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Chapter 7

Summary and general discussion
SUMMARY AND GENERAL DISCUSSION

On the definition of thrombotic microangiopathy

Patients presenting with thrombotic microangiopathy (TMA) may provide a wide range of physicians with diagnostic and therapeutic challenges: the differential diagnosis of TMA is broad and includes multifactorial diseases affecting multiple organs; the clinical, laboratory or pathological features can be insufficiently sensitive or specific; advanced diagnostic tests may be unavailable; overlapping diagnostic criteria can complicate the diagnostic procedure; and even among patients with the same diagnosis, such as atypical hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), the clinical presentation, course, and outcome are variable.\textsuperscript{1-10} It is important to overcome these challenges because rapid identification of not only the presence of TMA, but also of the underlying mechanism is required for targeted treatment, which can be life-saving and may prevent irreversible renal failure in some of these patients.\textsuperscript{5,11-13}

It has to be noted, however, that the term TMA is used with slightly different connotations in different contexts. As was mentioned in the introduction of this thesis (Chapter 1), TMA is used as a term which refers to a syndrome characterized by thrombocytopenia, microangiopathic hemolytic anemia and signs of organ injury (systemic TMA, also described as clinical TMA);\textsuperscript{5, 6, 14} as a term which refers to a histopathologic pattern of lesions (morphologic TMA) which would typically be present in the affected organs of a patient with systemic TMA, but can also be observed in the absence of such clinical features (local TMA);\textsuperscript{15-19} and as an ‘umbrella term’ for a broad category of disorders that can present with evidence of severe microvascular endothelial injury and microthrombi (the thrombotic microangiopathies; TMAs).\textsuperscript{3, 9, 20, 21} It is likely that systemic and local TMA are manifestations of specific causes, such as mutations in complement regulatory genes, deficiencies in A Disintegrin-like And Metalloprotease with ThromboSpondin type 1 motif, member 13 (ADAMTS-13) or the presence of antiphospholipid antibodies, which can be found if they are meticulously looked for, but are not be routinely tested in all patients.

These issues should be taken into consideration in the evaluation of
the patient groups with TMA described in this thesis. Several patients were diagnosed with ‘HUS’, ‘atypical HUS’ or ‘HUS/TTP’, rather than TTP, even if the underlying causes that define these diseases, such as the levels of ADAMTS-13 or genetic deficiencies of complement regulatory proteins, were not examined in the clinical setting. These diagnoses likely reflect the historic association between HUS and the clinical predominance of renal manifestations, as well as the contemporary difficulties in distinguishing HUS from TTP. Furthermore, as all patients described in this thesis were selected from the renal pathology databases, there was a selection bias towards morphologic TMA. Patients who, for various reasons, did not undergo renal biopsy were not included in our studies. This may have excluded patients with a straightforward clinical presentation, a self-limiting course, and patients with contraindications to renal biopsy, such as children with Shiga toxin-producing Escherichia coli-associated HUS (STEC-HUS), patients with TTP who lack renal manifestations, or patients with an unacceptably high risk of bleeding due to thrombocytopenia.

Throughout the study period of this thesis, various definitions of morphologic TMA have been suggested, but at the present time, no gold standard exists to identify morphologic TMA. Although the characteristic light microscopic and ultrastructural features of morphologic TMA are well documented, the definition of morphologic TMA remains based on expert opinion and there is no consensus on the criteria that could be used to define the histopathologic spectrum of lesions. Like in many other fields of immunopathology, what is regarded as TMA is influenced by inter-observer variation and local practices. A survey among pathologists diagnosing renal biopsies with morphologic TMA has shown that there are differences in the use of stains, histopathological criteria, value attribution to clinical features and family history, and availability of advanced diagnostic tools such as genetic analysis of complement proteins, ADAMTS-13 levels, and electron microscopy.

The setting of research may also give nuances to the definition of TMA and this is the reason why different definitions of morphologic TMA have been used in the work described in this thesis. In the work described in Chapter 2, we focused on active lesions. The definition of TMA in Chapter 2 required the presence of one or more microthrombi obstructing vessel lumina on renal biopsy and patients typically had multiple microthrombi throughout the renal tissue specimen. Concomitant chronic microangiopathic lesions were also observed.
For example, eight cases also had a duplicated glomerular basement membrane, and organizing microthrombi with recanalization were occasionally observed. In the work described in Chapter 3, we aimed to compare our results to findings by El Karoui et al, who described that 53% of biopsies with IgA nephropathy had morphologic TMA. In their study, TMA was described as “acute TMA”, defined by the presence of fibrin deposits, or as “organized TMA”, which did not require microthrombi but was defined by the presence of “evident fibrosis and recanalization and narrowing of the lumen at the arterial and arteriolar levels”. We made a similar distinction between acute and chronic cases, but we followed the recent conclusions from the “Kidney Disease: Improving Global Outcomes” (KDIGO) Controversies Conference. Consequentially, we used the term ‘microangiopathy’ rather than ‘TMA’ and distinguished cases with active and chronic lesions. In the work described in Chapter 4, we focused on chronic glomerular changes in the setting of transplant glomerulopathy. We included a control group of native biopsies diagnosed with chronic morphologic TMA which had glomerular basement membrane duplication; several cases also had microthrombi in glomeruli, arterioles, or both, but these lesions were not systematically studied.

