE, P–aHUS (+2 d)	–	CKD G4	16	35	ESKD
		G4	7/7	PE, bleeding	N/a	ESKD			
M08316	–	–	1/1	P–aHUS (+0 d), bleeding	PEX, Ecu	–	3	30	–
M06019	–	–	1/1	PE, P–aHUS (+0 d)	PEX, Ecu	–	1	30	–
B01	–	–	1/2	PE	N/a	HT	13	39	–
			2/2	HELLP, P–aHUS (+0 d)	PEX	–			
B06	–	–	1/1	PE, P–aHUS (+0 d)	PEX	–	1	30	–
B46	*CFI* c.772G>A[2]	–	1/1	PE, P–aHUS (+1 d)	PEX, Ecu	ESKD	1	32	ESKD
Primary atypical HUS after last pregnancy									
M00004	*CFI* c.1420C>T,[1] *C3* c.463A>C[1]	–	0/1	–	N/a	–	36	62	G5+/T
M00105	*C3* c.481C>T[1]	–	1/1	PE	N/a	Unknown	14	49	G3/T
M04010	*CFH* c.2558G>A[1]	–	0/1	–	N/a	–	10	40	G3/T
M02715	*C3* c.481C>T[1]	–	1/1	HELLP	N/a	Unknown	7	32	G2/T
M01416	–	–	1/4	PE	N/a	None	45	74	G4
M04516	–	–	0/4	–	N/a	–	21	46	ESKD
M06518	–	–	3/3	HT	N/a	HT	49	74	G3
B12	*CFH* c.3486delA[1]	–	0/1	–	N/a	–	2	32	–
B26	–	–	0/2	–	N/a	–	46	73	G3
Asymptomatic relative									
M04306.P	*CD46* c.811_816delGACAGT[1]	–	0/1	–	N/a	–	50	82	G3
B00.RI	*CD46* c.811_816delGACAGT[1]	–	0/2	–	N/a	–	6	37	–

*Follow–up after the first pregnancy. Rare variants in complement genes were classified as [1](likely) pathogenic or [2]uncertain significance.

CKD, chronic kidney disease (T, transplantation). Ecu, eculizumab. ESKD, end–stage kidney disease. HELLP, hemolysis, elevated liver enzymes, low platelets. HT, (gestational) hypertension. P, pregnancy. PE, preeclampsia. PEX, plasma exchange.

***Statistical analysis*.** Continuous variables were presented as mean (±SD) or median (interquartile range [IQR]) as appropriate. Descriptive statistics were used to analyze the cohort. *Ex vivo* C5b9 formation on HMEC–1 was compared with normal human serum by the paired sample *t* test or Wilcoxon signed rank test as appropriate.

RESULTS

***Patient population*.** Twenty–five women with primary atypical HUS and 5 asymptomatic relatives were recruited from the Limburg Renal Registry (*n*=18) and Cliniques universitaires Saint–Luc (*n*=12). Eleven nulliparous women (asymptomatic relatives, *n*=3) were excluded; 1 of them with 3 episodes of primary atypical HUS on the background of a pathogenic variant in *C3* remained intentionally childless to lower the risk of relapse.

Thus, 19 women at–risk for complement dysregulation and a total of 39 pregnancies were analyzed (Table 1). Rare variants in complement genes were found in 8 (47%) out of 17 patients with primary atypical HUS; combined variants were identified in 1 case. Five variants were considered pathogenic (carriers, *n*=6; Table 2). Two asymptomatic relatives had a pathogenic variant in *CD46* identified (patient no. M04306.P, B00.RI). The at–risk *CFH*–H3 and MCP_{GGAAC} were found in 3 and 2 patients with primary atypical HUS, respectively, but not in asymptomatic carriers. The homozygous genomic deletion of *CFHR1* and *CFHR3* but not factor H autoantibodies were identified in 1 patient with primary atypical HUS. Massive *ex vivo* C5b9 formation on HMEC–1 confirmed unrestrained complement activation in 9 patients with no variants identified at the time of acute primary atypical HUS. The patients' disease course can be found in Item S1.

***Maternal complications of pregnancy*.** We analyzed 39 pregnancies, all of whom were managed with no prophylactic measures.

P–aHUS developed in 7 (18%) out of 39 pregnancies at the time of delivery (*n*=4) or postpartum (*n*=3); 4 episodes were linked to the first pregnancy. Patients invariably presented with severe kidney failure (median serum creatinine 492 µmol/L; IQR, 194–557), including 6 patients who needed dialysis. Low platelets and Coombs negative microangiopathic hemolytic anemia were observed in 5 patients. Major bleeding, requiring blood and platelet transfusions, precipitated P–aHUS in 1 patient. Preeclampsia and HELLP were clinically inferred prior to the recognition of P–aHUS in 4 and 1 patient, respectively. Plasma exchange with fresh frozen plasma was started in 6 patients and associated with a complete clinical response in 2 cases, that is, normalization of kidney function. Eculizumab, a potent C5 inhibitor, was started in 3 refractory patients; 2 patients who initially required dialysis recovered kidney function and improved to CKD stage G2, while the other patient

Table 2. Detailed characteristics of the variants in complement genes.

Gene	Variant	Protein	MAF, %	*In vitro* defect	Significance
CFH	c.2558G>A	Cys853Tyr	0	LOF[64]	Pathogenic
CFH	c.3486delA	Lys1162Asnfs*7	0	Unknown	Pathogenic
CFI	c.772G>A	Ala258Thr	<0.03	Unknown	VUS
CFI	c.1420C>T	Arg474*	<0.01	LOF[110]	Pathogenic
CD46	c.811_816delGACAGT	Asp271_Ser272del	0	LOF[43]	Pathogenic
C3	c.481C>T	Arg161Trp	<0.01	GOF[66]	Pathogenic
C3	c.463A>C	Lys155Gln	0.2–0.4	GOF(?)[90]	VUS
C3	c.3125G>A	Arg1042Gln	0	Unknown	VUS

GOF, gain–of–function. LOF, loss–of–function. MAF, minor allele frequency. VUS, variant of uncer–tain significance.

progressed to ESKD. The patient not treated with plasma exchange was diagnosed with preeclampsia, but proved to have acute TMA on kidney biopsy, and progressed to CKD G4; ESKD developed after a subsequent pregnancy complicated by preeclampsia and major bleeding.

Five (16%) out of 32 pregnancies not linked to P–aHUS were complicated by preeclampsia (*n*=4, 12.5%) and HELLP (*n*=1, 3%). Furthermore, 1 patient had gestational hypertension. No maternal complications occurred in 26 (67%) out of 39 pregnancies, including 10 (71%) out of 14 pregnancies from carriers of rare variants in complement genes (5 patients with primary atypical HUS and 2 relatives with 11 and 3 pregnancies, respectively).

***Fetal outcomes*.** Fetal outcomes of all the 39 pregnancies have been depicted in Table 3. Thirty–five (90%) out of 39 pregnancies resulted in live birth, 3 pregnancies resulted in a spontaneous abortion, and 1 pregnancy was terminated at week 14 for unknown reasons. Twenty–two (63%) out of the 35 live births occurred at full term, 10 (29%) at preterm, and 2 (6%) at postterm. Eight of the preterm deliveries were induced because of preeclampsia, HELLP, and/or P–aHUS; the extremely preterm infant (i.e., gestational week 26+2) died from infantile respiratory distress syndrome 2 days after delivery. Pregnancies complicated by P–aHUS resulted in 8 newborns. Three (38%) were small for gestational age and 1 died from asphyxiation.

***Long–term kidney outcome after pregnancy*.** The women were followed for a median of 13 (IQR, 3–36) and 3.1 (IQR, 1.5–7.9) years after their first pregnancy and the onset of primary atypical HUS, respectively. At last follow–up, 6 patients and 1 asymptomatic relative had normal kidney function, that is, an estimated glomerular filtration rate >60 mL/min/1.73m^2. Two patients had progressed to CKD G3, 1 to CKD G4, and 8 patients to ESKD; 1 asymptomatic relative had progressed to CKD G3 but primary aHUS never developed.[112] The rate of ESKD in all 19 women after the first pregnancy was 2.95 (95% CI, 1.37–5.61) per 100 person–years; after

excluding both asymptomatic relatives, the rate of ESKD was 3.72 (95% CI, 2.75–7.16) per 100 person–years. In total, 7 kidney donors were transplanted in 5 recipients, all of whom had a high estimated risk for primary atypical HUS to reoccur.[11] None of the recipients became pregnant.

