On the pathology of focal segmental glomerulosclerosis

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General discussion

A paradigm shift – minimal change disease and focal segmental glomerulosclerosis as a spectrum of podocyte disease

A long standing discussion is whether minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are two separate disease entities or different manifestations of the same disease spectrum. Because of the different names and definitions, MCD and FSGS can be diagnosed as separate entities on renal biopsies. However, in biopsies with relatively few glomeruli, pathologists tend to be cautious to use the term MCD, because of the reasonable chance that an FSGS-lesion could have been missed due to sampling error. The clinical findings in MCD and FSGS largely overlap, although patients with FSGS tend to have additional symptoms next to nephrotic syndrome, such as hypertension, mild renal function disturbance and an active sediment.[1] Electron microscopy findings are similar in MCD and FSGS, although there is some evidence that podocyte changes may be more extensive in FSGS.[2] In general, patients with FSGS have a less favorable prognosis compared to patients diagnosed with MCD. Taking into account this difference in prognosis and also in responsiveness to therapy, it is sometimes considered that MCD is the milder and FSGS the more progressive manifestation of the same disease entity. In this section of the general discussion, we provide a detailed overview of the evidence supporting the concept of MCD and FSGS as separate disease entities and the counter hypothesis that both are different manifestations of the same disease process. We will evaluate newly proposed concepts for the diagnosis of MCD and FSGS and discuss new players that could contribute to the progression of MCD to FSGS.

In current textbooks on glomerular diseases, MCD and FSGS are described as separate entities underlying idiopathic nephrotic syndrome, sustained by the apparent difference in clinical presentation and prognosis.[1,3] MCD is currently considered a single disease entity, characterized by overt proteinuria with nephrotic syndrome in the absence of glomerular lesions by light microscopic evaluation with the exception of minimal mesangial expansion in a minority of patients.[3] Complete remission of proteinuria on corticosteroid treatment is almost always achieved and kidney function is usually maintained throughout the course of the disease.[1] FSGS is thought to be a separate disease entity characterized by typical lesions that are absent in MCD. FSGS is associated with a higher probability of steroid resistance and renal function loss compared to MCD.[4] Steroid resistance and renal function deterioration in patients originally diagnosed with MCD raise the possibility of sampling error by which FSGS lesions were missed in the biopsy, and therefore a diagnosis of MCD was erroneously given.

From a clinical perspective, MCD is predominantly a disease of the young.[5] It is the most common cause of nephrotic syndrome in children, whereas FSGS is the most common cause of nephrotic syndrome in adults and far less common in children.[4,6] MCD is occasionally observed in the context of lymphoproliferative disorders in adults, such as Hodgkin and non-Hodgkin lymphomas.[5] FSGS is not associated with lymphoproliferative disorders. It is not clear whether the etiology in these cases of MCD is different from idiopathic MCD.

Although much about the etiology and pathogenesis of both MCD and FSGS is unknown, some claim that MCD has a distinct etiology and pathogenesis. Research on the pathogenesis of MCD has mainly focused on the involvement of T cells, which is remarkable considering how little inflammation is encountered in the renal tissue of these patients. A substantial body of evidence has emerged that implicates different aspects of T cell regulation and dysfunction as drivers of podocyte injury in MCD. This includes an imbalance of T cell populations, cytokine production and activation of T cells by *de novo* expression of T cell costimulatory receptors on podocytes.[5] Moreover, the development of MCD, and in particular idiopathic nephrotic syndrome in children, is strongly associated with respiratory tract infections, implying that MCD is more prone to develop in an environment of T cell activation due to various causes, such as infections, in which the immune system is activated.[1,7] However, none of the associated findings with respect to T cells are unique to MCD and may also overlap with findings in FSGS. For instance, the T cell costimulatory receptor CD80, which was briefly coined as the culprit of MCD disease development,[8,9] later appeared to be overexpressed in FSGS as well.[10] Likewise, upper respiratory tract infections can also trigger relapses in children with nephrotic syndrome due to FSGS.[7] Moreover, the putative circulating permeability factors and cytokines in FSGS, as discussed in the introduction of this thesis, show overlap between the two disease entities.[11] In conclusion, a distinct pathogenetic factor that is exclusively involved in the pathogenesis of either MCD or FSGS has thus far not been identified.

Support for the notion that MCD and FSGS are different manifestations of the same disease process originates both from animal and human studies. Several animal models have been used to study the development of MCD and FSGS. Interestingly, almost all of these animal models have demonstrated that a continuum between MCD and FSGS exists. For instance, puromycin aminonucleoside (PAN) nephrosis has been used as a model for both MCD and FSGS. A single low dose injection of PAN leads to transient podocyte injury and proteinuria, whereas a high dose and repeated injections lead to both proteinuria and the development of FSGS lesions.[12] These dose-dependent effects on FSGS development have not only been observed

in the PAN model, but also in other models of podocyte injury and proteinuria such as the diphteria toxin model.[13] The dose-dependent development of either an MCD or an FSGS phenotype after podocyte injury supports the hypothesis that MCD and FSGS may be part of the same disease spectrum that is characterized by initial podocyte injury. In addition to models in which FSGS is induced later in life, models in which proteinuria and FSGS spontaneously develop are also available, such as the buffalo/mna rat model and the Munich Wistar Frömter (MWF) rat model.[14-16] In both models, rats undergo a stage that resembles human MCD prior to the development of FSGS lesions. In conclusion, all the models seem to support the idea that MCD and FSGS are manifestations of the same disease process, and that they reflect earlier and later stages of the same disease.

In humans, there is also evidence for a disease continuum. Historically, the term lipoid nephrosis was used for all patients with nephrotic syndrome, and histologically, those with advanced lipoid nephrosis showed sclerotic lesions.[17] In 1957, Rich observed that the severity of the lesions was associated with the duration of the disease.[18] He described the clinical course of events "with the gradual clinical transition from the pure nephrotic stage to the one with increasing damage" to be linked to the extent of pathological abnormalities. It was only in 1970 that MCD and FSGS became separate disease entities.[19] In this publication by Churg, the difference in clinical outcome and therefore the importance of distinguishing the two entities was elegantly demonstrated. Nevertheless, reports of MCD progressing to FSGS continued to emerge as well.[20-23] In 1964, McGroven *et al.* were the first to draw attention to morphological transition of MCD to FSGS in a later stage of the disease, which was confirmed by Heyslett *et al.* in 1969.[20,21] In a report by Tejani on 48 children with minimal change nephrotic syndrome and repeat biopsies, 88% of patients showed morphological transition.[22] Of these patients, 25% were in need of renal replacement therapy, 27% continued to have nephrotic syndrome with renal function deterioration and only 35% reached complete remission at the end of the study period. Because this study only described children with an indication for repeat renal biopsy, it is not representative of the transition rate of all children with MCD. However, it does show that in patients with a relatively unfavorable clinical course, morphological transition is common and is associated with poor renal outcome. Importantly, it was shown that clinical parameters at disease onset did not predict morphological transition, indicating that there were no means of identifying this patient group with poor renal prognosis upfront. More evidence for a disease continuum comes from patients with recurrent FSGS after transplantation. These patients initially present with a phenotype that resembles MCD: heavy proteinuria with podocyte foot process effacement and no glomerular abnormalities on light microscopy. Yet, in repeat biopsies of the renal transplant, these patients often show morphological transition to FSGS.[24,25]

Although classical text books still regard MCD and FSGS as two separate disease entities, several suggestions for a unifying view have been proposed in recent years. Barisoni *et al.* proposed a new taxonomy for podocytopathies that is based on the extent of podocyte damage.[26] However, the final taxonomy was based on histopathology and etiology and still considered MCD and FSGS as two separate diseases. The most far-reaching proposal for classifying MCD and FSGS as different manifestations of the same disease process comes from a review by Maas *et al.* from 2016.[27] In this review, the authors describe a new concept in which MCD and FSGS are viewed as different manifestations of the same podocytopathy. Their concept is built upon two pillars: the intensity of initial podocyte injury, and the extent of podocyte depletion. The intensity of initial injury determines the amount of histological abnormalities observed, the sensitivity to steroids and the likelihood of remission. Continued injury will lead to increased podocyte depletion. Once a critical amount of podocytes is lost, a patient will reach the point of no return and develop end stage renal disease. In this concept, the authors also took into account the different histological variants of FSGS. They suggest that the different variants may represent different stages of podocyte injury: the tip variant of FSGS represents mild podocyte injury (the stage after MCD), whereas collapsing FSGS represents more severe initial podocyte injury. Eventually, all variants will lead to FSGS not otherwise specified (NOS) if podocyte injury persists.