The grey area of morphologic TMA includes biopsies with local TMA, biopsies with a single microthrombus, and biopsies with chronic microangiopathic lesions that lack microthrombi. Several patients described in this thesis had local TMA, and the microangiopathic lesions of the cases described in Chapter 3 were generally more difficult to identify than the microangiopathic lesions in the cases with disseminated microthrombi, as described in Chapter 2. Nevertheless, the findings described in Chapter 3 suggest that patients with local microangiopathy can have a substantial risk for disease progression, even if the lesions are scarcely present. In comparison with ‘full-blown’ morphologic TMA, scarcely present microangiopathic lesions could possibly reflect a less severe phenotype, an earlier stage of a disease process, or sampling error. Local TMA has been described in relation to disease progression in several kidney diseases, including lupus nephritis, anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis, and diabetic nephropathy. In a study among kidney transplant recipients, de novo local TMA was compared to systemic TMA. In nearly all patients with local TMA, renal function improved with reduction, conversion, or temporary discontinuation of calcineurin inhibitors,
and patients with local TMA had a better short-term prognosis than patients with systemic TMA. However, local TMA was not benign: patients with local TMA also had a worse prognosis than patients without TMA, and long-term graft survival of grafts with local or systemic TMA was similarly poor. In a study on biopsies from deceased donor kidneys taken before engraftment, the presence of fibrin thrombi was a significant risk factor for the development of reduced graft function at 6 months after transplantation and graft loss within the first year after transplantation.\textsuperscript{36} Two other studies had a longer follow-up period and found no significant differences in renal outcome after 1 year of transplantation between cases with and without microthrombi in donor kidneys.\textsuperscript{37, 38} Although these studies may differ with regard to the clinical setting, study population, causal mechanism, and therapeutic management, the findings suggest that local TMA is an important risk factor for disease progression among various kidney diseases, but that this injury is potentially reversible.

In the studies described in this thesis, microangiopathy with or without thrombosis was scored as a histopathological pattern. Key questions that remain unanswered include: which of the individual microangiopathic lesions are clinically relevant and which criteria should define TMA? Which lesions indicate further examination of risk factors such as deficiencies in genes encoding complement regulatory proteins or ADAMTS-13 levels? What is the optimal treatment strategy for patients with local TMA? To what extent do active and chronic microangiopathic lesions share morphological features, signaling pathways, and mechanisms of injury and repair among patients with different diseases? Differences in the definitions of TMA limit the comparison between the cases reported across individual studies. Therefore, there is room for improvement. Refined definitions may lead to clearer scientific dialogue, improved accuracy for diagnosis and prediction of prognosis, a better understanding of the etiologies underlying similar manifestations, and advances in therapeutic management. For these definitions to be useful, there is a need for consensus among pathologists on what establishes morphologic TMA, and a need for multidisciplinary studies on the clinical outcome and response to therapy in patients with systemic and local TMA, integrating genetic profiles, clinical features, laboratory parameters, and tissue pathology in both native and allograft tissue samples.
On complement-mediated microangiopathy

The work described in this thesis provides evidence for the involvement of complement activation in the development or progression of various renal microangiopathies. Compared to control groups, complement deposits along the renal microvasculature, including C4d deposits, were observed more frequently in patients with various renal diseases that presented with systemic or local TMA (described in Chapters 2, 3, and 4), transplant glomerulopathy (described in Chapter 4), preeclampsia, (described in Chapter 5) and diabetic nephropathy (described in Chapter 6). Our findings demonstrate that complement activation has taken place along the renal microvasculature of these patients, as demonstrated by the presence of C4d which is a cleavage product that follows from C4 activation that remains covalently bound to the surrounding tissue long after complement-pathway initiating factors have dissociated. Deposits of C5b-9 were also observed in approximately 75% of patients with microangiopathy with or without thrombosis and diabetic nephropathy, suggesting that complement activation via any of the complement pathways proceeded to the formation of terminal complement complexes in these patients. Nevertheless, we also found C5b-9 deposition in a substantial proportion of control samples, which complicates the clinical use of this staining and will be discussed below. Our findings suggest that activation of the lectin and classical pathways could possibly contribute to the development of renal microvascular injury. Furthermore, our data suggest that in a subset of patients, complement-mediated microangiopathy is involved in disease progression, as renal complement deposits were correlated with clinical progression towards end-stage renal disease (described in Chapter 3), or the severity of histopathological lesions (described in Chapters 3, 4 and 6). We hypothesize that these patients are particularly susceptible to complement-mediated microangiopathic injury due to defective complement regulation and that complement-modifying therapy could be beneficial to a wider group of patients than is currently recognized. However, the molecular mechanisms underlying our findings remain to be determined. Future studies need to address whether complement activation along the renal microvasculature is a cause or consequence of injury, if complement-mediated microvascular injury is related to an underlying genetic or acquired defect in diseases characterized by endothelial injury or dysfunction, and if complement staining patterns are
useful in the workup of patients who may benefit from complement-inhibiting therapy.