DISCUSSION

Pregnancy is a critical condition in women predisposed to complement dysregulation as it can precipitate primary atypical HUS with the attendant risk of sequalae. The first episode of primary atypical HUS can be linked to pregnancy, that is, P–aHUS, in up to 20% of women.[120] P–aHUS can occur as often in the first pregnancy as subsequent pregnancies. Numerous women at–risk therefore decided not to become pregnant. Here, we demonstrate that the risk of pregnancy in women predisposed to complement dysregulation may be too pessimistic. P–aHUS occurred in <20% of pregnancies in the setting of additional potential precipitants, whilst the burden of preeclampsia and HELLP appeared lower than appreciated. Rare variants in complement genes did not predict the course of a given pregnancy.

The clinical course of P–aHUS resembles primary atypical HUS and has been linked to the first pregnancy in ~50% patients,[120] suggesting a high burden of complicated pregnancies in women with primary atypical HUS. Previous studies, however, did not report on uncomplicated pregnancies in detail. In our study, the incidence of P–aHUS as well as preeclampsia and HELLP appeared lower than anticipated. Gaggl and colleagues corroborated our findings,[149] indicating that uncomplicated pregnancies are common among women predisposed to complement dysregulation, including those with pathogenic variants in complement genes. Most of these variants have been linked to complement dysregulation on the endothelium and require a precipitating factor[12] before primary atypical HUS can manifest. We confirm that rare variants per se cannot predict the risk of P–aHUS in a given pregnancy, underscoring the key role of additional precipitants, such as bleeding and hypertension.[149]

Rare variants in complement genes were found in half the patients with primary atypical HUS, identical to two large registries.[8,9] DNA testing of genes encoding complement proteins showed that variants can also be found in women with preeclampsia[150] and HELLP,[114,151] although conflicting results have been reported.[101,205] Most of these studies, however, report on variants in complement genes of either uncertain or no significance, overestimating the prevalence of disease causing variants.[89] Moreover, preeclampsia or HELLP may develop in pregnant women on eculizumab treatment.[116,206] Placental release of antiangiogenic factors, such as soluble Fms–like tyrosine kinase 1, appeared more relevant for both conditions to develop.[207]

It should be emphasized that women with preeclampsia or HELLP may in fact

Table 3. Fetal outcome of the 39 pregnancies.

Patient no.	P	P–aHUS	Year	Outcome	Sex	Delivery	Weight, g	Gestational wk
M00503	1	–	2000	Live birth	F	Vaginal	2,480†	38
	2	–	2001	Live birth/died from IRDS (3 d)	M	Vaginal	890	26+2
	3	+	2002	Live birth	M	Vaginal	2,975	37
M00004	1	–	1982	Live birth	F	Vaginal	2,450†	40
M00105	1	–	2004	Live birth	M/M	Vaginal*	2,655/2,580	36+5
M04306.P	1	–	1966	Live birth	M	ND	ND	ND
M04010	1	–	2009	Live birth	M	Vaginal	3,640	40
M04813	1	–	2002	Live birth	M	Vaginal*	3,435	39
	2	–	2005	Live birth	M	Vaginal	3,600	Full term
	3	–	ND	Provoked abortion (14 wk)				
	4	–	2007	Live birth	M	Vaginal	3,290	Full term
	5	–	ND	Spontaneous abortion (ND)				
	6	+	2013	Live birth, IUGR	M/M	Vaginal*	1,120†/1,300†	33+0
	7	–	2014	Live birth, IUGR	F	CS*	1,001†	31+2
M02715	1	–	2011	Live birth	M	CS*	1,460†	31+5
M01416	1	–	1973	Live birth	M	Vaginal	Normal	Full term
	2	–	1974	Live birth	M	Vaginal	Normal	Full term
	3	–	ND	Spontaneous abortion (6 wk)				
	4	–	1978	Live birth	M	Vaginal	Normal	Full term
M04516	1	–	1997	Live birth	M	Vaginal	Normal	39
	2	–	1999	Live birth	M	Vaginal	Normal	38
	3	–	2004	Spontaneous abortion (13 wk)				
	4	–	2005	Live birth	M	Vaginal	Normal	42
M08316	1	+	2016	Live birth	F	Vaginal	3,255	39+5
M06518	1	–	1969	Live birth	M	Vaginal	Normal	Full term
	2	–	1970	Live birth	M	Vaginal	Normal	Full term
	3	–	1975	Live birth	F	Vaginal	Normal	Full term
M06019	1	+	2019	Live birth/died from asphyxia (4 d)	M	CS	Normal	Full term
B01	1	–	2005	Live birth	M	CS*	1,500†	32+0
	2	+	2016	Live birth	F	CS*	1,250†	31+3
B06	1	+	2017	Live birth	F	CS*	2,350	35+6
B12	1	–	2016	Live birth	M	Vaginal	2,675	35+5
B26	1	–	1971	Live birth	M	Vaginal	3,500	Full term
	2	–	1978	Live birth	F	Vaginal	3,200	Full term
B39	1	–	2014	Live birth	M	Vaginal	3,885	42+0
	2	–	2017	Live birth	F	Vaginal	3,370	38+0
B46	1	+	2018	Live birth	F	CS*	1,380	31+0
B00.RI	1	–	2012	Live birth	F	Vaginal	2,850	39+2
	2	–	2013	Live birth	F	Vaginal	3,060	40+2

*Induced labor or CS. †Small for gestational age, defined as a birth weight below the 10th percentile for gestational age.

CS, caesarean section. IRDS, infantile respiratory distress syndrome. IUGR, intrauterine growth restriction. P, pregnancy.

have P–aHUS. This is particularly the case in patients with severe kidney disease not improving after delivery. In one–third of patients with P–aHUS, ESKD can develop within 3 months after presentation,[120] contrasting the low risk of ESKD

associated with preeclampsia.[208] Kidney tissue specimens can aid the differential diagnosis as acute TMA and, in particular, glomerular thrombosis, favor a diagnosis of P–aHUS.[209] The correct recognition of patients with P–aHUS is of utmost importance given the potential benefit of therapeutic complement inhibition.[13-15]

The outcome of pregnancies appeared favorable, although the long–term kidney outcome resembled primary atypical HUS with high rates of ESKD.[8,9] Management of pregnant women with primary atypical HUS or asymptomatic relatives has not been delineated in current guidelines.[202] Prophylactic plasma infusions have been proposed during pregnancy,[149] identical to thrombotic thrombocytopenic purpura.[147] Prophylactic treatment, however, is debatable as pregnancy is a predictable event, the penetrance of primary atypical HUS in normal pregnancy is low, and the typical occurrence in the postpartum period. Eculizumab, however, has been proven safe, both for mother and child,[116] and effective[120,145] for the treatment of P–aHUS. Pregnancy is therefore not contraindicated in women predisposed to complement dysregulation, although close and careful monitoring in centers of expertise is warranted for at least 3 months after delivery. In patients with active disease, eculizumab should be immediately available. These data, however, cannot be extrapolated to patients diagnosed with primary atypical HUS and sequalae, such as hypertension and CKD. Future prospective studies are therefore needed to optimize the management of women predisposed to complement dysregulation.

Placentation and immunologic adaptation of the mother are key processes for pregnancy to succeed. *In vivo* studies linked complement dysregulation to growth restriction and fetal loss.[48,49] Most of the newborns, however, were appropriate for gestational age. Of note, placental complement regulation depends on membrane–bound CD55 and CD59.[210] Both proteins have not been implicated in the mechanism of primary atypical HUS, suggesting a normal fetomaternal crosstalk.

In conclusion, our data emphasized the high frequency of successful pregnancies in women predisposed to complement dysregulation. Rare variants in complement genes cannot be used to predict the risk of a given pregnancy as additional potential precipitants are often needed for P–aHUS to manifest.