Maas *et al.* describe an intriguing concept, yet a few questions remain. In particular, how to find compelling evidence for the transition of FSGS variants over time and how to investigate the possibility of a 'second hit.' Some of the recent findings and considerations that may shed light on these issues will be discussed here, starting with the morphological transition of FSGS variants and whether these variants reflect different stages of a disease continuum. The notion that the tip and collapsing variant of FSGS represent different stages of podocyte injury is appealing, but the evidence is still inconclusive. Canaud *et al.* and IJpelaar *et al.* studied the morphological transition of recurrent FSGS after transplantation and found little support for the transition of collapsing and tip variants to FSGS NOS.[24,25] Canaud *et al.* found that only 1 of 8 patients with collapsing FSGS progressed to FSGS NOS in repeat biopsies, and the single patient in this study with the tip variant progressed to the perihilar variant. Similarly, IJpelaar *et al.* showed evolution of collapsing FSGS towards FSGS NOS in 1 of 6 cases with collapsing FSGS. It has to be emphasized that although the results of these studies do not support the hypothesis of collapsing FSGS or tip lesions transforming to FSGS NOS, they do not contradict this notion either. According to the current classification, a single glomerulus with collapsing FSGS overrules all other forms, even though other glomeruli in the same biopsy may show other manifestations of FSGS such as FSGS NOS. Several observations

do support the notion that the tip and collapsing variant of FSGS represent more advanced stages of podocyte injury compared to MCD, eventually evolving to the NOS variant. Zhong *et al*. showed morphological transition in repeat biopsies in 11 patients with FSGS, 9 of which progressed from other variants to FSGS NOS.[28] Deegens *et al*. showed that podocyte foot process width is higher in patients with the tip variant of FSGS compared to patients with MCD, suggesting more advanced podocyte injury in the tip lesion.[2] In addition, Taneda *et al.* reported that both the collapsing and the tip variant show more extensive foot process effacement compared to MCD.[29] There are additional arguments in favor of the hypothesis that the glomerular tip lesion represents an early lesion in the development of FSGS and that a lesion with hyalinosis and sclerosis is representative of a later stage. The morphological transition of the tip lesion to a lesion characterized by hyalinosis and sclerosis has been described in native biopsies with FSGS in a report on the nature of the tip lesion shortly after its discovery.[30] Interestingly, findings in animal models are also suggestive of the transitional capacities of FSGS lesions, e.g. the progression from collapsing FSGS to FSGS NOS lesions. In p21 knockout mice, an experimental model for FSGS, hyperplastic lesions resembling human collapsing FSGS developed five days after induction of FSGS. Yet, 14 days after induction of FSGS, the predominant lesion had changed to a sclerotic lesion resembling human FSGS NOS.[31] Likewise, in an transgenic rat model for human immunodeficiency virus 1 (HIV-1) associated nephropathy, early changes in glomeruli included collapse of the glomerular tuft while late changes were characterized by extensive mesangial matrix expansion and sclerosis.[32,33] Thus, these studies support the hypothesis that the tip and collapsing lesions represent different stages of podocyte injury.

A second consideration concerning the model of MCD and FSGS as a disease continuum is the possibility of a 'second hit' as an important factor in the transition from MCD to FSGS. The model as proposed by Maas *et al.*, is based on the intensity of the initial podocyte injury and on the duration of podocyte injury leading to podocyte depletion. Although these factors are important determinants of disease progression, they might not fully correspond with observations in animal models and the clinic. What argues in favor of this concept is the study by Wiggins *et al.* (2005). The authors clearly showed in a diphtheria toxin model of direct podocyte injury, that the amount of podocytes lost is associated with the development of glomerular sclerotic lesions. The authors found that the number of podocytes inversely correlated with glomerular periodic acid-Schiff-positive areas (sclerotic areas of the glomerular tuft).[13] However, it is important to note that their method of podocyte counting did not adjust for sclerotic area. The presence of sclerosis excludes the presence of viable podocytes in the same area, which may be the obvious reason for the observed correlation. Additionally, in animals with little

podocyte loss, proteinuria was also minimal. This illustrates that this model does not accurately reflect the situation in humans, since MCD presents with overt proteinuria without significant podocyte loss. Other reports in favor of this concept are those showing that the extent of foot process effacement is higher in patients with FSGS compared to patients with MCD. However, some discrepancies can be found among the different studies. Deegens *et al.* reported that foot process width was significantly higher in primary FSGS NOS compared to MCD, whereas Taneda *et al.* found no differences between primary FSGS NOS and MCD.[2,29] Additionally, Deegens *et al*. showed that foot process width was similar or even lower in cases with secondary FSGS compared to MCD.[2] Whether the observed differences reflect a difference in initial podocyte injury or a difference in disease duration remains unclear.

The above mentioned observations suggest that the intensity of original podocyte injury or duration of this injury are important, but often incompletely explain the tendency of glomerulosclerosis to develop in patients with FSGS. The inclusion of a 'second hit' in the hypothesis of MCD and FSGS as a disease continuum could clarify the observations that are still unaccounted for in the current theory. The 'second hit' theory originates from oncology research.[34] It states that two 'hits' to the DNA are necessary for a malignancy to develop. Although initially intended to explain tumor pathology in relation to DNA changes, the 'second hit' theory found applications in numerous fields of biology. A 'second hit' in the context of FSGS would not necessarily have to describe a genetic factor predisposing for FSGS development, but could also describe a systemic factor or another biological factor in the local microenvironment of the glomerulus. Several clinical and pathological findings support the idea of a 'second hit' in the progression of MCD to FSGS. For example, the study by Canaud *et al.* shows that a substantial number of patients with recurrent "FSGS" never progresses to develop FSGS lesions.[25] The morphological phenotype of these patients remains that of MCD with extensive foot process effacement in the absence of segmental glomerulosclerosis, up to a year after disease recurrence. The absence of a 'second hit' might explain the low transition rate in these patients with former biopsy proven FSGS. Findings in animal models that are used to study the pathogenesis of FSGS support the 'second hit' hypothesis as well. In a model characterized by direct podocyte stress induced by continuously active transforming growth factor β signaling, it was shown that continuous podocyte stress itself is not sufficient to trigger podocyte detachment from the basement membrane.[35] Dysfunction of the glomerular endothelial cell is also necessary for podocyte apoptosis and glomerulosclerosis to develop in this model. Other experimental studies showed that interventions targeting the complement system could hamper the development of glomerulosclerosis,

whereas podocyte injury itself was not affected.[36,37] In **chapter 5** of this thesis we argue that complement activation is likely a downstream mechanism contributing to the formation of sclerotic lesions. In other words, it might serve as a 'second hit' in the development of FSGS lesions after initial podocyte damage. Our data in animals and patients show that complement deposition is most common after podocyte damage, but before the development of sclerosis. These data are in line with the experimental studies by Strassheim *et al.* and Morigi *et al.* and support the hypothesis that a 'second hit' can contribute the development of FSGS.[36,37] In conclusion, current patient and experimental data suggest that an additional factor to direct podocyte injury might explain the tendency of some individuals to progress to glomerulosclerosis.