The work described in Chapter 2 is focused on the role of complement activation in kidneys from patients with various renal diseases, including atypical HUS, who presented with morphologic TMA in the renal biopsy specimens. The data described in Chapter 2 show that C4d deposition is associated with the presence of microthrombi in renal biopsies from patients with various renal diseases that presented with systemic or local TMA. Complement deposits along the renal microvasculature were studied in 42 renal tissue samples obtained from a heterogeneous patient group with morphologic TMA in the renal biopsy and in 53 renal control samples without TMA. C4d deposits were observed in nearly 90% of the kidneys with TMA, indicating that complement activation took place in most kidneys with microthrombi, regardless of the underlying clinical condition. To explore which complement pathway could have led to C4d deposition and whether complement activation led to C5b-9 deposition, deposits of mannose-binding lectin (MBL) (lectin pathway), C1q and IgM (classical pathway), and C5b-9 (terminal pathway) were studied. In most patients, evidence for classical pathway activation was observed, but we also observed evidence for lectin pathway activation. C5b-9 deposits were observed in more than 75% of kidneys with TMA, suggesting that complement-inhibiting therapy could be beneficial for these patients.

The findings on complement deposition in the controls described in Chapter 2 warrant discussion. The control group consisted of healthy control samples without TMA from donor kidneys that were unsuitable for transplantation because of technical deficits, and biopsies from patients with focal segmental glomerulosclerosis (FSGS), Alport syndrome, and kidney transplant recipients with a variety of lesions other than antibody-mediated rejection. C5b-9 deposits were significantly more prevalent in kidneys with TMA than controls, and co-localized with microthrombi. However, C5b-9 deposits were also observed in 40% of controls, predominantly in arterioles. This suggests that the presence of C5b-9 staining alone cannot discriminate cases with microthrombi from cases without microthrombi. In contrast, C4d deposition was infrequently observed in controls, suggesting that a combination of C4d and C5b-9 could possibly be useful in the diagnostic workup of patients with TMA because it could help identify lesions that may have been missed.
otherwise, and possibly indicate patients with complement-mediated TMA. C4d deposits were not observed in any of the controls, except for 3 of the 19 control patients with FSGS. In routine diagnostics, complement deposits in FSGS are typically considered a consequence of non-specific entrapment in sclerotic lesions.\textsuperscript{40} However, our cases also had staining in glomerular segments that appeared non-sclerotic. This observation is consistent with recent studies demonstrating that IgM and C3 deposits can be observed in unaffected glomeruli of patients with primary FSGS,\textsuperscript{41} and that plasma and urine levels of complement factor Ba, C4a, and sC5b-9 are significantly higher in patients with primary FSGS than control groups.\textsuperscript{42} Following the observations described in Chapter 2, we studied the glomerular deposition of C4d in native biopsies obtained from patients with primary FSGS, allograft biopsies from patients with native FSGS who would develop recurrent FSGS, and the Munich Wistar Frömter rat model of FSGS. We found that glomerular C4d deposition is associated with FSGS lesions.\textsuperscript{43} Moreover, we found that glomerular C4d deposition can precede the development of FSGS and indicate early lesions of FSGS. These data suggest that complement activation is involved in the pathogenesis of FSGS.

The study described in Chapter 2 validates previous findings from our group indicating that C4d deposition can be useful to identify morphologic TMA in renal biopsies from patients with systemic lupus erythematosus (SLE) and patients with antiphospholipid syndrome.\textsuperscript{25} Antiphospholipid antibodies and immune-complexes in lupus nephritis can activate the classical pathway, causing endothelial injury and local inflammation.\textsuperscript{44, 45} Complement activation has been recognized as an important mechanism for antiphospholipid syndrome-associated thrombosis in experimental models and in patients.\textsuperscript{45} Others have shown that renal TMA in patients with SLE was associated with poor renal outcome, and that renal outcome was even poorer among SLE-associated TMA patients with glomerular C4d deposition and decreased serum levels of complement factor H.\textsuperscript{32, 46} These data suggest that activation of both the classical and alternative pathways of complement might be involved in the development of TMA in these patients. It is possible that immune-complexes or auto-antibodies activated the classical pathway, but that uncontrolled alternative pathway activation amplified endothelial cell injury. The hypothesis that complement can be involved in SLE-associated TMA is also supported by case
reports describing the efficacy of the anti-C5 monoclonal antibody, eculizumab, and the anti-inflammatory and anticoagulant agent recombinant human soluble thrombomodulin, in patients with lupus nephritis-associated TMA; these findings are promising because some of the patients described in these case reports were unresponsive to conventional therapy.\textsuperscript{46-50}

A similar mechanism of antibody-induced and complement-mediated injury might be responsible for a subset of patients with \textit{de novo} TMA in kidney transplant recipients. A study on \textit{de novo} TMA following kidney transplantation found a mutation in complement factor H, factor I, or both in 29\% of patients with \textit{de novo} TMA, and in none of the control kidney transplant recipients or healthy controls, suggesting that a subset of patients with \textit{de novo} TMA in the renal allograft may be particularly susceptible to complement-mediated injury.\textsuperscript{51} Several case reports describe a beneficial effect of complement-inhibiting therapy in kidney transplant recipients with \textit{de novo} TMA, including patients with antibody-mediated rejection as well as drug-induced TMA that is refractory to other treatments.\textsuperscript{52,53} Future studies need to address the role of complement activation in the development of \textit{de novo} TMA in allograft kidneys and develop optimal therapeutic guidelines for \textit{de novo} TMA after kidney transplantation.