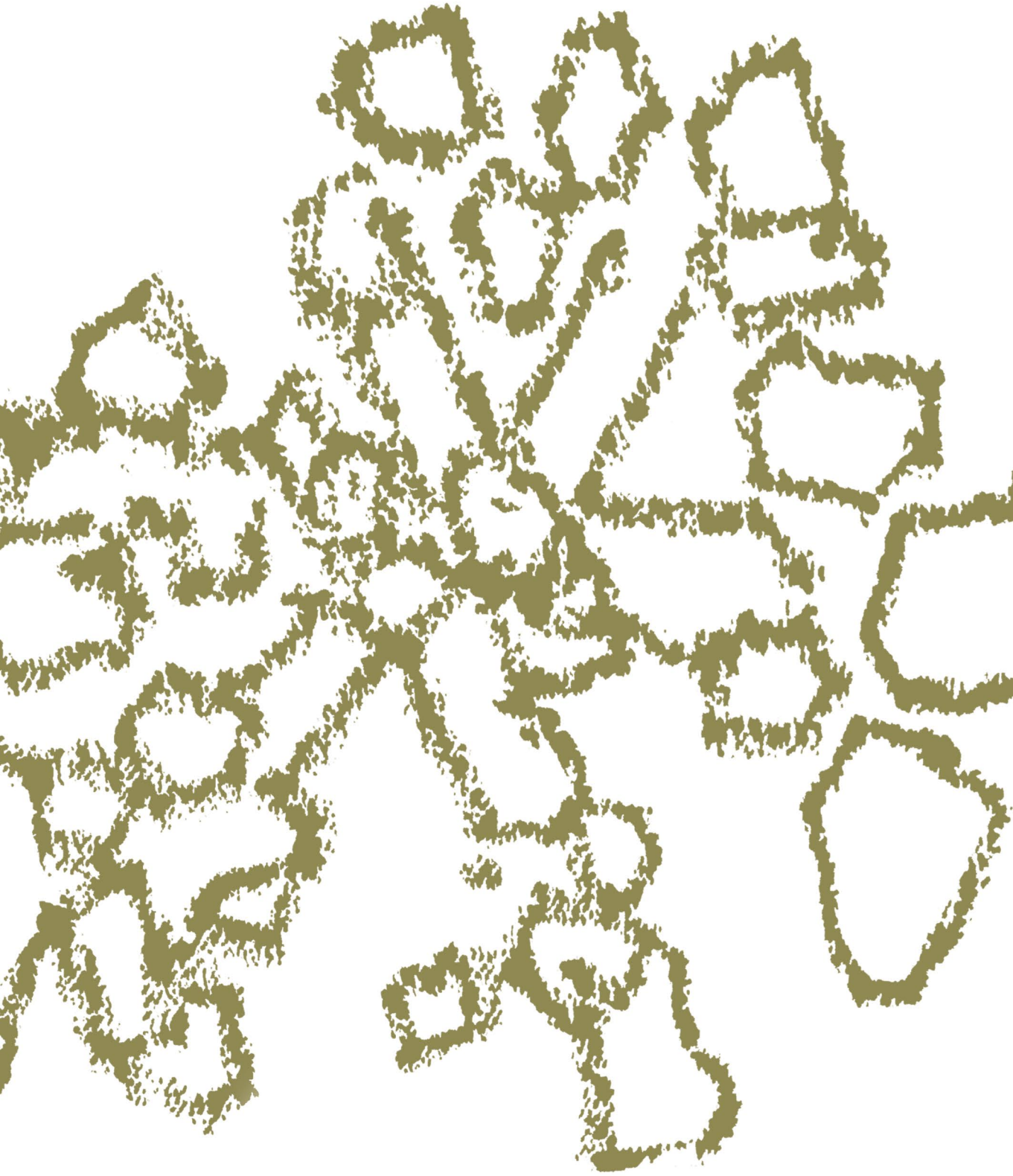
Item S1. Patients' disease course.

Patient no.	Age, yr	CAC	SCr, µmol/L	MAHA	Platelets, $\times 10^9$/L	*Ex vivo* C5b9, % control	Variant(s)	Treatment	Follow–up,* yr	ESKD/ relapse
Primary atypical HUS prior to first pregnancy										
B39	25	–	469	+	7	301	*C3*[2]	PEX	3.5	–/–
P–aHUS										
M00503	32	P	1,388	–	212	366	*C3*[1]	PEX	15.1	+/+
M04813	30	P	240	+	92	295	–	–	5.6	+/–
M08316	28	P, bleeding	492	+	47	366	–	PEX, Ecu	2.9	–/–
M06019	30	P	>500	+	36	317	–	PEX, Ecu	1.0	–/–
B01	37	P	194	–	32	424	–	PEX	2.0	–/–
B06	29	P	559	+	25	238	–	PEX	0.6	–/–
B46	31	P	557	+	77	338	*CFI*[2]	PEX, Ecu	0.8	+/–
Primary atypical HUS after last pregnancy										
M00004	49	–	>800	+	<150	ND	*CFI*[1], *C3*[1]	PEX	14.2	+/+
M00105	38	HE	1,730	–	228	310	*C3*[1]	–	11.4	+/+
M04010	32	HE	1,138	+	142	339	*CFH*[1]	PEX	8.6	+/–
M02715	28	HE	1,065	–	228	373	*C3*[1]	PEX	3.8	+/–
M01416	72	HE	356	+	75	245	–	PEX, Ecu	2.7	–/–
M04516	44	HE	649	–	339	198	–	PEX, Ecu	2.6	+/–
M06518	74	Surgery	375	+	24	364	–	PEX, Ecu	0.6	–/–
B12	31	–	359	+	23	ND	*CFH*[1]	PEX, Ecu	1.3	–/–
B26	72	–	321	–	246	202	–	–	3.3	–/–

*Follow–up after the first episode of primary atypical HUS. Rare variants in complement genes were classified as [1](likely) pathogenic or [2]uncertain significance.

CAC, complement amplifying condition. Ecu, eculizumab. ESKD, end–stage kidney disease. HE, hypertensive emergency. HELLP, hemolysis, elevated liver enzymes, low platelets. MAHA, microangiopathic hemolytic anemia. ND, not determined. P, pregnancy. PEX, plasma exchange. SCr, serum creatinine.

Summary and general discussion



10

Recent travels (summary)

The kidney donor recipient with a diagnosis of “hypertensive” end–stage kidney disease (ESKD) who presented with graft failure in the kidney from his mother, as presented in **Chapter 1**, intrigued me because morphologic features of chronic thrombotic microangiopathy (TMA), identical to those found on native kidney biopsy, developed in the graft. Blood pressure, however, was well–controlled after kidney transplantation, suggesting a mechanism independent of hypertension. The genetic studies, requested a decade after the patient had progressed to ESKD, indicated hereditary complement–mediated (C–)TMA rather than secondary atypical hemolytic uremic syndrome (HUS) related to hypertension. During the patient’s disease course, none of the attending physicians considered C–TMA because systemic hemolysis, as seen in primary atypical HUS (i.e., prototypic C–TMA), was not present. Thus, if one assumes that coexisting conditions reflect the etiology of disease, C–TMA can be missed, having impact on treatment and prognosis in the era of complement–specific drugs. We therefore studied the role of complement dysregulation along the spectrum of so–called “secondary” atypical HUS.

This thesis illuminates that complement dysregulation is common in a subset of patients with TMA, coexisting conditions, and severe kidney disease not responding to standard of care and/or relapsing disease, resembling primary atypical HUS. This, in particular, is the case in patients with coexisting hypertensive emergency, pregnancy, and *de novo* TMA after kidney transplantation. Our studies highlight the need for an updated and true etiology–based nomenclature that makes so much more sense. The term C–TMA was introduced to define patients with TMA on the background of complement dysregulation, either with coexisting conditions or not. The presence of hereditary and/or acquired factors can inform the risk of TMA recurrence in patients with C–TMA. We developed and validated a specific serum–based *ex vivo* test that enables us to recognize complement dysregulation on the endothelium. This *ex vivo* test facilitates the identification of C–TMA and may guide treatment decisions and monitoring during follow–up. Thus, our data are a first step to pursue precision medicine.

HYPERTENSIVE EMERGENCY AND C–TMA

The incidence of hypertensive emergency, defined as impending or progressive target organ dysfunction secondary to severe hypertension, has declined over the last decades. Also, the prognosis has improved from a “malignant” disease, with mortality rates up to 80% within 2 years from diagnosis[211] to a 10–year survival of >95%.[74] Kidney disease, both acute and chronic, however, has been linked to morbidity and mortality.[212] Most patients with acute kidney injury (i.e., ~75%) respond to rapid blood pressure control, whereas ~20% patients progress to ESKD.[74] Patients with microangiopathic hemolytic anemia had highest levels of

serum creatinine (median of 690 versus 120 µmol/L),[79] pointing to TMA as a potential factor associated with ESKD.