The differentiation between MCD and FSGS, whether they are considered different syndromes or manifestations of the same disease, has profound implications for treatment and patients' prognosis. In the management of MCD it remains a challenge to identify those patients that are prone to become steroid resistant and progress to renal insufficiency. Looking at MCD and FSGS as a disease continuum might help us in this matter. There is an ongoing need for tools that assist in predicting the prognosis of patients with MCD and FSGS. As was illustrated by the study by Tejani, clinical presentation at baseline is poorly associated with a morphological transition from MCD to FSGS and renal function deterioration.[22] Therefore, clinical and histological biomarkers to identify those patients with worse clinical outcome are warranted. The authors that proposed the new hypothesis of MCD and FSGS as different manifestations of the same disease, published a study in 2014 in which they investigated biopsies with histologically proven FSGS that did not contain advanced lesions; MCD cases were used as controls.[38] They found that cells positive for the parietal epithelial cell activation marker CD44 were present in cases with early FSGS, but not in MCD. As parietal epithelial cell activation is thought to be an early event in the formation of sclerotic lesions in FSGS, this may suggest that CD44 could be used to distinguish MCD from early FSGS.

In **chapter 2** of this thesis, we investigated whether loss of the podocyte molecule nephrin could be used as a biomarker to determine adverse renal prognosis in patients with MCD. The segmental loss of important podocyte molecules has been reported in early stages of FSGS development in animal models.[14] In these animals, segmental loss of podoplanin expression, probably reflecting podocyte stress, occurred concomitantly with podocyte foot process effacement and proteinuria development. Segmental nephrin loss was also previously described in histologically confirmed cases of FSGS.[39] Our findings show that segmental nephrin loss in patients with MCD predicts worse renal outcome and is possibly associated with the development of FSGS lesions.

The study described in **chapter 2** of this thesis, as well as studies by Smeets *et al.* and Fatima *et al*. illustrate that looking at MCD and FSGS as a continuum might lead to valuable insights in disease progression and help to better distinguish between those patients with a good and those with an unfavorable prognosis.[38,40] The benefits of this different view could also reach further, towards approaches in experimental studies, clinical trials and patient follow-up.

The new players in FSGS

Transmembrane protein 63c and prostaglandin reductase 2: new players in podocyte injury and proteinuria.

Research *in vitro*, *in vivo* and in humans has tremendously increased our understanding of podocyte biology in health and disease. Our view of the podocyte has evolved from a cellular sieve to a complex signaling hub that is indispensable for glomerular integrity. In the past decades, important intrinsic drivers of podocyte injury have been identified. These discoveries have greatly increased our understanding of podocyte injury, but have not led to major breakthroughs concerning the treatment of associated diseases. Many aspects of podocyte (patho) physiology remain unknown territory and the discovered drivers of podocyte disease are often unsuitable therapeutic targets. Moreover, the heterogeneity of injury patterns in podocyte disease and overlap between different disease phenotypes complicate the identification of individual factors that initiate and/or sustain podocyte injury. A combination of several factors is likely to be involved in the progression of podocyte injury.

In **chapter 3 and 4** of this thesis, we used a bottom-up approach to identify new, promising genetic targets for podocyte pathophysiology in the MWF rat, an animal model known for the spontaneous development of podocyte pathology and FSGS lesions. Previous research on the genetic basis of FSGS development has clarified that the renal phenotype of MWF rats is largely confined to a quantitative trait locus (QTL) on chromosome 6.[41] The more detailed mapping of the identified QTL on chromosome 6 led to the prioritization of transmembrane protein 63c (TMEM63c) and prostaglandin reductase 2 (PTGR2) as novel targets for the development of the MWF phenotype (in **chapter 3** of this thesis). It should be noticed that the approach taken is not strictly limited to the identification of novel genetic targets of FSGS development, but rather to the development of albuminuria in general. The identification of these genes was based on the comparison of the genetic background of MWF rats with spontaneously hypertensive rats (SHR). Both MWF rats and SHR spontaneously develop hypertension, but in contrast to the MWF rats,

SHR do not develop albuminuria or glomerulosclerosis. Interestingly, the consomic MWF strain MWF-SHR6 displays significantly reduced levels of albuminuria in comparison to the 'normal' MWF strain while still being hypertensive. Moreover, podocyte injury is less prominent in this consomic strain than in the non-consomic strain.[42,43] This indicates that the QTL on chromosome 6 makes MWF rats vulnerable to the development of albuminuria and podocyte injury. Therefore, the results of **chapter 3** could be applicable to other renal diseases characterized by albuminuria and podocyte injury. Likewise, although podocyte injury in the setting of FSGS has been the initial basis of the work described in **chapter 4,** the focus of this chapter is podocyte injury in general. PAN-induced nephrosis is commonly referred to as an *in vivo* model for FSGS and MCD, but is first and foremost a model for direct podocyte injury. It is used to induce podocyte injury in the context of other glomerular diseases as well.[44] The results of the works described in **chapter 3** and **chapter 4** of this thesis illustrate that TMEM63c and PTGR2 likely play a role in podocyte pathophysiology. Additionally, Eisenreich *et al.* recently reported pro-survival properties of TMEM63c in human cultured podocytes.[45] Apart from FSGS, podocyte pathophysiology plays an important role in many other diseases characterized by impaired glomerular filtration barrier (GFB) integrity. In general, podocyte injury is a key factor in the development of chronic kidney disease due to various underlying causes. Therefore, it would be of great interest to investigate whether the observed TMEM63c reduction in patients with FSGS is disease specific or whether TMEM63c levels are also reduced in other glomerular diseases characterized by proteinuria and podocyte injury. Both outcomes would provide equally interesting insights.

Although **chapter 3** and **chapter 4** of this thesis focus on TMEM63c and PTGR2 function in podocyte pathophysiology, the genetic analysis with which these targets were identified does not limit their possible involvement to albuminuria development to podocyte pathophysiology. It is possible that these two genes affect the GFB via mechanisms that are not necessarily linked to the podocyte. Especially PTGR2 is likely to affect the GFB via extrinsic pathways, due to its involvement in metabolic and systemic processes such as lipid metabolism and septic inflammation. As was discussed in **chapter 4** of this thesis, PTGR2 is involved in biological processes involving peroxisome proliferator-activated receptor γ (PPARγ) and prostaglandins. Both PPARγ transcriptional regulation and prostaglandin E2 metabolism affect many cell types in a variety of organ systems, which implies that PTGR2 might exert effects throughout the body. In fact, we showed that PTGR2 knockdown in zebrafish, using a morpholino construct, resulted in severe developmental malformations of the entire body of the larvae (data unpublished). Thus, not only glomerular PTGR2 could play a role in podocyte injury, but systemic effects might play a role as well. In addition, the phenotype of the MWF rat has characteristics beyond the kidney.

Several studies have shown endothelial malfunction of the aorta and coronary arteries in these animals.[46,47] Ulu *et al.* suggested that these vascular changes were not merely due to kidney disease-associated cardiovascular injury.[48] In **chapter 5** of this thesis, we report on complement activation in glomeruli of MWF rats from the onset of proteinuria development, which might go beyond sole local production of complement components. Taken together, these data suggest that systemic processes are involved in the pathogenesis of albuminuria and glomerulosclerosis in the MWF rat model. In conclusion, the characterization of the genomic architecture of the MWF rat strain described in **chapter 3** of this thesis provides a rich pool of data that should be investigated more thoroughly and beyond the biology of the podocyte.