The mechanisms of endothelial injury in TMA following hematopoietic stem cell transplantation (HSCT-TMA) are poorly understood. HSCT recipients may be exposed to multiple endothelial insults, including chemotherapy, radiation, infections, and calcineurin inhibitors to prevent graft-versus-host-disease.\textsuperscript{54} We observed C4d in the arterioles of patients with HSCT-TMA. Other studies on renal biopsies from HSCT-recipients reported similar findings and suggested that arteriolar C4d deposition might be useful to discriminate HSCT-TMA from other causes of renal injury in HSCT recipients.\textsuperscript{54-60} Given that HSCT-TMA has been associated with graft-versus-host disease,\textsuperscript{61} it is possible that complement-mediated TMA is a manifestation of chronic graft-versus-host disease in HSCT recipients.

In most patients with TMA, C4d deposits were associated with C1q and IgM deposits, and we observed co-localization of these deposits on sequentially sectioned kidney samples. These data suggest that the classical pathway was activated, possibly via the IgM subtype. We found similar associations in patients with microangiopathic lesions in the setting of IgA nephropathy (IgAN) or IgA vasculitis with nephritis (IgAVN), preeclampsia, and diabetic
nephropathy (described in Chapters 3, 5 and 6). We hypothesize that endothelial damage triggered the binding of natural IgM antibodies to injured cells, thereby activating the classical pathway and leading to C1q and C4d deposition. Natural IgM antibodies play an important role in the clearance of damaged cells by binding to hypoxic, necrotic, and apoptotic cells through intracellular antigens that become externalized under these conditions. As was reviewed previously, natural IgM antibodies can prevent harmful autoimmune reactions from damaged cells by generating an immunoregulatory milieu and promoting the resolution of inflammation, in part by activating the complement system.

Furthermore, the complement system may also be activated by antibody-independent mechanisms in renal microangiopathies. Experiments have shown that endothelial cells that were stimulated in vitro by inflammatory cytokines or high levels of shear stress can activate the classical pathway up to the formation of C5b-9 by demonstrating the deposition of C4d and C5b-9 on endothelial cells, independent of IgM or IgG. Another in vitro study demonstrated that complement activation could be initiated by exposure to subendothelial extracellular matrix, independent of endothelial cell activation. Similarly, platelets that were activated either by chemical means or shear stress were found to induce activation of the alternative pathway involving P-selectin, and the classical pathway involving C1q binding proteins. It has been suggested that the recruitment, adhesion, and activation of platelets during injury and inflammation is mediated by C1q. Recently, a new complement activation pathway was discovered: in C3-deficient mice, thrombin was demonstrated to cleave C5. Subsequently, activated components of the coagulation cascade have also been shown to activate the complement system: plasmin, FIX, FX, and FXI can activate C3 and C5, activated FXII can activate C1 causing classical pathway activation, and both ficolin and MBL can interact with fibrinogen and fibrin, enhancing lectin pathway activation. Other triggers that could activate the classical pathway in the setting of renal microangiopathies include the acute phase C-reactive protein (CRP), danger-associated molecular patterns (DAMPs), neutrophil extracellular traps (NETs), free-heme following intravascular hemolysis, and molecules that become accessible after cell apoptosis, necrosis, or ischemia.

Data showing that numerous mechanisms can activate the complement
system may complicate the interpretation of our data, but they are likely a realistic representation of complex biological processes. As was reviewed previously, there is a continuous and dynamic interaction in the microvasculature between the endothelium and the inflammation, coagulation, and immune systems. Excessive activation or inadequate regulation of any one of these processes may disturb homeostasis, leading to complement-mediated endothelial injury, or endothelial dysfunction. Regardless of the initiating factor that causes complement activation, complement dysregulation can cause a positive feedback loop that perpetuates endothelial injury. It is possible that a subset of our patients has an increased susceptibility to complement-mediated injury due to inherited or acquired complement defects. Mutations in genes encoding proteins of the complement system can be identified in more than 50% of patient with atypical HUS. These include genes encoding complement factor H, CD46, factor I, C3, factor B, and thrombomodulin. However, disease penetrance is variable in patients with mutations in complement regulatory genes, not all mutations may be pathogenic, and in many patients with atypical HUS, a complement abnormality cannot be identified. A mutation in complement regulatory genes is often a predisposing factor rather than a direct cause of local or systemic TMA, requiring a trigger for atypical HUS. These triggers include autoimmune diseases, transplantation, pregnancy, infections, and drug-toxicity; several of these clinical conditions were also identified in our TMA cohort. Although mutations in complement regulatory genes were documented in a few of our patients as part of their diagnostic workup, we could not determine the causes of complement activation in most of our patients, due to the unavailability of DNA, serum and urinary samples. Therefore, in most of the patients described in Chapter 2, it is unknown if they have mutations in complement regulatory genes, auto-antibodies against factor H, or other auto-antibodies that can cause endothelial injury and complement activation. This limitation also applies to the work described in Chapters 3-6.