The hypothesis that complement dysregulation, pathognomonic for C–TMA, is key for poor outcomes and, in particular, ESKD, was tested in a pilot cohort, including 9 patients with TMA and coexisting hypertensive emergency (**Chapter 2**). Patients invariably presented with severe kidney disease; all but 1 patient required dialysis despite rapid blood pressure control. Most patients presented without profound hematologic abnormalities and thus, a diagnosis of so–called malignant nephrosclerosis was inferred. Notably, genetic studies demonstrated a high prevalence (i.e., *n*/*N*=6/9, 67%) of pathogenic variants in complement genes linked to C–TMA.

Patients with C–TMA who start eculizumab early have the best possible chance to recover kidney function.[13,14] Genetic studies, however, are time–consuming and lack sensitivity; thus, the decision to start treatment should not await genetic test results.[11] Routine complement measures, such as C3, soluble C5b9, and functional assays, also lack sensitivity and specificity.[51,67,86] Therefore, a specific serum–based *ex vivo* test using endothelial cells was developed to recognize patients with complement dysregulation in the earliest possible stage of disease (**Chapter 3**). Patients with TMA, coexisting hypertensive emergency, and severe kidney disease often presented with massive *ex vivo* C5b9 formation, whereas normal *ex vivo* C5b9 formation was found in patients with dense deposit disease (i.e., complement dysregulation in the fluid phase)[213] and patients with biopsy–proven arterionephro–sclerosis not presenting with hypertensive emergency. Thus, *ex vivo* C5b9 formation reflects the dynamics of complement activation on the endothelium (i.e., solid phase). At the time of quiescent disease, *ex vivo* C5b9 formation normalized on the resting endothelium; pre–incubation with adenosine diphosphate that causes endothelial perturbation[51] resulted in massive *ex vivo* C5b9 formation, indicating that a precipitating factor is needed for unrestrained complement activation to occur. Serum samples from patients treated with eculizumab attenuated C5b9 to form. *Ex vivo* C5b9 formation on the endothelium can therefore aid the recognition of C–TMA and may guide treatment during follow–up.

Next, additional patients were recruited from the Cliniques universitaires Saint–Luc, Brussels, Belgium, to study diagnostic and risk factors for complement dys–regulation in patients with TMA, coexisting hypertensive emergency, and severe kidney disease (**Chapter 4**). Again, profound hematologic abnormalities appeared uncommon, underscoring the need for a kidney biopsy to detect the TMA. Neither morphologic nor immunologic features on kidney biopsy, however, can be used to define etiology. Massive *ex vivo* C5b9 formation was found in 68% tested patients and associated with rare variants in complement genes. Also, patients with massive *ex vivo* C5b9 formation seem to benefit from eculizumab, with a renal response in

most of the treated patients. It is important to stress that *ex vivo* C5b9 formation reflects the dynamic process of complement on the endothelium, whereas rare variants in complement genes indicate the predilection for disease. Pathogenic variants in complement genes, indeed, were associated with relapsing disease. Thus, assessment of *ex vivo* C5b9 formation and screening for rare variants in complement genes can better categorize the TMA into different groups with therapeutic and prognostic implications.

Two independent cohort studies of patients with TMA and coexisting hypertensive emergency showed a high prevalence of rare variants in complement genes (i.e., ~55%) and moreover, eculizumab induced a renal response in >70% treated patients,[97,119] validating our observations.

THE RECOGNITION OF C–TMA

C–TMA has been linked to poor outcomes, with high rates of ESKD and TMA recurrence,[8,9] whereas most patients with secondary atypical HUS respond to treatment directed towards the coexisting condition.[139] Based on our clinical experience in patients with TMA and coexisting hypertensive emergency, the hypothesis that complement dysregulation is key for poor (kidney) outcomes in patients with “secondary” atypical HUS was tested in a well–defined cohort of 65 patients with TMA (**Chapter 5**). At baseline, massive *ex vivo* C5b9 formation on the endothelium was associated with severe kidney disease, rare variants in complement genes, and a favorable response to eculizumab, validating that the *ex vivo* test can aid the recognition of C–TMA in patients with coexisting conditions. Massive *ex vivo* C5b9 formation was common in the setting of hypertensive emergency, pregnancy, and, to a lesser extent, *de novo* TMA after kidney transplantation. Most of these patients, indeed, did not respond to standard of care and rapidly progressed to ESKD, whereas eculizumab appeared effective and prevented ESKD in 86% patients. Prolongation of eculizumab’s interdose interval appeared to block *ex vivo* C5b9 formation on the perturbed endothelium and prevented TMA recurrence despite a functional activity of the classical pathway >10% (recommended activity for complement inhibition, <10%),[11] corroborating data from Giuseppe Remuzzi’s group.[92] Thus, the *ex vivo* test, when performed in a specialized laboratory, facilitates the identification of C–TMA in patients with coexisting conditions, guides treatment, and helps to monitor patients during follow–up. As expected, TMA recurrence was associated with rare variants in complement genes. This study provides a rationale for an updated nomenclature on TMAs, as is discussed later.

Patients with TMA and coexisting autoimmunity were not studied in **Chapter 5**. Patients with the antiphospholipid syndrome (APS), characterized by thrombotic and/or obstetric complications with persistent antiphospholipid autoantibodies, may

present with TMA. Murine data suggested that complement, at least partly, is involved in the development of APS–related TMA.[128] Preliminary data showed that patients' serum induced C5b9 formation on hybrid endothelial cells that lack glycosylphosphatidylinositol–anchored complement regulatory proteins (i.e., CD55 and CD59) and corresponding complement–depending cell killing.[129] Variants in complement genes (classified as benign or of unknown significance) were found in some patients. From a kidney point of view, however, ESKD is uncommon in patients with APS–related TMA[107] as compared to those with C–TMA not treated with therapeutic complement inhibition,[8,9] suggesting a pathogenic mechanism not linked to complement dysregulation. We therefore studied the role of complement dysregulation in APS–related TMA (**Chapter 6**). *Ex vivo* C5b9 formation on the perturbed endothelium did not differ from controls, kidney tissue sections did not show complement deposits, and the disease course differed from C–TMA (i.e., 12 [92%] out of 13 patients with APS–related TMA stabilized and/or improved kidney function without therapeutic complement inhibition), excluding complement dysregulation. The non–complement fixing IgG2, a "thrombotic" subclass of anti–β2 glycoprotein–1 and anti–cardiolipin autoantibodies,[132,133] was found on the endothelium after serum incubation; neither IgG1 nor IgG3 were found. Therefore, anti–phospholipid autoantibodies may exert direct effects on the endothelium[134] and/or cause so–called annexin A5 resistance,[135] leading to thrombophilia. Standard of care, that is, anticoagulation either with immunosuppressive drugs or not, should therefore be started instead of therapeutic complement inhibition. The life–threatening catastrophic APS (~1% patients with APS), defined as (microvascular) thrombosis in at least 3 organs that develop in less than 1 week, with mortality >40%[214] may be an exception as case reports suggest that add–on therapeutic complement inhibition offer survival benefit.[215-218] Prospective (un)controlled studies, however, are needed to test this premise.

Patients with C–TMA typically present with acute features of TMA on kidney biopsy, whereas a small subset of patients may present with chronic features of TMA, that is, double contour formation of the glomerular basement membrane. In general, the clinical phenotype of patients with chronic TMA is poorly understood.[11] Murine data showed that a C3 gain–of–function protein (i.e., p.Asp1115Asn) drives chronic rather than acute TMA with heavy proteinuria.[32] The C3 gain–of–function protein (p.Arg161Trp) is prevalent in the Limburg Renal Registry's C–TMA cohort and has been associated with nephrotic–range proteinuria in more than half the patients,[66] suggesting chronic damage. The genotype–phenotype correlation was studied to better understand the etiology and disease course of patients with chronic TMA (**Chapter 7**). C3 p.Arg161Trp and probably other C3 gain–of–function proteins commonly present with morphologic features of chronic TMA and heavy proteinuria, often with normal platelets (11 [73%] out of 15 events). This is particular the case in