Complement activation in FSGS

Complement components have been observed in biopsies of patients with FSGS since the earliest studies on FSGS histology. Possibly due to the lack of inflammation in the biopsies and the previously believed minimal role of the immune system in the development of FSGS, these deposits were usually regarded as nonspecific entrapments. However, recent experimental studies showed that complement components were involved in the development of glomerulosclerosis, raising the question whether these deposits might be of clinicopathological importance after all. The work described in **chapter 5** of this thesis provides evidence for a role of the complement system in the development of FSGS in patients. Compared to healthy control subjects and patients with MCD, deposits of the complement activation product C4d appeared to be more common in patients with FSGS. In addition, in both animals and humans with recurrent FSGS after transplantation, C4d deposits were present before the development of glomerular sclerotic lesions, indicating that complement is activated in an early stage of the disease. Finally, the presence of C1q and the absence of mannose-binding lectin (MBL) deposits in glomeruli of patients with FSGS suggests that complement is activated via the classical pathway. An important question remains: why do we observe C4d and C1q deposition in glomeruli of patients with FSGS? Based on the above-mentioned results, the most straightforward explanation is that these deposits are the result of local classical complement pathway activation. However, it remains unclear what causes the activation of the classical route of complement. The major activators in the kidney are aggregates of immunoglobulins, which are visible as electron dense deposits on electron microscopy. These aggregates may deposit in the glomerulus as a result of trapping of circulating immune complexes, deposition or binding of circulating antigens in the glomerulus followed by binding of immunoglobulins, or aggregation of immunoglobulins and intrinsic glomerular antigens.[49] FSGS is not generally considered an immune complex-mediated disease and immune deposits are thus

rarely seen by electron microscopy. In our cohort of patients with FSGS, we found only two patients with complement activation that also showed possible immune deposits by electron microscopy. Yet, dense deposits by electron microscopy together with the deposition of C1q can be encountered in cases with FSGS in the context of C1q nephropathy. Currently, it is unclear whether C1q nephropathy represents a separate disease entity or should be viewed as a subtype of FSGS.[50-52] One of the patients in our cohort met the criteria of C1q nephropathy and the presence of C4d suggested that the classical route of complement was activated. Taken together, immune complex deposition does not provide a satisfying explanation for classical pathway activation in FSGS, since most cases with C4d deposits did not show the presence of electron dense deposits.

Nonetheless, the classical route of complement could still be activated via immunoglobulins that do not form stable and detectable immune complexes. One of the mechanisms of complement activation in FSGS is via natural IgM antibodies, as has been discussed in **chapter 5** of this thesis. This hypothesis was first proposed by Strassheim *et al.* who showed that IgM depletion diminished glomerulosclerosis in an animal model for FSGS.[36] Natural antibodies are antibodies produced without a known history of exposure to the antigen. IgM natural antibodies are polyreactive, which means they can bind many different antigens, but they do generally have a lower affinity to the antigen, making the antibody-antigen complex unstable.[53] Nevertheless, due to its pentameric structure, even low affinity IgM has the capacity to activate complement.

Natural IgM antibodies have been demonstrated to protect against infections and autoimmunity, and to facilitate healing.[54,55] However, they are also known to provoke cellular injury via activation of the complement system.[56] In the study by Strassheim *et al.*, the authors hypothesize that IgM antibodies react with neoantigens exposed on injured podocytes, thereby activating the complement system and promoting the development of FSGS. In this scenario, complement activation would not be the initiator of podocyte injury, but rather exacerbate glomerular disease progression in FSGS. In a study by Panzer *et al*. from 2016, the authors compellingly show that natural IgM antibodies can contribute to existing cellular injury.[57] They investigated a factor H knockout mouse model, which develops glomerulonephritis via activation of the alternative complement pathway. In young mice, only C3 deposition was observed, indicating alternative pathway activation. However, in older mice with more advanced glomerular injury, the authors also observed deposits of IgM and C4, which are indicators of classical pathway activation. To determine whether these deposits of IgM and C4 were pathogenic, they investigated glomerular injury in factor H knockout mice that were depleted for IgM. No C4 or C1q deposits were

observed in these mice and glomerular disease progression was attenuated. Finally, both in this study and in the study by Strassheim *et al.*, purified IgM was found to bind to glomeruli of diseased and IgM depleted mice, suggesting that natural IgM might indeed bind to glomerular neoepitopes.[36,57] Together, these two studies support the idea of natural IgM antibodies as the initiators of complement activation in FSGS. Moreover, these results are in line with the idea that complement activation serves as a 'second hit' in the development of FSGS.

C1q can also directly bind to damaged cells without interference of antibodies. In the study described in **chapter 5** of this thesis, we made several observations confirming that C1q binding to self-cells could play a role in MCD and FSGS. In both patients with MCD and patients with FSGS, we observed cellular adhesions or synechiae that were positive for C1q and/or C4d. In addition, we detected these complement components at the site of early tip lesion formation (Figure 1). The presence of complement components in tip lesions that are mainly composed of cellular components rather than extracellular matrix, might indicate direct binding of C1q to damaged cells. C1q has the capacity to sense and engage a large variety of targets.[58] Apart from non-self-antigens, C1q is known to directly interact with various types of immune cells as well as several other cell types, including fibroblasts and endothelial cells.[59] The ability of C1q to directly bind to damaged cells was first described in the context of clearance of apoptotic T lymphocytes and endothelial cells as well as cell-derived blebs.[60,61] This process of C1q binding is thought to be essential in the clearance of apoptotic cells, without causing cell lysis or inflammation. Whether C1q binding to self-cells can also be harmful is currently unknown. Direct binding of C1q to endothelial cells has been shown to activate the complement cascade and to induce the formation of C5b-9, but it is unclear whether and to what extent this contributes to cell damage.[62] An interesting finding regarding the direct binding of C1q to endothelial cells in the context of FSGS comes from Kreutz *et al*. in 2007.[63] They report differential expression of C1q in glomerular endothelial cells of MWF rats, the same FSGS animal model in which we showed C4d deposition in **chapter 5** of this thesis. The local production of C1q in these animals could contribute to excess binding of C1q to local cells such as endothelial cells or podocytes due to higher bioavailability of C1q.

Complement activation in FSGS could also be the result of reduced local complement regulation. Loss of regulating factors of complement has been observed in FSGS. In a cohort of 7 patients with FSGS, glomerular expression of the extracellular portion of the complement receptor 1 (CR1) was reduced.[64] Barisoni *et al.* showed that cases of collapsing FSGS displayed reduced expression of CR1 in podocytes.[65] Loss of

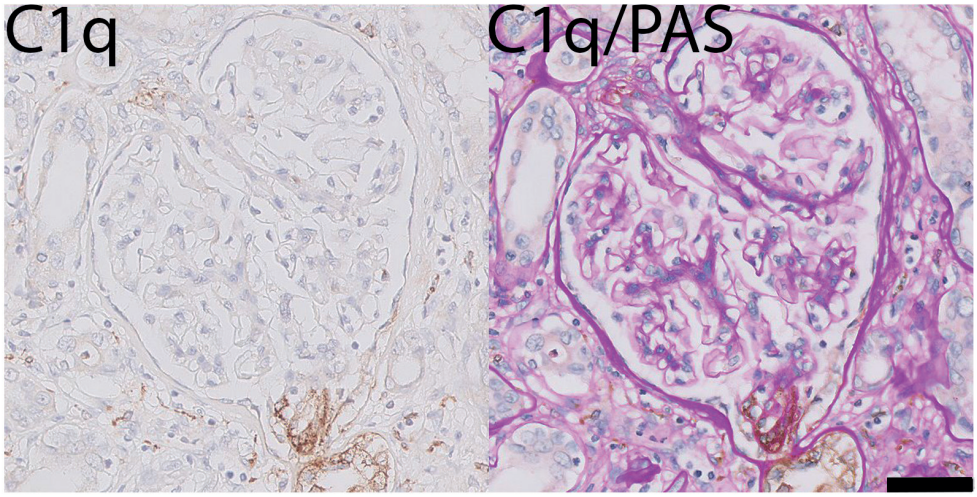

Figure 1. C1q staining co-localizing with an FSGS tip lesion.