The work described in Chapter 3 is focused on complement-mediated microangiopathy in the renal biopsies of patients with IgAN and IgAVN. It was previously shown that in patients with IgAN, disease progression was associated with microangiopathic lesions, and with C4d deposition in separate studies that did not address microangiopathic lesions. These studies and the observations described in Chapter 2, led to the hypothesis that there could be
an intricate relationship between complement activation and microangiopathic lesions in patients with IgAN and IgAVN, possibly denoting a subgroup of patients with a relatively poor clinical outcome. We studied 128 renal biopsies from adult and pediatric patients who presented with IgAN or IgAVN. Biopsies were re-evaluated histologically with particular attention for the presence of microangiopathy with or without thrombosis and stained for C4d, C1q, MBL, and C5b-9. Re-examination of the renal biopsies revealed microangiopathic lesions in 20% of the biopsies, suggesting that microangiopathy, with or without thrombosis, could possibly be overlooked in clinical practice. In line with findings from Chapter 2, the presence of microangiopathic lesions in renal biopsies was associated with the presence of C4d and C5b-9 deposits along the renal microvasculature, with evidence for activation through the classical complement pathway.

Interestingly, in the cohort described in Chapter 3, patients with a combination of C4d positivity and microangiopathy comprised a clinical subgroup with a higher number of chronic lesions, lower eGFR, and poorer renal survival compared to patients without microangiopathy or C4d deposits. These findings provide evidence for the involvement of complement activation in the development of severe microvascular injury and suggest that complement-mediated microangiopathy contributes to disease progression in at least a subgroup of patients with IgAN and IgAVN. These findings are in line with previous studies suggesting that complement activation is involved in the pathogenesis of IgAN and that complement-inhibiting therapy may be promising for several patients with IgAN. However, the predictive value of microangiopathy and C4d deposition in our cohort should not be overstated, as the study was underpowered to examine the incremental effect of these variables in addition to other markers of poor prognosis, including parameters of the Oxford classification. Future multi-center studies on prognostic factors in IgAN or IgAVN should include C4d staining patterns and microangiopathic lesions as parameters.

Possible explanations for complement activation in relation to microangiopathic lesions in IgAN or IgAVN are similar to those described above. In addition, complement activation may result from processes that are intrinsic to the pathogenesis of IgAN and IgAVN and involve the lectin and alternative complement pathway, as was reviewed previously. This may explain why
complement deposition was also observed in patients without microangiopathic lesions. Several studies reported on glomerular C4d deposition in IgAN.\textsuperscript{83, 85, 89, 90} In a study on complement deposition in fresh-frozen renal biopsies of patients with IgAN, C4d deposition was exclusively associated and co-localized with proteins of the lectin pathway.\textsuperscript{85} Other studies on C4d deposition in IgAN assumed lectin pathway activation based on the absence of C1q but did not examine the deposition of lectin proteins.\textsuperscript{83, 89, 90} In contrast, in our study C4d was associated with C1q but not MBL deposition. However, it is still possible that C4d was activated via the lectin pathway because we did not examine ficolins, or mannose-associated serine protease 1-3 (MASP1-3). Similarly, the MBL-negative patients with TMA, preeclampsia, and diabetic nephropathy described in Chapters 2, 5 and 6 may also have had C4d deposition resulting from the lectin pathway. Nevertheless, it is very well possible that C4d deposition reflects classical pathway activation in IgAN. Although evidence for classical pathway activation is infrequently reported in IgAN,\textsuperscript{84} IgG and C1q deposition have been described in association with disease progression and the prevalence of C1q in some cohorts ranges from 10-45\%.\textsuperscript{91-102} It has to be noted that the sensitivity of detecting C1q varies between techniques. For example, C1q is detected more often by immunoperoxidase staining on formalin-fixed, paraffin-embedded tissue than by immunofluorescence staining on fresh-frozen tissue, possibly because fixed tissue detects antigens that may be lost or removed in the washing steps of immunofluorescence staining on unfixed tissue.\textsuperscript{103} Proteomic analysis of laser-captured microdissected glomeruli found that components of the classical pathway C1q, C1r, and C1s were significantly increased in patients with progressive IgAN as compared with non-progressive IgAN.\textsuperscript{104} Taken together, several datasets indicate that C4d is a valuable biomarker for disease progression in IgAN, but in addition to being a footprint of lectin pathway activation, C4d may also reflect classical pathway activation as it does in other areas such as in the setting of renal transplant pathology. Future studies should determine the molecular mechanisms leading to complement activation in IgAN.

