On the pathology of focal segmental glomerulosclerosis

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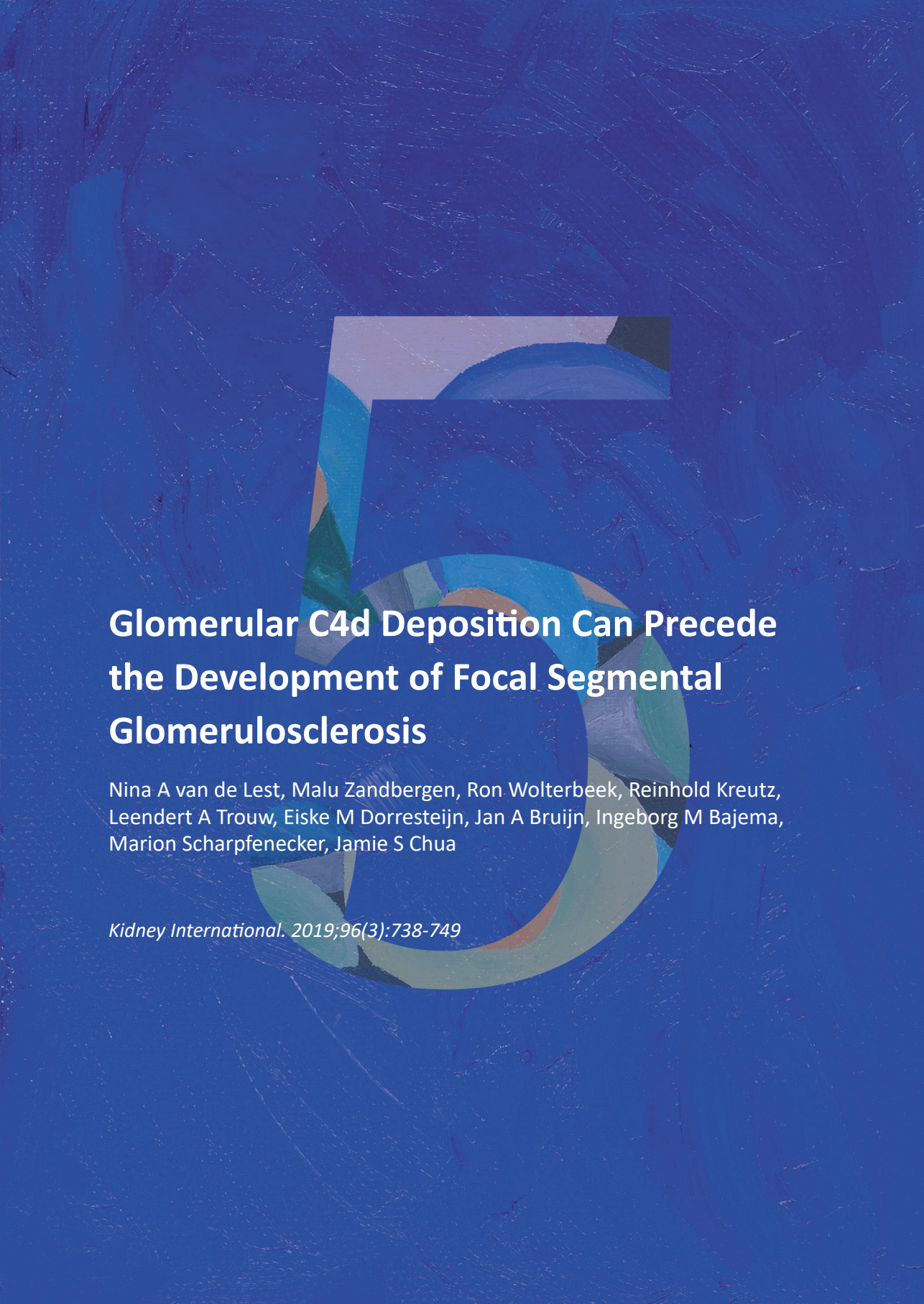