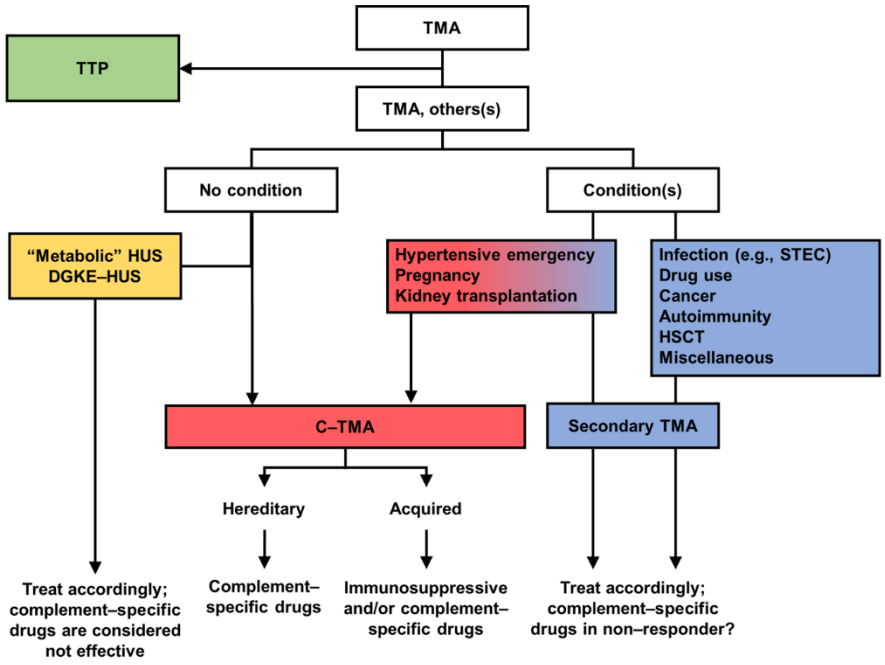
"late" TMA recurrence after kidney transplantation; all patients progressed to graft loss. Morphologic features on kidney biopsy and the clinical course of disease resembled the so–called C3KI mice. C5 inhibition rescued affected C3KI mice with chronic TMA.[32] Prospective controlled studies are needed to test whether patients with chronic C–TMA in isolation benefit from therapeutic complement inhibition or not. To state the obvious, early recognition is of utmost clinical importance. Proteinuria >1 g/day, although aspecific, may be a marker of TMA recurrence and should prompt a diagnostic work–up.

Taken together, our studies provide a rationale to update HUS International's nomenclature on the TMAs,[10,11] focusing on the correct recognition of complement dysregulation in patients presenting with coexisting conditions (**Chapter 8**). Moreover, HUS indicates hemolysis and uremia, whereas profound hematologic abnormalities can be lacking in patients with "organ–limited" TMA and coexisting conditions. Thus, primary atypical HUS, indicating a diagnosis of exclusion, should be replaced by C–TMA to improve the recognition of complement dysregulation in patients with TMA and coexisting conditions. C–TMA should be considered in patients with TMA and severe kidney disease not responding to standard of care and/or relapsing disease. Provocative studies, including our observations,[101,105,140] demonstrated that C–TMA is prevalent in patients with coexisting hypertensive emergency,[97,119] pregnancy,[120,145] and *de novo* TMA after kidney transplantation.[39] In contrast, C–TMA is uncommon in patients with other coexisting conditions.[100] Patients with TMA, excluding thrombotic thrombocytopenic purpura, at higher risk for C–TMA should be screened for unrestrained endothelium–restricted complement activation, rare variants in complement genes, and autoantibodies that inhibit complement regulation to better categorize the TMA (Figure 1). (Of note, patients with thrombotic thrombocytopenic purpura and severe kidney disease may present with complement dysregulation.[219]) Rare variants in complement genes and, to a lesser extent, factor H autoantibodies inform the long–term prognosis. This information should be added to the classification, that is, hereditary and acquired C–TMA. Our retrospective analyses suggest that patients with severe kidney disease not responding to standard of care and, in particular, those with massive *ex vivo* C5b9 formation, should be selected for therapeutic complement inhibition, either with eculizumab or other complement–specific drugs under development. We therefore initiated the "*Complement Prospective Evaluation of TMA on the Endothelium*" (COMPETE; NCT04745195) study to test this premise in patients with TMA and coexisting conditions.

IMPROVING PATIENT CARE BEYOND THE TMA

In women (~20%), pregnancy is an important precipitating factor for C–TMA to manifest.[47] C–TMA often develops late in pregnancy or postpartum,[120] whereas

Figure 1. Pragmatic approach to diagnosis and treatment of TMA.



Patients with TMA should be tested for the enzymatic activity of ADAMTS13 (i.e., >10% excludes thrombotic thrombocytopenic purpura [TTP]). Patients with a normal activity of ADAMTS13 should be screened for coexisting conditions. Of note, patients with coexisting hypertensive emergency, pregnancy, or *de novo* TMA after kidney transplantation may have C–TMA rather than secondary TMA. Most patients with no coexisting conditions have C–TMA, although primary TMA related to recessive variants in *DGKE* and metabolic causes should be considered in children.

DGKE, diacylglycerol kinase epsilon. HSCT, hematopoietic stem cell transplantation. STEC, Shiga toxin–producing E. *coli*.

thrombotic thrombocytopenic purpura is more common in the second and third trimester[147] due to a rise in von Willebrand factor multimers.[220] The counseling of women predisposed to complement dysregulation, both patients and asymptomatic carriers of rare variants in complement genes, who wish to start pregnancy is difficult as data are scarce. Numerous women at–risk therefore decided not to become pregnant. Maternal and fetal outcomes were studied to better understand the natural course of pregnancy in women predisposed to complement dysregulation (**Chapter 9**). The risk in such women appeared to be too pessimistic as C–TMA occurred in <20% pregnancies, often in the setting of additional precipitants. Of note, all but 1 patient had normal kidney function prior to pregnancy. Eculizumab recovered kidney function in all but 1 treated patient, corroborating previous studies.[120,145] Rare variants in complement genes per se cannot predict the risk of C–TMA in a given pregnancy.

Also, the burden of preeclampsia and HELLP (hemolysis, elevated liver enzymes, low platelets) appeared lower than anticipated. Previous studies linked HELLP[114,151] and, to a lesser extent, preeclampsia[150] to (rare) variants in complement genes. Four patients with HELLP from the Limburg Renal Registry presented with mild–to–moderate acute kidney injury (serum creatinine ranged from 54 to 227 μmol/L) and normal *ex vivo* C5b9 formation on the perturbed endothelium, 1 of whom had a variant of unknown significance identified in *CFHR2* (data not shown); after delivery, kidney function recovered in all without sequalae. In contrast to C–TMA, ESKD–related to preeclampsia and HELLP is uncommon,[208,221-223] most variants in complement genes are of unknown significance, and morphologic features reflect defects in vascular endothelial growth factor's function, that is, endotheliosis,[207] rather than thrombi. Moreover, preeclampsia and HELLP have been reported in pregnant women treated with eculizumab.[116,206] Thus, whether preeclampsia and HELLP fall within the spectrum of complementopathies remains debatable.

Pregnancy should be considered individually and carefully planned in women predisposed to complement dysregulation. Monitoring for at least 3 months after delivery is warranted in centers of expertise. In patients with active disease, eculizumab should be immediately available. Future studies need to assess the risk of pregnancy in women with a history of C–TMA and sequalae, such as hypertension and chronic kidney disease.

11

New roads ahead (impact paragraph)

In the era of complement–specific drugs, the diagnostic approach of patients with thrombotic microangiopathy (TMA) should focus on the recognition of complement–mediated (C–)TMA in the earliest possible stage of disease. C–TMA, although considered a diagnosis of exclusion (i.e., primary atypical hemolytic uremic syndrome),[10,11] appeared prevalent along the spectrum of TMA and, in particular, among patients presenting with coexisting hypertensive emergency, pregnancy, and *de novo* TMA after kidney transplantation.[101,140] Our understanding of deregulated or excessive complement activation in patients with TMA and coexisting conditions highlights the need for a new and practical approach to diagnose C–TMA. Most patients with C–TMA, either with coexisting conditions or not, present with severe kidney disease not responding to standard of care and/or relapsing disease. It is important to stress that systemic hemolysis can be absent in up to 60% patients with coexisting conditions, requiring a kidney biopsy to detect the TMA.[140] *Ex vivo* C5b9 formation on the resting endothelium (i.e., HMEC–1 test), as described in this thesis, appears a promising method to diagnose C–TMA and thus, select patients for treatment with complement–specific drugs. Our data provide the background for the "*Complement Prospective Evaluation of TMA on the Endothelium*" (COMPETE; NCT04745195) study. The aim of this prospective observational cohort is to study the prevalence of C–TMA in patients with TMA and coexisting conditions. The application of the HMEC–1 test or other (high–throughput) methodologies is imperative to select patients for treatment. The COMPETE cohort will be used to assess the HMEC–1 test's performance for the diagnosis and monitoring of C–TMA.