The work described in Chapter 4 is focused on C4d deposition along peripheral glomerular capillaries with a duplicated glomerular basement membrane (GBM). In the clinical setting of renal transplantation, C4d along the peritubular capillaries of the allograft kidney is an important biomarker for the diagnosis and classification of antibody-mediated rejection.\textsuperscript{10, 39} In contrast,
the significance of glomerular C4d deposits in the allograft kidney is not fully understood. In Chapter 4, 319 consecutively obtained diagnostic kidney allograft biopsies from 219 patients are described, including 100 follow-up biopsies. C4d staining along the GBM (GBM-C4d) was associated with C4d deposition along the peritubular capillaries, suggesting that GBM-C4d could be a manifestation of antibody-mediated rejection. GBM-C4d was also associated with GBM duplication and subendothelial new lamina densa formation, the defining features of transplant glomerulopathy, even after correcting for parameters that indicate antibody-mediated rejection. We confirmed the association between GBM-C4d and GBM duplication in a setting without donor-specific antibodies by studying native biopsies with GBM duplication in the setting of chronic TMA and native biopsies without GBM duplication in the setting of minimal change disease. The GBM-C4d staining pattern mirrored the degree of GBM remodelling and the staining intensity was correlated with the severity of GBM duplication. By immunogold labeling, C4d deposits were mainly detected in architecturally altered subendothelial zones, as well as under podocytes, possibly reflecting subepithelial capillary wall turnover and restructuring originating from visceral epithelial cells. These findings suggest that in transplant biopsies, pseudo-linear GBM-C4d deposits can be interpreted as etiologic markers for active ongoing antibody-induced renal graft rejection, especially when observed with concurrent C4d deposition along the peritubular capillaries, or as structural markers for microangiopathy-like GBM duplication, independent of antibody-mediated rejection.

Our findings were recently concurred by von der Thusen et al., stating that isolated pseudo-linear C4d along the GBM in renal allograft biopsies frequently marks ultrastructural alterations to the GBM, even if it the staining is scant. They also report that this staining pattern can be the only sign marking the necessity for further electron microscopic analyses that might be skipped otherwise, since significant hematuria and proteinuria may be absent. Graft survival in patients with transplant glomerulopathy is particularly poor and identifying patients at an early stage may be an important step towards preventing transplant glomerulopathy and prolonging graft survival.

The mechanism of complement activation in GBM duplication remains to be determined. C5b-9-induced endothelial injury can cause leakage and exposure of the subendothelial cell matrix, activation of platelet aggregation
and activation of the coagulation cascade; the morphological manifestation of this process can be observed as mesangiolysis and microthrombi.\textsuperscript{107} Sublytic quantities of C5b-9 can induce endothelial cell activation, causing an upregulation of adhesion molecules such as P-selectin, E-selectin, ICAM-1, and VCAM-1, and cytokines such as IL-6, IL-8, and MCP-1.\textsuperscript{77} Endothelial cells exposed to C5b-9 can release heparan sulfate, basic fibroblast growth factor, and platelet-derived growth factor which have been suggested to contribute to the chronic microangiopathic lesions, such as GBM duplication and multilayering of the basal lamina of peritubular capillaries.\textsuperscript{107}

The work described in Chapter 5 is focused on the role of complement activation in kidneys from patients with preeclampsia, a pregnancy-specific microangiopathy that affects 5-7\% of all pregnancies and is a major cause of maternal, fetal, and neonatal morbidity and mortality worldwide.\textsuperscript{8, 20, 108, 109} The pathogenesis of preeclampsia is incompletely understood, but there is compelling evidence that it is caused by an imbalance of circulating angiogenic factors, which results in endothelial dysfunction.\textsuperscript{109} This angiogenic imbalance is caused by increased levels of the anti-angiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin, and by decreased levels of pro-angiogenic factors, such as placental growth factor and vascular endothelial growth factor A (VEGF-A). Recent studies also provide evidence for complement activation in the development of preeclampsia and our group previously showed that patients with preeclampsia have classical pathway activation in the placenta.\textsuperscript{110-114} Based on these findings, we hypothesized that the classical pathway is also activated in the kidneys of women with preeclampsia.

In Chapter 5, we report on the glomerular deposits of complement and immunoglobulins in a unique cohort of renal autopsy samples from 11 women with preeclampsia, 25 pregnant controls, and 14 hypertensive controls who were not pregnant. Preeclampsia was significantly associated with glomerular C4d, C1q, and IgM deposition, suggesting that the classical complement pathway is activated in the kidneys of patients with preeclampsia, possibly via antibody-mediated injury of the IgM-subtype. These findings were validated in the sFlt-1 mouse model for preeclampsia: compared with control-treated mice, sFlt-1 injected pregnant mice had higher serum levels of circulating activated C3 fragments, and significantly more C4-positive glomeruli, which were co-localized with endothelial cells, suggesting that complement activation can result
from endothelial dysfunction caused by an angiogenic imbalance. Together with previously reported studies,\textsuperscript{111-115} the findings described in Chapter 5 suggest that complement activation might contribute to renal injury in preeclampsia and that inhibiting the complement system could possibly reduce both the renal and placental manifestations of preeclampsia.