A kidney section of a patient with FSGS was immunostained for C1q and subsequently subjected to periodic acid-Schiff staining. The example image illustrates that C1q deposition co-localizes exactly with an FSGS tip lesion. As the tip lesion mainly consists of cellular components, this direct colocalization might indicate binding of C1q to damaged cells. Scale bar represents 50 µm

CR1 expression in podocytes has also been shown in typical complement-mediated diseases such as lupus nephritis and membranous glomerulonephritis.[64] CR1 is a receptor for the complement components C3b and C4b, and to a lesser extent for C1q.[66] CR1 is a versatile inhibitor of both the alternative and classical pathway of complement activation. It has decay-accelerating activity for both alternative and classical pathway C3 and C5 convertases and possesses co-factor activity for C3b/C4b cleavage. In the healthy glomerulus, CR1 is only expressed by podocytes. However, little is known about the function of CR1 in podocytes and its role in glomerular complement activation.

Although our findings point towards classical complement pathway activation, complement activation via the lectin pathway is also a possibility. In **chapter 5,** we evaluated lectin pathway activation by immunohistochemistry and found a low prevalence of MBL deposits and no differences between FSGS cases and control subjects. However, MBL is not the only activator of the lectin pathway: ficolins and collectins are newly discovered activators of the lectin pathway.[67] Since we did not

investigate the deposition of ficolins and collectins, we cannot state with absolute certainty that the lectin pathway is not activated, although the absence of MBL deposits makes this less likely.

The final question that remains to be answered is whether classical pathway activation in FSGS leads to activation of the final common pathway and formation of the membrane attack complex (MAC) or C5b-9. Increased levels of soluble C5b-9 have been reported in urine and plasma of patients with FSGS, indicating that the final common pathway might be activated.[68] However, these results do not provide evidence of local formation of MAC in glomeruli of patients with FSGS. MAC formation is generally associated with thrombotic injury and necrosis of the surrounding tissue. However, sublytic levels of MAC formation also influence fibrotic processes and have been shown to cause DNA damage in cultured podocytes.[69,70] In several renal diseases, C5b-9 deposition has been shown to be associated with disease severity and/or disease progression.[71,72] Moreover, treatment with eculizumab, an antibody that inhibits the cleavage of C5 into C5a and C5b, resulted in reduced C5b-9 staining in all studied patients with C3 glomerulopathy.[73] Thus, determining C5b-9 deposition in patients with FSGS could help to substantiate whether the final common pathway is activated. We have previously shown C5b-9 deposition in several renal diseases characterized by microvascular injury, such as diabetic nephropathy, lupus nephritis and thrombotic microangiopathy (TMA).[74-76] However, C5b-9 deposition was also frequently present in controls and in patients that did not show deposition of complement factors of any of the three initiating pathways. These findings question the specificity of C5b-9 deposits. Immunohistochemical evaluation of C5b-9 could provide valuable insights concerning MAC formation in FSGS, but experimental studies are needed to verify whether there is a role for the final common pathway in the development of FSGS.

Endothelial injury and endothelial crosstalk

Podocyte injury is a hallmark of FSGS. There is a sizable amount of data supporting the concept of podocyte injury as the main pathogenic event in FSGS. However, studies also suggest that podocyte injury might be essential, but not sufficient to develop FSGS, and that other cell types are involved in disease progression and outcome. For instance, in the absence of parietal epithelial cells, models for collapsing FSGS result in a much milder phenotype.[77] Indeed, the standard therapy of glucocorticoïd treatment might in part exert its effects via the parietal epithelial cells, as was shown by Kuppe *et al.* in their glomerulonephritis model.[78] Thus, although podocyte injury might be the hallmark of FSGS development, dysfunction of other cell types may be as important for disease progression and outcome.

The endothelial cell has been largely neglected in FSGS research. One explanation might be that the histopathology of FSGS does not resemble the pattern that is usually seen in diseases characterized by endothelial damage. In response to injury, endothelial cells generally become activated, leading to a proinflammatory and procoagulant state characterized by the expression of the surface molecules vascular cell adhesion protein 1, intercellular adhesion molecule 1 and E-selectin or tissue factor, respectively. Signs of inflammation and/or thrombosis are seldom seen in patients with FSGS, apart from the occasional presence of a limited number of inflammatory cells.[3] Only recently, an association between FSGS and thrombotic renal damage was proposed by Buob *et al.*, who investigated the prevalence of secondary FSGS in cases with renal TMA.[79] In **chapter 7** of this thesis, the landscape of microvascular changes in FSGS, including signs of TMA, was investigated. In this detailed histopathological analysis, we observed that thrombotic abnormalities are indeed very rare in patients with FSGS, which is in line with previous findings. However, we did observe microvascular abnormalities – such as endothelial hypercellularity, endothelial swelling or vascular pole hyalinosis – in a substantial number of patients and in all histological variants of FSGS. Studies on endothelial alterations in patients with FSGS have occasionally emerged over the last decades, most of which focused on ultrastructural changes. Endothelial vacuolization and subendothelial widening were reported in both primary and secondary cases of FSGS.[29,80,81] In general, the endothelial changes described in these papers were not accompanied by inflammation or thrombosis. Similar observations are found in other glomerular diseases, such as diabetic nephropathy, a disease in which overt glomerular inflammation or thrombosis are not generally observed in glomeruli, but endothelial activation and glycocalyx loss are important factors contributing to disease progression.[82-84] An experimental model of FSGS also showed that glycocalyx loss contributes to the development of glomerulosclerosis.[85] Although the classical features of endothelial injury such as thrombosis and inflammation are not apparent in FSGS, the histological endothelial abnormalities that we observed across patient with FSGS, and in particular collapsing FSGS, could still reflect a pathogenic role for the endothelial cell in disease development.

Although the observed structural abnormalities in the endothelium are compelling, they do not provide direct evidence for involvement of endothelial cells in the pathogenesis of FSGS and could merely be a consequence of existing podocyte injury. Yet, the occurrence of endothelial abnormalities early in the disease process suggests that endothelial cells do play a pathogenic role.[86,87] Studies investigating the pathogenic involvement of the endothelial cell in FSGS are limited and have only recently emerged. In 2014, Daehn *et al.* were the first to show that the endothelial cell is required for the progression of podocyte injury and the development of

glomerulosclerosis in a model for FSGS.[35] They proposed that the altered interaction between podocytes and endothelial cells plays a significant role in disease progression and that endothelin 1 (ET-1) is the key player in this altered interaction. In **chapter 6** of this thesis, we show that the mechanism of altered podocyte-endothelial crosstalk via the ET-1 system also holds true in patients with FSGS. The association between expression of the endothelin receptor A (ET_AR) in endothelial cells and the loss of nephrin in podocytes suggests that this mechanism contributes to podocyte damage. Altered podocyte-endothelial crosstalk via the ET-1 system is thus an interesting target for future research and will shed further light on the role of endothelial cells in FSGS. Nevertheless, both the results described in this chapter and the studies by Daehn *et al.* do not provide an answer as to why ET_AR expression is upregulated in glomerular endothelial cells. ET_AR expression in endothelial cells is observed almost exclusively in pathological conditions.[35,88,89] The simplest explanation for increased ET_AR expression is a response to increased production of ET-1 by the podocytes.[35,85] However, it might also be due to ET-1 independent changes of the endothelial cells. Since no research investigating this mechanism has been performed so far, we can only speculate about what these factors could be. Endothelial alterations might be the result of changes to the GFB as a whole. As was illustrated in the introduction, the GFB is a dynamic structure and signaling hub. Alterations to the GFB may change the microenvironment of the glomerular endothelial cells causing alterations in expression of surface molecules, similar to what occurs during endothelial activation. Another interesting hypothesis is that the endothelial cells might be primed by systemic factors, which makes them interact differently with the podocytes. This concept of altered podocyte-endothelial interaction has been described in diabetic kidney disease: endothelial cells exposed to a hyperglycemic microenvironment may react differently to paracrine signaling from podocytes leading to a vicious cycle of reactive oxygen species production.[90] Endothelial cells are the first line of contact with the circulation. In a substantial number of cases with primary FSGS, a systemic factor is involved in the disease process. It is possible that systemic factors that encounter the glomerular endothelial cells prime them to react differently to the injured podocyte. These speculations go beyond our current understanding of the role of the glomerular endothelial cell and podocyte-endothelial crosstalk in FSGS, but they do represent interesting future perspectives.