Glomerular C4d Deposition Can Precede the Development of Focal Segmental Glomerulosclerosis

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Abstract

Recent studies suggest that complement plays a role in the pathogenesis of focal segmental glomerulosclerosis (FSGS). Moreover, co-localization of IgM and C3 deposits with FSGS lesions has frequently been reported. Here, we investigated whether glomerular complement deposition precedes the development of FSGS and whether it represents local complement activation. Renal biopsies from 40 patients with primary FSGS, 84 patients with minimal change disease, and 10 healthy individuals were stained for C4d, C1q, and mannose-binding lectin. C4d deposits were also measured in renal allograft biopsies from 34 patients with native primary FSGS, 18 of whom subsequently developed recurrent FSGS. Lastly, we measured C4d deposits in the Munich Wistar Frömter rat model of FSGS. The prevalence of C4d-positive glomeruli was significantly higher among patients with FSGS (73%) compared to patients with minimal change disease (21%) and healthy individuals (10%). Moreover, segmental sclerosis was absent in 42% of C4d-positive glomeruli. Glomerular C1q was significantly more prevalent in FSGS compared to minimal change disease or healthy individuals, while mannose-binding lectin was infrequently observed. C4d deposition was significantly more prevalent in recurrent FSGS (72%) before the development of sclerotic lesions compared to control transplant samples (27%). Finally, at the onset of albuminuria but before the development of FSGS lesions, Munich Wistar Frömter rats had a significantly higher percentage of C4d-positive glomeruli (31%) compared to control rats (4%). Thus, glomerular C4d deposition can precede the development of FSGS, suggesting that complement activation may play a pathogenic role in the development of FSGS.

Introduction

FSGS is the most common cause of nephrotic syndrome in adults.[1] FSGS is classified as primary (idiopathic), hereditary, or secondary; primary FSGS is defined by the exclusion of any identifiable cause of secondary FSGS.[1,2] Despite the identification of numerous factors leading to FSGS, approximately 80% of cases remain idiopathic.[1,3] Experimental data suggest that complement activation plays a pathogenic role in the development of FSGS. For example, mice that lack the complement components C3 and factor D and mice that are depleted of IgM are protected from adriamycin-induced glomerulosclerosis and have significantly reduced levels of proteinuria.[4,5] In contrast, mice that lack factor H, which inhibits complement activation, are more susceptible to develop glomerulosclerosis compared with control mice.[6] Moreover, patients with FSGS have significantly higher urine and plasma levels of complement fragments compared with control subjects.[7] Furthermore, deposits of immunoglobulins such as IgM and complement proteins such as C3, C4, C1q, and mannose-binding lectin (MBL) have been found in renal biopsies from patients with FSGS.[5,8-12] In routine diagnostics, complement deposits in segmental sclerotic lesions are considered to be due to nonspecific entrapment.[13,14] Interestingly, however, both IgM and C3 deposits have also been reported in unaffected glomeruli of patients with FSGS, suggesting that complement deposits may actually play a pathogenic role in the development of FSGS.[8]

Based on these findings, we examined whether the complement system is indeed involved in the pathogenesis of FSGS. We therefore investigated whether the deposition of glomerular complement precedes the development of FSGS lesions and examined whether this deposition reflects complement activation at the glomerular level. In our study we measured the complement cleavage product C4d, because it provides a marker of complement activation long after the initial complement activators have been eliminated.[15] To study the putative relationship between glomerular C4d and the development of segmental glomerulosclerosis, we determined glomerular C4d deposition in biopsies obtained from both patients with FSGS and patients with minimal change disease (MCD). MCD resembles early primary FSGS in terms of proteinuria and foot process effacement; however, patients with MCD do not develop segmental sclerosis.[16,17] In addition, we investigated C4d deposition in allograft biopsies before the development of recurrent segmental glomerulosclerosis; specifically, biopsies of renal transplants were obtained from patients with primary FSGS in the native kidney who either developed recurrent FSGS in the transplanted kidney or did not develop recurrent disease. Finally, we confirmed that glomerular C4d deposits appear before the development of FSGS using a rat model of hereditary FSGS.