The experiments in sFlt-1 injected pregnant mice support the hypothesis that complement deposition in renal microangiopathies can result from endothelial dysfunction \textit{in vivo}. As was described in the introduction of this thesis (Chapter 1), sFlt-1 binds to vascular endothelial growth factor (VEGF) and placental growth factor, which reduces the bioavailability of these growth factors for their receptors, and causes endothelial dysfunction. If an angiogenic imbalance is induced, this can manifest as a preeclampsia phenotype with hypertension, proteinuria, and endotheliosis.\textsuperscript{116} The importance of VEGF is also shown in other renal microangiopathies. VEGF promotes angiogenesis and is essential for maintaining vascular integrity.\textsuperscript{117} Homeostasis is required: both overexpression and inhibition of VEGF can cause disease.\textsuperscript{118} Patients who were treated with systemic bevacizumab, a monoclonal VEGF inhibitor, have been reported to develop glomerular TMA and this phenotype could be reproduced in adult mice from a podocyte-specific conditional knock-out model for the VEGF gene.\textsuperscript{119} Likewise, podocyte-specific heterozygosity for VEGF-A results in renal failure, proteinuria, and endotheliosis and homozygous deletion causes perinatal death.\textsuperscript{120} Glomerular TMA has also been demonstrated in patients treated with VEGFR-2 inhibitors sunitinib and sorafenib, and in mice with reduced VEGFR-2 expression.\textsuperscript{121-124} Vice versa, in a rat model of TMA induced by anti-endothelial cell antibodies, VEGF-treatment accelerated renal recovery.\textsuperscript{125} Interestingly, a recent study demonstrated that VEGF increases complement regulator factor H synthesis by glomerular endothelial cells, whereas VEGF inhibition leads to a reduced expression of factor H and to increased glomerular deposition of activated complement components C3d and C4d.\textsuperscript{126} These data suggest that podocytes regulate local complement activation via VEGF in a paracrine way.

The work described in Chapter 6 is focused on the role of complement activation in patients with diabetic nephropathy, a microvascular complication of diabetes mellitus that affects 30-40\% of diabetic patients, making it the leading cause of end-stage renal disease.\textsuperscript{127-129} The presence of C4d, C1q, MBL, and C5b-9 deposits was examined in 159 kidney samples from autopsied diabetic patients
for whom the histopathological presence or absence of diabetic nephropathy was confirmed, and in a control group of 41 non-diabetic patients without renal pathology. Findings were validated in 12 kidney biopsies from patients with diabetic nephropathy and 10 kidney biopsies from healthy living transplantation donors. Kidneys from patients with diabetic nephropathy had a significantly higher prevalence of C4d and C5b-9 deposits in glomeruli and in arterioles than kidneys from diabetic patients without nephropathy, and kidneys from non-diabetic patients without renal disease. Moreover, C4d deposition was correlated with higher classes of diabetic nephropathy and C4d deposition was associated with chronic microvascular and interstitial lesions. These data provide evidence for complement activation along the renal microvasculature in the development of diabetic nephropathy.

Our findings in diabetic nephropathy are in line with recent studies, suggesting that complement activation is involved in the development of microvascular and macrovascular complications of diabetes mellitus, as was reviewed previously.\textsuperscript{130-132} In diabetic nephropathy, several mechanisms of complement activation were suggested. Under normal conditions, MBL and ficolins do not bind to their receptors on cell surfaces; however, hyperglycemia can cause glycation of proteins resulting in the production of advanced glycation end products and the generation of neo-epitopes to which lectin proteins can bind. Although our findings point towards classical pathway activation rather than lectin pathway activation, we cannot exclude that lectin pathway activation led to C4d deposition, as discussed above. Moreover, it was suggested that hyperglycemia can lead to glycation-induced inactivation of complement regulatory proteins such as CD59, which inhibits C5b-9 under physiological circumstances, and can cause C5b-9 induced injury. In our study, the prevalence of C5b-9 deposits was significantly higher in diabetic patients than in non-diabetic controls, but it was similar between diabetic patients with or without diabetic nephropathy. Within the group of patients with diabetic nephropathy, glomerular C5b-9 was correlated with the histologic markers of disease progression. These data suggest that the glycation-induced CD59 inactivation may indeed lead to the formation of C5b-9 as a common mechanism in diabetes mellitus that it is not specific to diabetic nephropathy, but may contribute to disease progression.

As was discussed above, natural IgM antibodies could explain the
association between C4d, C1q, and IgM in our cohort. In addition, diabetic nephropathy is associated with altered VEGF homeostasis and endothelial dysfunction.\textsuperscript{117, 118, 133-135} Hyperglycaemia and hypoxia can cause upregulated VEGF-A expression by the podocyte.\textsuperscript{135} Although VEGF upregulation may be a protective response that may limit endothelial injury and dysfunction, increased VEGF may also enhance the progression of diabetic nephropathy by causing foot process effacement, TGF-β activation and collagen IV synthesis in podocytes and mesangial cells, and inducing mesangial cell proliferation.\textsuperscript{118, 134} Renal VEGF levels are high at early stages but low at advanced stages of diabetic nephropathy in humans and rodent models, possibly due to podocyte dropout.\textsuperscript{135} A recent study showed that low VEGF expression was associated with TMA and end-stage renal disease in patients with diabetic nephropathy.\textsuperscript{34} Combined with the finding that the podocyte may regulate local complement activation using VEGF,\textsuperscript{126} these data suggest patients with severe diabetic nephropathy may have reduced local complement regulation due to lower VEGF levels, and that this contributes to the development of complement-mediated microvascular endothelial injury.
Future perspectives