Another interesting topic for future investigations is FSGS-associated endothelial injury in the context of complement activation. Complement activation is a common denominator in glomerular diseases characterized by endothelial injury. Previous work of our research group demonstrated that the complement cascade is activated in a variety of glomerular diseases with diverse etiology,

but with a common background of glomerular microvascular injury.[91] Chua *et al.* provided evidence for a role of the complement system in the development of microangiopathic lesions in IgA nephropathy, a disease otherwise/primarily characterized by changes to the glomerular mesangium.[74] Likewise, complement activation could be an indicator of microangiopathic injury in patients with FSGS as well. Based on the results described in **chapter 5** of this thesis, it is hypothesized that classical pathway activation in FSGS could be the result of binding of natural IgM antibodies to damaged cells. It has been previously proposed that endothelial cell damage can trigger the binding of natural IgM antibodies, thereby activating the classical complement pathway.[92,93] The deposition of IgM in FSGS patients and FSGS animal models has not been conclusively shown to locate at the podocyte. Thus, the IgM deposits and subsequent C1 and C4 activation could also reflect IgM binding to neoepitopes presented on injured endothelial cells. Lastly, regardless of the initiator of complement activation, complement dysregulation can cause a positive feedback loop that both triggers and sustains endothelial injury.[94-96]

Apart from glomerular endothelial injury, there appears to be an association between FSGS and microvascular injury of the renal arterioles. The connection between arteriolar thrombotic microangiopathic injury and secondary collapsing FSGS was already shown by Buob *et al*. (2016).[79] Although secondary collapse and visceral epithelial cell hyperplasia due to vaso-occlusive processes had been reported,[97] Buob *et al.* were the first to focus on the prevalence of the different FSGS variants occurring in the setting of histological TMA in native kidneys. They proposed that ischemia, endothelial injury or both could play a major role in the pathophysiology of collapsing FSGS. In response to this theory, critics might argue that the collapsing lesions due to thrombosis of the afferent arterioles do not truly represent FSGS, but rather a lesion that is the result of prolonged ischemia leading to shrinking of the glomerular tuft. This notion is supported by the fact that the clinical and histological presentation of these patients differs from the usual presentation of patients with collapsing FSGS: 1) nephrotic syndrome is uncommon and proteinuria levels are relatively mild and 2) dysregulation of the immunohistochemical phenotype of podocytes is less marked.[79] What argues against this is that the histological lesions do meet all the criteria for collapsing FSGS according to the Columbia classification, including epithelial cell hyperplasia; ischemic kidneys frequently show wrinkling of the glomerular basement membrane, but without prominent podocyte hyperplasia or damage. Moreover, studies reporting an association between arteriolar injury and secondary collapsing FSGS, observed overt proteinuria and/or loss of podocyte differentiation markers.[98-100] In **chapter 7** of this thesis, we report the presence of arteriolar lesions in cases of idiopathic collapsing FSGS and HIV-associated collapsing FSGS, disease entities in which the podocyte is primarily affected and proteinuria is

overt. Furthermore, we also observed arteriolar lesions in other histological variants of FSGS apart from collapsing FSGS. Even in cases with the tip variant of FSGS, which is generally considered to have mild histological changes, arteriolar lesions were observed. These finding do not support the notion that the association between arteriolar lesions and FSGS is merely based on an ischemic processes leading to collapse of the glomerular tuft. Whether the glomerular lesions would aid in the development of arteriolar lesions or vice versa remains unclear. Apart from lesions that occurred in the vascular pole, we were not able to determine the exact localization of most of the arteriolar lesions. Lesions in afferent arterioles could affect the downstream glomerulus. However, changes in the microenvironment of the glomerulus might also lead to altered feedback mechanisms to the afferent and efferent arterioles.

Endothelial cells are emerging as an interesting target in FSGS research and endothelial changes in FSGS are increasingly reported. Clarifying the role of the endothelium in the development of FSGS would benefit our understanding of its pathogenesis, which could aid in the development of new therapeutic options, as will be discussed later. Future studies will have to resolve whether altered podocyte-endothelial crosstalk is associated with clinical outcome. Importantly, studies do show that several endothelial alterations in FSGS are associated with disease outcome. Menon *et al.* recently reported that changes in the glomerular endothelial transcriptome correlate with decreased proteinuria remission rates in patients with FSGS.[101] The failure to reach remission is an important predictor for later renal function deterioration. Thus, these results reflect that endothelial changes could be of clinical significance. The reduced remission rate in patients with endothelial transcriptomic changes might be the result of a more severe manifestation of the disease, but could hypothetically also represent a subgroup of patients that do not respond to the standard therapy regiments. These patients might benefit from therapy that targets the glomerular endothelial cell.

Treatment options in FSGS: new insights based on this thesis

Glucocorticoids are the cornerstone of the treatment of most glomerulonephritides and glomerulopathies with the nephrotic syndrome, including FSGS. Glucocorticoids were first used in nephrology in 1950 to empirically treat 11 patients with the nephrotic syndrome.[102] At that time, the rationale for starting glucocorticoid treatment was that the overproduction of adrenal hormones aggravated the nephrotic syndrome and a reduction of adrenal cortical activity following cortisone

administration would affect the major manifestations of the nephrotic syndrome. This idea has long been abandoned and glucocorticoids are generally believed to act primarily via their immunosuppressive effects. In addition, some *in vitro* studies have shown direct effects of glucocorticoids on podocytes, which might contribute to the beneficial effects of glucocorticoids in less inflammatory glomerular diseases such as MCD and FSGS.[103-105] Nevertheless, evidence-based experimental studies providing insight in the primary mechanisms of action of glucocorticoids in glomerular disease were long missing. In 2017, Kuppe *et al.* conducted a study in which they investigated the effects of glucocorticoids on podocytes and parietal epithelial cells *in vivo*.[78] They developed a mouse model with a genetic inactivation of the glucocorticoid receptor in podocytes and parietal epithelial cells. In contrast to what was expected, genetic inactivation of the glucocorticoid receptor did not reduce the effects of glucocorticoid treatment. On the contrary: glucocorticoid receptor inactivation in itself protected the mice from developing glomerular disease independent from glucocorticoid treatment. Pharmacological inactivation of glucocorticoid receptor led to similar results. This study nicely illustrates how little is known about the primary mechanism of action of the cornerstone treatment in glomerular diseases, including FSGS. In an ideal situation, new therapeutic options are the products based on the unraveling of the underlying disease mechanism, extensively tested in animal models for their efficacy before continuing to the clinic. However, one might argue whether this approach always leads to the best results. Let us use the study by Kuppe *et al.* as an illustration. If this study had been performed in the 1950s, immediately after the discovery of glucocorticoids, we might never have started with treating glomerulopathies with glucocorticoids. It is true that glucocorticoid treatment in glomerulopathies has never been ideal: we do not know its primary mechanism of action, it has many side effects and does not work in all cases. However, it has proven to be an effective therapy in most cases of glomerulonephritis and nephrotic syndrome and has saved many kidneys and many lives. Ample studies have demonstrated that results from animal investigations often do not correspond with the human situation. In some fields, only 8% of drugs that enter into clinical trials will successfully pass phase 1.[106] Even targeted molecular approaches with remarkable success in mice are often futile in humans. Thus, a detailed molecular exploration does not ensure a ready-to-use therapy. Although a bottom-up approach, starting form an in-depth understanding of the molecular or cellular levels and working towards higher order systems, promises to lead to more specific and effective therapies, the majority of the most useful therapies to date were not discovered based on this methodology.[106,107] A top-down approach can be very effective in drug development, because in-depth knowledge on how a drug works is not strictly necessary to treat a patient. A dual top-down and bottom-up approach in biomedical research can result into an early translation of discoveries