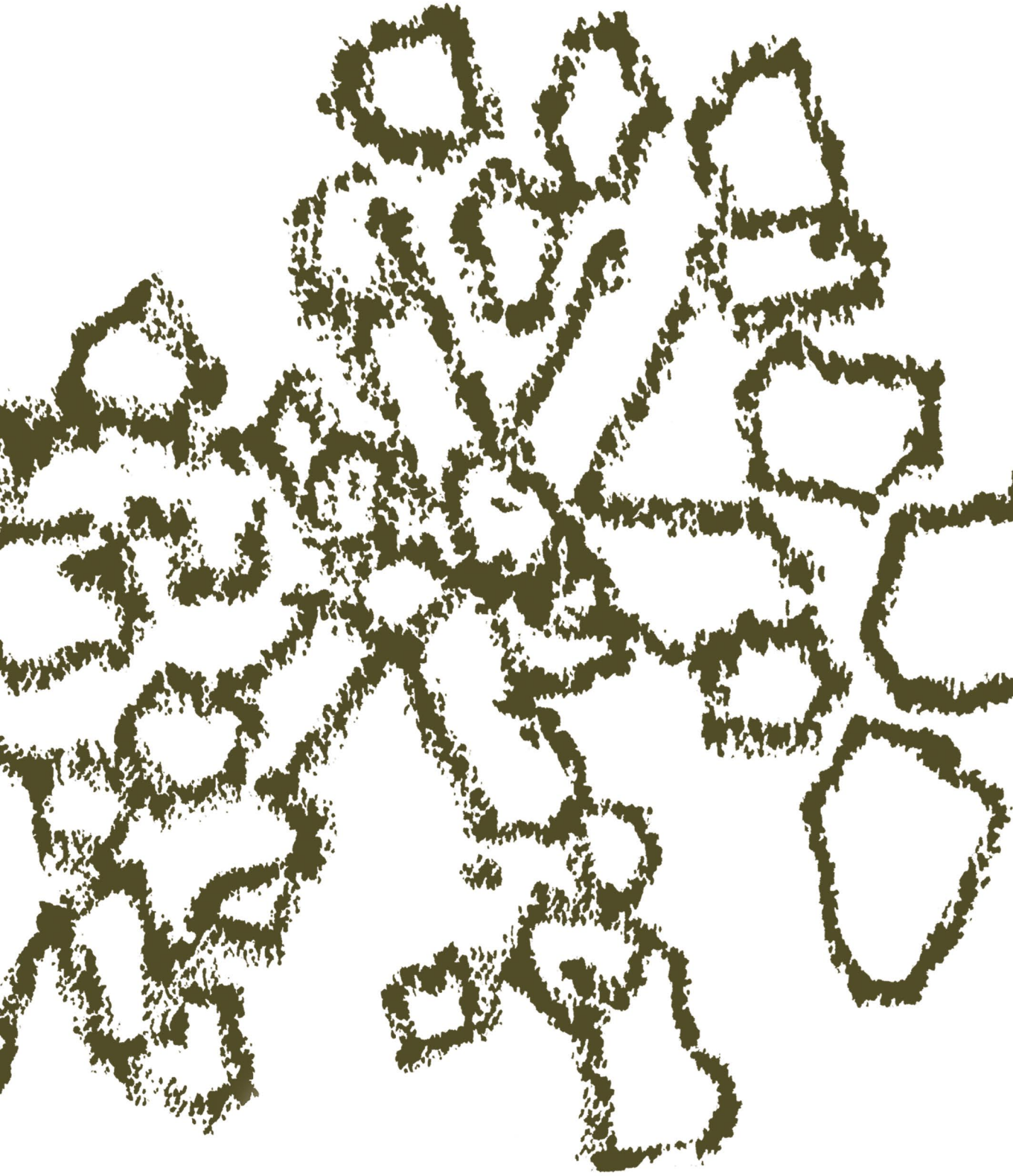
Therapeutic C5 inhibition, that is, eculizumab[13-15] or ravulizumab,[224,225] revolutionized the treatment of C–TMA. None of the clinical trials, however, included patients with TMA and coexisting conditions. Observational cohorts, including those from Maastricht and Brussels,[140] indicate that patients with TMA and coexisting conditions may benefit from therapeutic C5 inhibition. Of note, many of the responding patients had C–TMA rather than secondary TMA. A randomized, placebo–controlled, trial (ALXN1210–TMA–315; NCT04743804) will evaluate ravulizumab's efficacy in 100 adult patients with TMA and coexisting conditions, including but not limited to hypertensive emergency and kidney transplantation. The results of this long–awaited trial will aid the discussion whether or not therapeutic C5 inhibition should be used for the treatment of secondary TMA. Newer complement–specific drugs targeting complement activities upstream of C5 (e.g., C3, Factor D, Factor B) are under development, providing a choice in therapeutic target, modality, and route of delivery in the next few years. I hope that with the advent of newer compounds on the horizon, there will be an opportunity to study complement–specific drugs more widely and that the cost of this class of drugs will be more affordable.

The optimal dosing and treatment duration remains to be established. Our

observations indicate that TMA recurrence is common in hereditary C–TMA. To date, the safety of eculizumab discontinuation has been studied in a single prospective cohort, including patients with C–TMA treated for at least 3–6 months who achieved a complete clinical response (i.e., estimated GFR >60 mL/min/1.73m^2).[226] TMA re–occurred in patients with hereditary C–TMA but not in those without a germline complement gene variant, confirming retrospective observations.[227-229] Thus, eculizumab discontinuation appears to be reasonable and safe in patients with normal complement genetics and no renal sequelae, while a subset of patients with hereditary C–TMA [11] and, in particular, those with chronic kidney disease, may require long–term treatment. Less–intensive treatment, such as prolongation of eculizumab's interdose interval as guided by the HMEC–1 test,[92,140] potentiates a lower (economic) burden of disease and warrants further evaluation. Also, it remains to be established whether or not less–intensive treatment is safe in kidney donor recipients because the allograft's capacity to recover is limited.[96]

Altogether, recent advances have clearly changed the landscape of TMAs. Knowledge on complement dysregulation has enabled breakthroughs in diagnosis and treatment of C–TMA. Further progress can be expected in the coming years and will pave the road to our ultimate goal of precision medicine.

Appendices



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Nederlandse samenvatting voor de niet–ingewijde lezer

De titel “The syndromes of thrombotic microangiopathy (TMA): towards a true etiology–based approach” geeft aan dat het mechanisme van ziekte (in dit geval TMA) centraal dient te staan in de benadering van patiënten, zoals blijkt uit de studies die dit proefschrift hebben vormgegeven. Enige kennis van de geschiedenis helpt om de resultaten in het juiste perspectief te plaatsen.

TMA bestaat uit de woorden trombus (stolsel) en microangiopathie (ziekte van de kleine vaten), hetgeen beschrijft wat de in New York gevestigde patholoog Eli Moscowitz één eeuw geleden onder de microscoop zag. De kleine vaten, zoals haarvaten, zijn onder andere verantwoordelijk voor het transporteren van zuurstof naar het weefsel. Elk orgaansysteem beschikt over dergelijke kleine vaten. Het ontwikkelen van een trombus in zo’n vat (TMA) kan daarom verscheidene gevolgen hebben. TMA in de nier leidt veelal tot een ernstige nierinsufficiëntie, omdat trombosering van de “filters” (ook wel glomeruli) leidt tot een onvermogen om (voor)urine te produceren. Zonder behandeling blijven de “filters” verstopt, waardoor terminaal nierfalen met noodzaak tot nierfunctie vervangende therapie kan ontstaan. Naast ernstige orgaanschade presenteren patiënten zich veelal met lage bloedplaatjes en bloedarmoede; trombosering leidt namelijk tot verbruik van bloedplaatjes en een turbulente stroming van bloed, waardoor rode bloedcellen makkelijk stuk gaan. De aanwezigheid van deze trias is vrijwel diagnostisch voor TMA, echter zijn deze afwijkingen in een minderheid der gevallen aanwezig (~40%).