At the dawn of the 20th century, the significance of the complement system in humans was first recognized. More than a century after this discovery, there has been a profound shift in our perception of the complement system in health and disease. We have come to realize that over the evolutionary course of millions of years, the human complement system has developed an impressive versatility of functions that, by far, exceed the task of eliminating microbes. We have also come to realize that there is a ‘dark side’ of complement activation; complement activation has now been implicated in the pathogenesis of a wide range of disorders. One of the major future challenges will be to unravel the molecular mechanisms by which complement activation causes or aggravates microvascular diseases, as well as the mechanisms by which complement activation orchestrates repair. In addition, new therapeutic strategies are emerging to modulate the complement system, but it is still difficult to identify which patients will benefit from such therapy. Although the complement system is typically shown as a linear cascade of pathways, it is now known to be a dynamic, hub-like network of circulating, cell-surface expressed, and intracellular proteins, which are connected with other physiologic systems. Given the complexity of the complement system, studies addressing complement-mediated microangiopathies may benefit from systems biology approaches and technical advancements in the fields of complement assays and artificial intelligence. These efforts may not only improve our understanding of the pathophysiology of complement-mediated diseases but also help identify complement profiles in individual patients to facilitate patient-tailored therapy.

Complement-mediated microangiopathy in other fields

The findings on complement activation in the renal microangiopathies described in this thesis could be relevant to other renal disorders that are characterized by endothelial injury or dysfunction. For example, Timmermans et al. recently studied a cohort of patients with morphologic TMA attributed clinically to malignant hypertension; however, they found that 67% of patients had mutations in complement genes, concomitant evidence of complement activation in vivo, and poor renal outcome. Moreover, they validated our finding that C4d
deposition is associated with the presence of microangiopathic lesions, as was described in Chapters 2 and 3. Although we did not examine ADAMTS-13 levels, a recent study demonstrated that C4d deposition was also more prevalent in renal biopsies from patients with the Upshaw-Schulman syndrome, a congenital form of TTP associated with loss-of-function mutations in the ADAMTS13 gene than controls. These findings suggest that complement-mediated injury could also be involved in other diseases in which local TMA manifests, such as the recently described patients with microangiopathic lesions in the setting of ANCA-associated glomerulonephritis, diabetic nephropathy, and anti-GBM glomerulonephritis. It would be interesting to determine if the combined presence of complement deposits and microangiopathic lesions could identify patients with a poor prognosis who may benefit from complement-modulating therapy and if these patients have a genetic predisposition to complement-mediated injury. Furthermore, the finding that patients with preeclampsia and diabetic nephropathy have more glomerular complement deposits than controls, suggests that complement activation could be involved in the pathophysiology of various disorders other than TMA, in which endothelial injury or dysfunction is a key component, such as systemic sclerosis, diffuse intravascular coagulation, or variants of FSGS. In addition, both microangiopathic injury and complement-mediated injury are not limited to the kidney. For example, atypical HUS, TTP, systemic lupus erythematosus, and TMA following hematopoietic stem cell transplantation may affect multiple organs such as the lungs, brain, and gastrointestinal tract. Similarly, the microvascular complications of preeclampsia and diabetes mellitus may affect multiple organs. A recent study from our group demonstrated that C4d and C5b-9 deposits were associated with microthrombi in the brains of patients with systemic lupus erythematosus and neuropsychiatric involvement. Therefore, complement activation may also be involved in the development or progression of microangiopathic injury in the extra-renal manifestations of systemic diseases described in this thesis.

Complement-inhibiting therapy
Complement inhibition has greatly reduced morbidity and mortality in patients with atypical HUS, even in patients without demonstrated mutations in complement regulatory genes. Our observations suggest that treatment against components of the complement system could benefit a broad range of
patients with complement-mediated microangiopathy. As described in this thesis, complement activation up to C5b-9 deposition was observed along the renal microvasculature of a heterogeneous group of patients, but not in all, and it is tempting to speculate that patients with severe microvascular injury and complement deposition at the site of injury will benefit most from complement-inhibiting therapy. There is evidence that eculizumab, a monoclonal antibody against C5, can benefit patients with other renal microangiopathies than atypical HUS.\textsuperscript{53, 114, 153-169} Although these studies show encouraging efficacy in a subset of patients, there are important concerns preventing the justification of life-long complement-inhibiting therapy for a broader patient group. These concerns include publication bias, the harmful side effects and risks associated with complement-inhibiting therapy, and the financial impact on health care services. Moreover, for many renal microangiopathies, it is still unclear if complement activation is the primary cause of injury, a disease modifier that can contribute to disease progression, or an ‘innocent bystander’. Therefore, future studies should systematically address the potential use of complement-inhibiting therapy in various renal microangiopathies, including systemic or local TMA, and microangiopathies such as diabetic nephropathy, preeclampsia, and transplant glomerulopathy. Although the standard maintenance treatment of atypical HUS requires life-long eculizumab therapy, discontinuation seems to be possible in some patients with stable remission.\textsuperscript{170} This indicates that in patients with temporary complement-amplifying conditions, such as pregnancy, temporary inhibition could be sufficient to break the vicious cycle of complement-mediated injury. The work described in this thesis suggests that multiple pathways of complement activation could be involved in the pathogenesis of renal microangiopathies. It is, therefore, encouraging that new complement therapies are emerging, targeting various components of the complement system. As was reviewed recently,\textsuperscript{137, 171} these therapies cover an impressive scope of approaches, including local and systemic administration, treating acute, chronic and episodic disorders, focusing on initiation, amplification and effector phases of complement activation, targeting specific activation fragments, and intervening at the protein and gene levels.
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