at the molecular level to the clinical setting. Translational studies in an early stage of medical research could therefore guide further steps. In light of this historical and philosophical perspective, the next section will discuss the therapeutic insights provided in this thesis and the relevant next steps to be taken.

In the work described in this thesis, we have explored several new targets in FSGS development that might have implications for future therapies. In **chapter 4** of this thesis, we suggest that PTGR2 plays a role in podocyte pathophysiology partly by modulating PPARγ transcriptional activity. PPARγ agonists have been studied extensively in various diseases. Targeting PPARγ in proteinuric diseases became a topic of interest after it was shown to be a promising pharmacologic target in diabetes mellitus. Based on the important role of PPARγ in glucose and lipid metabolism and insulin sensitivity, PPARγ agonists were found to increase peripheral insulin sensitivity and glucose uptake in type 2 diabetes.[108] The beneficial effects of PPARγ agonists were not restricted to systemic glucose handling: they also improved diabetic kidney disease in both animals and patients.[108-111] Moreover, polymorphisms in PPARγ are compellingly linked to the development of diabetic nephropathy in diabetic patients.[112] These kidney-specific protective effects of PPARγ led to an increased interest for PPARγ in other renal diseases. The effects of PPARγ on podocytes, and in particular FSGS and the nephrotic syndrome, have been investigated *in vitro* and in several animal models.[113] PPARγ agonists are suggested to be protective in Adriamycin nephropathy, aldosterone-induced nephropathy, stretch-induced nephropathy and PAN-induced podocyte injury.[114-117] However, some critical notes are in place. The protective effects of the PPARγ agonist pioglitasone in PAN-induced podocyte damage were first described by Kanjanabuch *et al.* (2005).[114] They showed beneficial effects of pioglitazone on podocyte apoptosis, by measuring mRNA levels of antiapoptotic molecule *Bcl-xL* and protein levels of caspase-3. A subsequent study showed that treatment of PPARγ concomitantly with or after PAN stimulation improved proteinuria and glomerular morphology.[118] However, treatment with pioglitazone before stimulation with PAN worsened the disease phenotype showing that the effects of PPARγ agonist treatment are dependent on the stage of the disease. Although the possibility of treating a patient before the disease develops is small, this finding asks for caution. Exposing a patient in remission to PPARγ agonists might potentially be harmful instead of beneficial. The complex regulation of PPARγ in the podocyte was also illustrated in a study by Zhou *et al*., who showed that PPARγ phosphorylation causes β-catenin activation, which triggers epithelial-mesenchymal transition, loss of podocyte differentiation markers and podocyte apoptosis.[119] This adds to the complexity of PPARγ signaling, as it shows that PPARγ downstream effects could also be harmful to podocytes, dependent on the phosphorylation status. Lastly, several promising PPARγ agonists that had been

approved for clinical use have been taken off the market because of serious adverse events concerning fluid retention and congestive heart failure.[120,121] The wide range of PPARγ effectors poses a problem for the use of PPARγ agonists in clinical practice, because they elicit a large number of side effects. More specific PPARγ agonists are needed if this therapy is to be implemented in the clinic. As a natural agonist of PPARγ, 15-keto-prostaglandin E2 might be a promising future target. However, the investigation of PTGR2 and 15-keto-prostaglandin E2 in podocyte disease is still in its infancy and experimental studies investigating the effects of 15-keto-prostaglandin E2 on podocytes are warranted before any additional steps can be taken.

In **chapter 5**, we demonstrated that complement activation is a frequent phenomenon in glomeruli of patients with FSGS. This observation, and in particular activation in the early development of the lesions, suggests that the complement system plays a role in the pathogenesis of FSGS. Thus, complement inhibiting therapy provides an interesting future prospect. Several experimental studies have demonstrated the effectiveness of disrupting the complement cascade in reducing glomerulosclerosis in models of FSGS.[36,37,122] With the rapid rise in available complement inhibitors, complement inhibiting therapy might be on the horizon for patients with FSGS as well.[123] Eculizumab, a monoclonal antibody against C5, is the most widely used complement inhibitor today. Eculizumab targets the final common pathway of the complement system and the formation of MAC. Other inhibitors target the initiation of the complement cascade. Several inhibitors of the classical pathway are currently being investigated. Already in the 1990s, plasma-purified C1INH was used in the handling of hereditary angioedema. In the management of this disease, the effects of C1INH are thought to be mainly via the Kinin system. Its efficacy in controlling complement activation is currently being investigated.[124,125] Pharmacological candidates are antibodies binding C1q (ANX005) and C1s (BIVV020) that prevent the initiation of the classical complement cascade and are currently being evaluated in phase 1/2 trials.[123,126] These antibodies block the interaction of C1q and C1s with multiple substrates thereby also inhibiting all downstream components of the classical cascade.[126] An important benefit of specifically targeting the classical complement pathway is that it leaves the lectin and alternative complement pathways intact, thus reducing the severe side effects of blocking the whole complement cascade.

In the work described in **chapter 5**, evidence for complement activation via the classical pathway is reported. However, we did not investigate whether this also resulted in the subsequent activation of the final common pathway. Evidence supporting C5b-9 formation or anti-C5 efficacy in FSGS is lacking from animal studies. Thus, it remains unclear whether the MAC, the target of eculizumab, is

formed in FSGS. Based on our current knowledge on complement activation in FSGS, classical pathway inhibition might represent the most promising anti-complement target. Yet, the complement system is a notoriously difficult therapeutic target: it consists of proteins with high concentrations in the circulation and high turnover, necessitating high concentrations of inhibitors or frequently repeated treatment.[127] The high costs of complement inhibiting therapy pose another limitation. In conclusion, complement inhibiting therapy appears to be a field worthy of interest to explore in FSGS, but the complicating factors might necessitate a more detailed investigation in experimental models before we can continue to the clinic.

In **chapter 6** of this thesis, we investigated podocyte-endothelial crosstalk via the endothelin system in patients with FSGS. We showed that in most cases with FSGS, ET_AR is expressed in a pattern indicating glomerular endothelial cell expression. ET_AR receptor antagonists are being investigated in clinical trials concerning chronic kidney disease since 2009. Since then, they have emerged as promising therapy for various types of chronic kidney disease.[128,129] Several phase 2 and phase 3 trials have been conducted to test the safety and efficacy of ET_AR blockers in treating diabetic nephropathy. Although early trials with avosentan, a relatively non-specific ET_AR blocker, showed beneficial effects on reduction of urinary albumin, trials had to be prematurely terminated due to severe side effects concerning fluid retention.[130,131] In retrospect, the frequent occurrence of these severe side effects was attributed to too high doses of avosentan and poor patient selection, such as patients with a higher risk of heart failure.[132] Fortunately, later trials with lower doses of atrasentan and improved methods for patient selection showed that ET_AR blockers given in lower doses are effective in reducing proteinuria with less severe side effects.[133,134] Nevertheless, side effects such as fluid retention and heart failure still occurred despite careful patient selection. This indicates that continued watchfulness for fluid retention during the clinical use of ET receptor antagonists will likely be necessary.