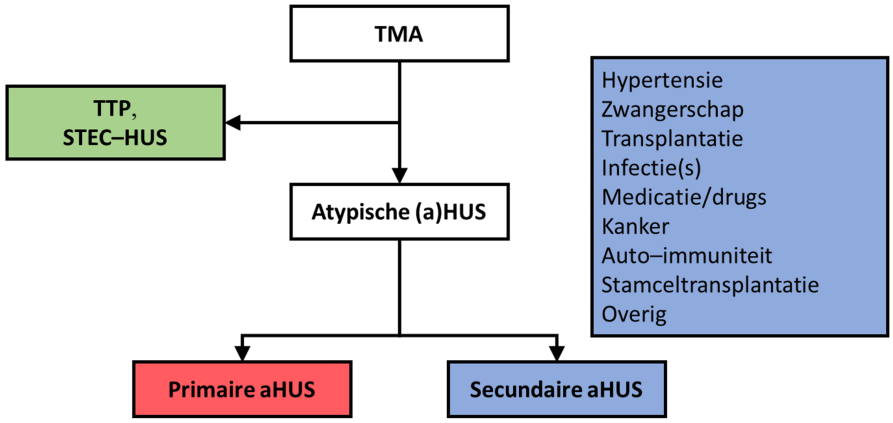
Eli Moscowitz suggereerde dat het ziektebeeld werd veroorzaakt door een krachtig vergif. Inmiddels weet men dat vele oorzaken aanleiding kunnen geven tot het ontwikkelen van TMA (bijvoorbeeld: [maligne] hypertensie, zwangerschap of niertransplantatie). Deze oorzaken, hoewel divers, beschadigen de wand van de kleine vaten, bekleed door endotheelcellen. Een belangrijke oorzaak van TMA werd in de jaren tachtig en negentig ontrafelt: een te actief aangeboren afweersysteem en meer specifiek het complementsysteem. Dit systeem, bestaande uit >30 eiwitten, is actief in het bloed en staat derhalve in nauw contact met de endotheelcellen. Het complementsysteem surveilleert als eerste afweer tegen indringers, zoals bacteriën en virussen. Enkele eiwitten van het complementsysteem binden aan de indringer, waarna een cascade aan reacties in gang wordt gezet. Deze reacties rekruteren en stimuleren witte bloedcellen om de indringer in de eigen cel op te nemen en af te breken. Tevens kunnen bepaalde eiwitten een porie vormen in de celwand en in het bijzonder van bacteriën, waarna de bacterie ten gronde gaat en het gevaar geweken is. Endotheelcellen worden, in tegenstelling tot indringers, normaliter beschermd tegen de vorming van dergelijke poriën. Poriën, mits overmatig gevormd op het endotheel, kunnen aanleiding geven tot trombosering met alle gevolgen van dien. Deze specifieke vorm van TMA, complement gemedieerde (C–)TMA genaamd,

wordt niet zelden veroorzaakt door aangeboren (ook wel genetische) variaties en/of verworven factoren, waardoor de regulatie van het complementsysteem niet goed kan plaatsvinden. Dergelijke genetische variaties en/of verworven factoren leiden uitsluitend tot C–TMA als de balans tussen complement activatie en remming op het endotheel kantelt ten faveure van complement activatie, dit fenomeen wordt aangeduid met de term complement dysregulatie. C–TMA is uiterst zeldzaam en verantwoordelijk voor <5% der TMA gevallen, met jaarlijks 2 nieuwe gevallen per miljoen personen.

De prognose van niet (h)erkende c.q. onbehandelde patiënten met C–TMA is zeer somber en wordt gekenmerkt door terminaal nierfalen en/of sterfte in >50% der gevallen gedurende het eerste jaar na presentatie. Door de introductie van het geneesmiddel eculizumab (een specifieke remmer van het complementsysteem) is de (nier)overleving verbeterd tot >90% en de ziekte behandelbaar geworden. De kostprijs van eculizumab is enorm (minstens €250.000,00 voor een volledig jaar), waardoor het correct selecteren van patiënten voor behandeling uiterst belangrijk is. Er bestaat consensus dat TMA zonder andere aanwijsbare oorzaak per definitie veroorzaakt wordt door een te actief complementsysteem (Figuur 1); C–TMA wordt daarom gezien als een diagnose per exclusionem. Deze benadering, hoewel praktisch, blijkt op basis van studies beschreven in dit proefschrift niet absoluut.

Het eerste deel van deze thesis focust op patiënten met TMA en maligne hypertensie. Hypertensie is als maligne te beschouwen op het moment dat er acute en ernstige orgaanschade optreedt als gevolg van de hoge bloeddruk. TMA is een voorbeeld van vasculaire (orgaan)schade door hypertensie, hetgeen niet zelden gepaard gaat met ernstig nierfalen. Het is goed voor te stellen hoe een hoge bloeddruk door druk c.q. rek op de vaatwand kan leiden tot schade aan het endotheel en dus, TMA en nierfalen kan veroorzaken. Hoewel meerdere dier–modellen deze zogenaamde “druk hypothese” hebben bevestigd, werd deze theorie in de jaren zeventig bekritiseerd door Jan Möhring: “*Pressure, if it is great enough, will eventually disrupt any structure. Obviously, this is also true of blood pressure. It is therefore not surprising that an experimentally induced great increase in pressure disrupts the integrity of the blood–vessel wall.*” Met deze woorden gaf hij aan dat een beter begrip van het onderliggende mechanisme (leidende tot hypertensie en orgaanschade) nodig is. Het is belangrijk om te benadrukken dat nieren een zeer belangrijke rol spelen in de bloeddrukregulatie. Nieren die niet goed doorbloed worden, zoals voor kan komen in het geval van TMA, kunnen op meerdere manieren hypertensie veroorzaken. De aanname dat TMA (en nierfalen) het gevolg is van maligne hypertensie, zoals klinisch veelal gebeurd, kan hypothetisch leiden tot een onderschatting van C–TMA als behandelbare oorzaak van nierfalen. Deze hypothese werd getoetst in een pilot studie (**Hoofdstuk 2**). Patiënten met TMA

Figuur 1. De (mogelijke) oorzaken van TMA en huidige nomenclatuur.



STEC–HUS, Shiga toxine producerende E. *coli* infectie gecompliceerd door het hemolytisch uremisch syndroom. TTP, trombotische trombocytopene purpura.

(bewezen in nierweefsel) en maligne hypertensie werden retrospectief gescreend op genetische variaties en verworven factoren gelinkt aan C–TMA. Zes (67%) van de 9 patiënten, allen met terminaal nierfalen, bleken genetisch gepredisponeerd voor C–TMA. Eén patiënt met terminaal nierfalen werd behandeld met eculizumab, waarna de nierfunctie herstelde. Deze pilot studie maakte onze hypothese aannemelijk. Na uitbreiding van het cohort tot 26 patiënten hield de hypothese stand (**Hoofdstuk 4**); bovendien zijn de resultaten door onafhankelijke groepen gevalideerd. Kort samengevat blijkt dat complement dysregulatie/C–TMA en niet maligne hypertensie de oorzaak is van ziekte in ~50% der gevallen; dergelijke patiënten werden vaak (onder)behandeld zonder eculizumab met alle gevolgen van dien.

C–TMA is daarom géén diagnose per exclusionem. Het vroegtijdig opsporen en behandelen van C–TMA is onontbeerlijk. Een robuuste test om C–TMA op te sporen is noodzakelijk, temeer omdat screening op genetische variaties en/of verworven factoren in de praktijk 6–8 weken duurt en een negatieve uitslag de diagnose niet verwerpt. C–TMA gaat ten tijde van actieve ziekte gepaard met een te actief complementsysteem op het endotheel. De dynamiek van het complementsysteem kan men visualiseren door opgekweekte endotheelcellen bloot te stellen aan serum (compartiment van bloed met complement eiwitten) gedurende enkele uren om nadien de mate van complement activatie te kwantificeren middels het kleuren van “poriën” (**Hoofdstuk 3**); de zogenaamde HMEC–1 test. Massale complement activatie (gebaseerd op de HMEC–1 test) is geassocieerd met de aanwezigheid van genetische variaties en een ernstig klinisch beloop, overeenkomend met C–TMA en

niet maligne hypertensie als oorzaak van TMA. Normale complement activatie (gebaseerd op de HMEC–1 test) is daarentegen indicatief voor TMA veroorzaakt door maligne hypertensie getuige de afwezigheid van genetische variaties en een goede respons op bloeddrukregulatie (**Hoofdstuk 3**, **4**); routine complement testen in bloed en nierweefsel bleken niet geschikt om dit onderscheid te maken.