Table 1. Clinical characteristics of patients with FSGS or MCD and control subjects

	FSGS (n=40)	MCD (n=84)	Control (n=10)
Age, years, median (IQR)	45 (16-55)	19 (9-39)	52 (46-61)
Sex, male, n (%)	20 (50)	53 (63)	2 (20)
Protein-creatinine-ratio, mg/mmol, median (IQR)	806 (215-1137)	514 (154-813)	13
Protein excretion rate, g/24h, median (IQR)	5.9 (3.0-8.4)	9.1 (3.6-13.9)	0.11 (0.09-0.20)
eGFR, ml/min/1.73 m^2, mean ± SD	72 ± 40	103 ± 47	93 ± 16
Medication, n (%)			NA
No medication	0 (0)	2 (3)	
Conservative medication	12 (30)	14 (17)	
Prednisone	8 (20)	44 (52)	
Cyclosporine/ Cyclophosphamide	7 (18)	7 (8)	
Unknown	13 (32)	17 (20)	

eGFR, estimated glomerular filtration rate; IQR, interquartile range; NA, not applicable.

Results

Glomerular C4d deposition in native kidney biopsies obtained from patients with primary FSGS and patients with MCD

First, we scored glomerular C4d in the native kidneys of 40 patients with FSGS, 84 patients with MCD, and 10 healthy control subjects. Table 1 shows the clinical features and demographic data of the patient groups and control subjects. Figure 1 shows representative examples of C4d-stained sections. Glomerular deposits of C4d were observed in 73% of biopsies from patients with FSGS, which was significantly higher than in the biopsies from both patients with MCD (21%; $p<0.001$) and healthy control subjects (10%; $p<0.01$) (Figure 2A). Moreover, the percentage of C4d+ glomeruli was higher in patients with FSGS compared with both the MCD and healthy control groups (Figure 2B). When we examined only the C4d+ biopsies, we found that the median percentage of C4d+ glomeruli was similar between the FSGS (40%; IQR 21%–57%) and MCD (34%; IQR 9%–57%; $p=0.33$) groups. Segmental C4d staining was the predominant staining pattern in both the FSGS and MCD patient groups. We examined correlations between the percentage of C4d+ glomeruli and proteinuria levels both in patients with FSGS and patients with MCD. Our analysis revealed no significant correlation among patients with FSGS. With respect to patients with MCD, we found that a higher percentage of C4d+ glomeruli was correlated with a higher protein-to-creatinine ratio (rho=0.44, $p=0.021$). Supplementary Table S1 shows differences in proteinuria between C4d+ and C4d- patients.

Co-localization between C4d and sclerotic and nonsclerotic areas in FSGS and MCD

In patients with MCD, C4d deposition was observed in unaffected glomeruli based on light microscopy, with C4d positivity observed along the glomerular capillary wall (Figure 1B and I). Furthermore, we identified 3 cases with C4d+ synechiae between Bowman's capsule and the glomerular tuft (Figure 1J).

In patients with FSGS, the percentage of glomeruli with segmental sclerosis and C4d+ glomeruli in a given biopsy was significantly correlated (Figure 2C). However, C4d was also present in unaffected areas in 14% of glomeruli with segmental sclerosis (Figure 2D). Moreover, 42% of C4d+ glomeruli were nonsclerotic, with no segmental or global sclerosis (Figure 2E). To further investigate the association between C4d positivity and segmental sclerosis, we used generalized estimating equation (GEE) analysis, which revealed that the probability of a segmentally sclerotic glomerulus being C4d+ was 76% ($p<0.001$). This finding indicates that a glomerulus with segmental sclerosis is more likely to be C4d+ than C4d–. On the