Interestingly, several studies showed that ET_AR blockade seemed to be most effective in dual therapy with a renin–angiotensin–aldosterone system (RAAS) blocker.[128] The RAAS system and the endothelin system display many similarities and complex interactions. For instance, angiotensin II stimulates renal ET-1 production in rats and ET-1 increases aldosterone secretion by the zona glomerulosa.[135] Moreover, ET_AR inhibiting therapy also effects the RAAS system by reducing the hypertensive response induced by angiotensin II.[136] Attempts to therapeutically block ET_AR in FSGS have focused on the synergistic effects of RAAS and ET_AR blocking. The phase 2 DUET trial investigating the efficacy and safety of sparsentan, a dual ET_AR and angiotensin receptor blocker, in patients with FSGS showed that sparsentan was

more effective in reducing proteinuria than a single angiotensin receptor blocker.[137] Serious adverse events were similar between the two study arms, although the development of edema was more common in the sparsentan group. It appears that the severe side effects observed in previous studies using a single ET_AR blocker are less apparent in combination with RAAS system blockers. The follow-up phase 3 DUPLEX trial investigates the long-term nephroprotective potential of sparsentan compared to an angiotensin receptor blocker in patients with primary and genetic FSGS.[138] This trial is currently being conducted and will provide us with additional information on the possibilities for endothelin receptor blocking in FSGS. At present, the rationale for ET_AR inhibition in FSGS is mainly based on experimental studies indicating a role for ET-1 in podocyte injury and to a lesser extent on its hemodynamic and vasoactive properties. The hypothesis of ET-1 playing a key role in impaired podocyte-endothelial crosstalk in FSGS (**chapter 6**) reveals a possible additional pathophysiological mechanism of action that was previously not recognized in patients with FSGS. Studies investigating the possibilities of ET_AR inhibiting therapy in FSGS should take this new mechanism of action into account. ET_AR inhibiting treatment has already been shown to be more effective in dual treatment with RAAS system inhibitors.[128] Targeting both the podocyte and the glomerular endothelial cell to address altered podocyte-endothelial crosstalk could synergistically increase the effects of ET_AR treatment alone. In the light of the serious adverse events that can occur due to ET_AR inhibition, a dual approach might lead to less side-effects with sustained effectiveness. Biopsies obtained in the current DUPLEX trial are a valuable source of information that could help us determine whether ET_AR inhibition with sparsentan affects altered podocyte-endothelial crosstalk via the endothelin system, thereby reducing podocyte and endothelial injury.

It is not in the name: focal segmental glomerulosclerosis that is neither focal nor segmental nor sclerotic – an evaluation of the classification and nomenclature of FSGS

The term focal segmental glomerulosclerosis was coined by Rich to describe the advanced glomerular lesions they observed in children who died due to nephrotic syndrome.[18] The segmental character was shown to be of importance, because global sclerosis could also be observed in healthy kidneys. In addition, only patients with nephrotic syndrome and segmental glomerulosclerosis, but not with global glomerulosclerosis, showed worse clinical outcome.[3,139] Noteworthy, the hallmark paper of Churg *et al.* already recognized that patients with advanced disease can show completely sclerosed glomeruli.[19] Moreover, they described cellular

components of the segmental lesions such as visceral epithelial cell hyperplasia, meaning that sclerosis was not always the sole defining feature. In the years that followed, new descriptions of the lesions seen in cases with FSGS were reported that moved further away from the original name. The term 'glomerular tip lesion', which was first used in 1984, described a segmental but not necessarily sclerotic lesion.[30] The same holds true for the cellular and collapsing lesions that were first described in 1985 and 1986, respectively.[140,141] In addition to not being sclerotic, the collapsing lesions were not necessarily segmental either; global collapse of the glomerular tuft is observed in patients with the collapsing variant of FSGS as well. Lastly, the use of serial sections and three-dimensional morphometric analysis in FSGS animal models and patients has shown that the number of affected glomeruli is much greater than initially thought.[142,143] Some reported that up to 90% of glomeruli could be affected.[142] This illustrates that FSGS can actually be diffuse, which is disguised by the segmental character of most of the lesions.

Thus, the name FSGS stands for lesions that do not necessary have to be focal, not necessarily have to be segmental and not necessarily have to be sclerotic. The only accurate aspect of the name is that the lesion affects the glomerular tuft. A name that does not accurately describe the corresponding lesion does not have to lead to any misunderstanding as long as everyone agrees on what is meant with the term. Many diseases are named after their original discoverer and these names do not in any way represent the corresponding lesions. However, ambiguity in the name can affect the way the lesions are considered, the relationship among them and the pathogenic routes behind them.

Difficulties with the nomenclature can also be found in the definitions of primary and secondary FSGS. Primary or idiopathic FSGS originally identified patients with FSGS of unknown origin. This group of patients generally had a typical clinical presentation with massive proteinuria and nephrotic syndrome. These clinical associations are now often presented as diagnosis criteria. Whether this is justified is debatable, since there are patients with FSGS of unknown origin who do not present with these typical clinical symptoms. Historically, studies evaluating clinical characteristics have suggested that 54–90% of patients with primary FSGS present with nephrotic syndrome.[144-148] In addition, a substantial number of patients with secondary FSGS also present with massive proteinuria and nephrotic syndrome.[4,148-150] Illustrative examples are patients with HIV-associated FSGS and patients with a genetic form of FSGS. These cases would have been considered primary FSGS before the genetic basis of FSGS was known and genetic testing was possible. Indeed, a recent study by Sadowski *et al.* showed that the genetic burden of (primary) FSGS could be much higher than previously recognized.[151] Currently,

a circulating factor is often considered to be the cause of all cases with primary FSGS and nephrotic syndrome.[152] However, in the light of the increasing genetic burden in cases previously described as primary FSGS, this might very well not be the case. Moreover, many patients, who received a kidney transplantation due to primary FSGS, do not develop recurrent FSGS in their allograft kidney, even without prophylactic plasmapheresis.[153] Essentially, primary or idiopathic FSGS does not exist. It is a term brought to life to describe those patients of which we do not understand the underlying cause of the disease. As long as a universal circulating factor remains unidentified, it is difficult to say with certainty that the same pathogenic factor underlies all cases with primary FSGS.

Conclusion

FSGS stands for a complex clinicopathological entity that is uniformly characterized by proteinuria and podocyte injury. In 1975, the discovery that FSGS was associated with severe damage to the podocyte led to an era of research focused on podocyte pathophysiology. Many studies have shown us that the podocyte is the pacemaker of FSGS development and new discoveries concerning podocyte pathophysiology in FSGS are still being made. Nonetheless, a profound shift in our understanding of the complexity of the disease and its many faces has taken place. Factors that lie beyond the podocyte are now acknowledged to play a role in the development and progression of this clinicopathological entity. The pathogenesis of FSGS comprises a complex interplay between the podocyte and other glomerular resident cells along with systemic factors. It has become clear that the complement system and the glomerular endothelial cell have received too little attention in our attempts to unravel the complexity of FSGS. A better understanding of how these factors interact with injured podocytes will result in new insights regarding the progression of podocyte injury and glomerulosclerosis in FSGS. These insights in different pathogenic pathways could also provide new therapeutic opportunities. Future research should focus on elucidating which patients might benefit from new therapeutic regimens that are based on different therapeutic targets. Research strategies in which primary FSGS is viewed as an advanced state of MCD might help to unravel prognostic markers that can guide these (new) therapeutic strategies and improve the clinical management of these patients.

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