De eerder beschreven resultaten werden in het tweede deel van deze thesis geëxtrapoleerd naar TMA op basis van andere oorzaken (Figuur 1), ook wel secundaire TMA genaamd. De dynamiek van het complementsysteem werd middels de HMEC–1 test bestudeerd in een cohort van 65 patiënten met TMA, waarvan het merendeel zich presenteerde met een aanwijsbare oorzaak (secundaire TMA; **Hoofdstuk 5**). Het serum van patiënten geclassificeerd als secundaire TMA bleek in ~60% der gevallen massale complement activatie te induceren op gekweekt endotheel suggestief voor C–TMA. Een aanzienlijk deel van deze patiënten met massale complement activatie had genetische variaties en/of verworven factoren aantoonbaar (40% versus 0% in het geval de HMEC–1 test normale complement regulatie suggereert) en bovendien kwam het klinisch beloop overeen met C–TMA (ernstige nierinsufficiëntie en hoog recidief risico); de nierfunctie stabiliseerde en/of herstelde pas nadat patiënten werden behandeld met eculizumab, dat de rol van complement dysregulatie verder onderschrijft. De “secundaire oorzaak” bleek meestal maligne hypertensie, zwangerschap of niertransplantatie. Het (h)erkennen van complement dysregulatie als oorzaak van de TMA heeft een belangrijke impact op de behandeling en prognose, zoals reeds bediscussieerd. Het cohort van 65 patiënten met TMA bleek niet representatief voor TMA gepaard gaande met auto–immuunziekten. Het antifosfolipiden syndroom (APS), een auto–immuunziekte geassocieerd met trombosering en TMA, bleek niet geassocieerd met massale complement activatie c.q. complement dysregulatie (**Hoofdstuk 6**). APS wordt gekenmerkt door autoantistoffen gericht tegen onder andere het endotheel, waardoor trombosering kan ontstaan; de binding van deze autoantistoffen aan het endotheel konden we middels een variant op de HMEC–1 test visualiseren. APS patiënten werden behandeld met bloedverdunners en/of immunosuppressieve medicatie, maar géén complement gerichte therapie. De nierfunctie stabiliseerde en/of verbeterde in het overgrote deel der gevallen. Er bestaat (zeer waarschijnlijk) géén plaats voor complement gerichte behandeling in het geval van APS.

Hoofdstuk 8 beschrijft de resultaten van een kritisch literatuuronderzoek betreffende de rol van complement dysregulatie in het geval van secundaire TMA, want zoals Albert Einstein zei: “*A man should look for what is, and not for what he thinks should be*.” De meeste studies dateren uit het laatste decennium en tonen vergelijkbare resultaten als vergeleken met de Maastrichtse observaties. Een substantieel deel van TMA patiënten met maligne hypertensie, zwangerschap en

niertransplantatie ontwikkelt ziekte op basis van complement dysregulatie (ergo, C–TMA). C–TMA wordt klinisch gekenmerkt door ernstige nierinsufficiëntie en hoog recidief risico; de nierfunctie stabiliseert en/of herstelt veelal na het starten van complement gerichte behandeling. Er bestaat echter géén onderbouwing om aan te nemen dat complement dysregulatie een rol speelt in het geval de TMA bestaat op basis van een (bacteriële) infectie, medicatie, kanker, auto–immuunziekte, stam–celtransplantatie of andere oorzaak; dergelijke casuïstiek betreft veelal een acute niet–recidiverende ziekte, die per definitie als secundair beschouwd dient te worden. De HMEC–1 test kan helpen om C–TMA (vroegtijdig) van secundaire ziekte te differentiëren, zoals reeds beschreven. De aanduiding hereditair en verworven kan men gebruiken om de aanwezigheid van respectievelijk genetische variaties en verworven factoren te benadrukken. Het is belangrijk om de ziekte in het licht van dergelijke factoren te benaderen (**Hoofdstuk 7**), daar het recidief risico c.q. prog–nose hiermee samenhangt.

Het gros van de onderzoeken, incluis de studies die dit proefschrift hebben vormgegeven, hebben een retrospectief karakter. De COMPETE studie, een prospectief cohort onderzoek, loopt in Maastricht om de rol van complement (dysregulatie) in verscheidene TMA's nader te bestuderen.

Het derde en laatste deel van deze thesis is voortgekomen uit een regelmatig terugkerende vraag van vrouwelijke patiënten (**Hoofdstuk 9**). Hoe groot is het zwangerschapsrisico ten aanzien van de moeder en het (ongeboren) kind? Het is goed om te beseffen dat 20% van de vrouwelijke C–TMA patiënten voor het eerst ziek wordt tijdens of kort na de zwangerschap. In totaal hebben we 39 zwangerschappen van 19 vrouwen gepredisponeerd voor C–TMA geanalyseerd; vrouwelijke patiënten met C–TMA en asymptomatische draagsters van genetische variaties (zonder ziekte) werden geïncludeerd. Het risico op C–TMA tijdens of kort na de zwangerschap bleek <20%, dat minder pessimistisch is dan gedacht. Nagenoeg alle patiënten hadden een goede nierfunctie tijdens de zwangerschap, waardoor het niet mogelijk is de observaties te extrapoleren naar vrouwen met chronische nierschade (bijvoorbeeld: na eerdere C–TMA activiteit). Eculizumab stabiliseert en/of herstelt de nierfunctie in een groot deel der gevallen en lijkt, op basis van ander onderzoek, veilig voor het (ongeboren) kind. Kortom, er zijn succesvolle zwangerschappen mogelijk.

Het complementsysteem speelt ook een rol in de genese en ontwikkeling van de placenta. Een (te) actief complementsysteem ter hoogte van de placenta leidt in muizen tot een lagere kans op succesvolle afloop. ~90% van de geobserveerde zwangerschappen bleek succesvol. Vroeggeboorte, veelal gelinkt aan zwanger–schapsvergiftiging, kwam relatief vaak voor.

Het plannen van de zwangerschap is complex doch niet onmogelijk. De

zwangere vrouw dient nauwlettend gemonitord te worden om determinanten van vroeggeboorte en complicaties, incluis C–TMA, vroegtijdig te detecteren en behandelen. Het staat buiten kijf dat dit in een expertisecentrum dient te geschieden.

Zoals de titel van dit proefschrift impliceert is begrip van het mechanisme leidende tot TMA onontbeerlijk om gerichte therapie te initiëren. C–TMA dient men niet te zien als een diagnose per exclusionem, daar dit (onnodig) tot terminaal nierfalen en andere complicaties kan leiden. De HMEC–1 test heeft potentie om de diagnose C–TMA te bespoedigen, hoewel men dit prospectief dient te bevestigen. Het is goed mogelijk dat de HMEC–1 test daarnaast gebruikt kan worden om complement gerichte behandeling te titreren. De COMPETE studie gaat nieuwe inzichten opleveren om deze vraagstukken te beantwoorden. Het is tevens goed om te weten dat nieuwe geneesmiddelen gericht op het complementsysteem de behandeling van C–TMA in de nabije toekomst zullen uitbreiden en bovendien, kosten reduceren. Het is en blijft dan ook een enerverende tijd!

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Curriculum vitae

Sjoerd A.M.E.G. Timmermans was born on October 14, 1990, in Sittard, The Netherlands. He attended pre–university education at the Connect College Echt (previously known as Bisschoppelijk College Echt) and graduated in 2009, after which he studied medicine at Maastricht University. Before obtaining his bachelor's degree, intrigued by the kidneys and immune system, he started studying the role of anti–phospholipase A2 receptor autoantibodies in primary membranous nephropathy using a cohort from the Limburg Renal Registry, which he combined with medical school.

After obtaining his medical degree with honors in 2015, he started studying the role of complement along the spectrum of thrombotic microangiopathy, as described in this thesis, under supervision of Dr. Pieter van Paassen (Dept. Nephrology and Clinical Immunology, Maastricht UMC), Prof. Dr. Chris P. Reutelingsperger (Dept. Biochemistry, Cardiovascular Research Institute Maastricht), and Dr. Jan G.M.C. Damoiseaux (Central Diagnostic Laboratory, Maastricht UMC). He received 2018's young investigator award of the Dutch Federation of Nephrology. As of January 1, 2019, he started as a medical resident in internal medicine at the Maastricht UMC and continued research. During this clinical and scientific journey, he also published on small vessel vasculitis, immune–mediated kidney disease, and Covid–19 and served as a guest editor for the Journal of Clinical Medicine.

Currently, he continues medical training in nephrology and clinical immunology at the Maastricht UMC, combined with research.