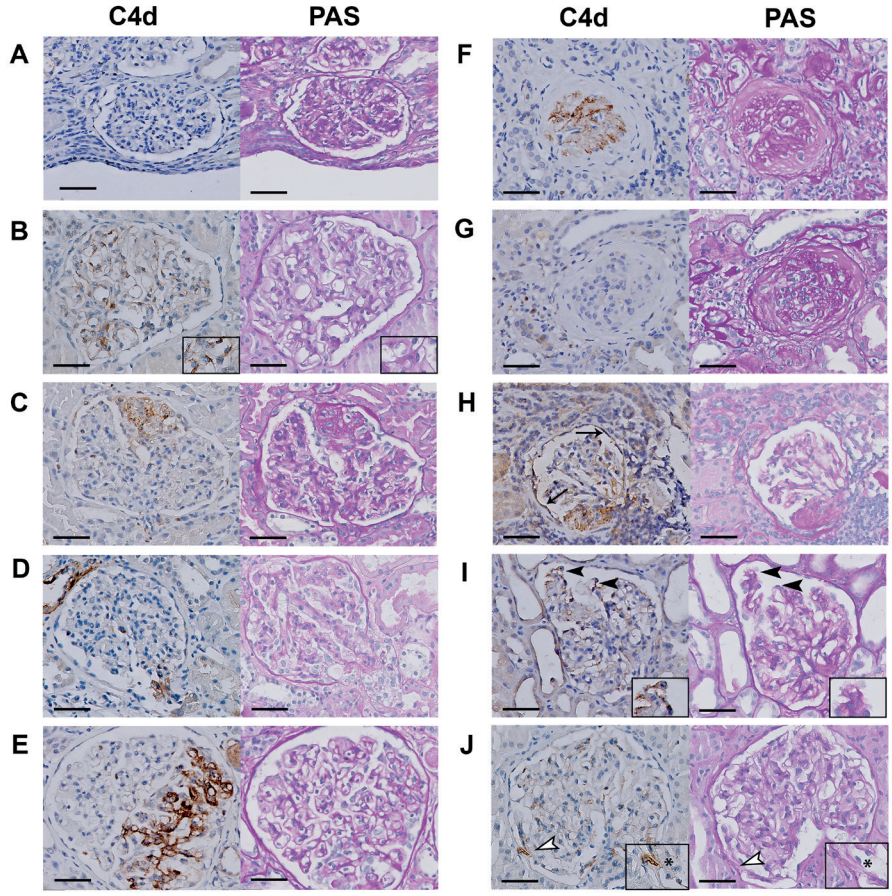


Figure 1. Sequential biopsy sections stained for C4d and periodic acid–Schiff (PAS) staining in patients with focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD).

(A) A C4d-negative (C4d–) glomerulus in a patient with FSGS. (B) Endothelial C4d staining in a patient with MCD. Insets show a detail of a C4d-positive (C4d+) capillary. (C, D) C4d deposits co-localized with FSGS lesions: (c) a C4d+, not otherwise-specified FSGS lesion, and (D) a C4d+ glomerular tip lesion. (E) C4d positivity in a patient with FSGS, predominantly in a mesangial pattern, in a glomerulus that does not show a clear FSGS lesion. (F, G) Example images of global sclerosis in FSGS, showing both C4d+ (F) and C4d– (G) staining. (H) C4d positivity at Bowman's capsule (arrows) in a patient with FSGS. (I) C4d staining in a podocyte-like pattern (arrowheads) in a patient with MCD (enlarged in insets). (J) A patient with MCD showing a C4d+ small cellular adhesion (white arrowheads, enlarged in insets), which was also observed in patients with FSGS. Bars = 50 µm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

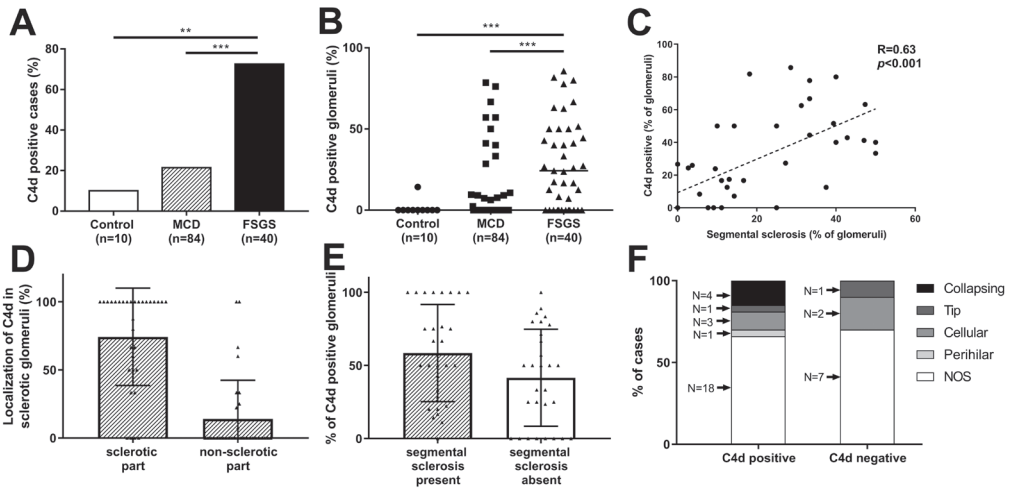
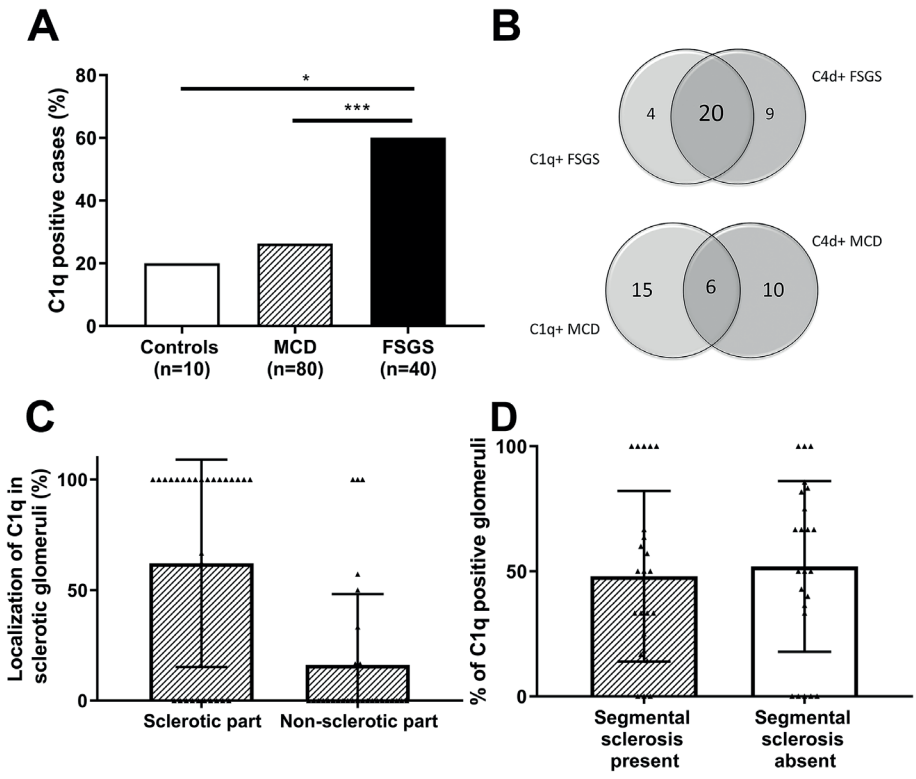


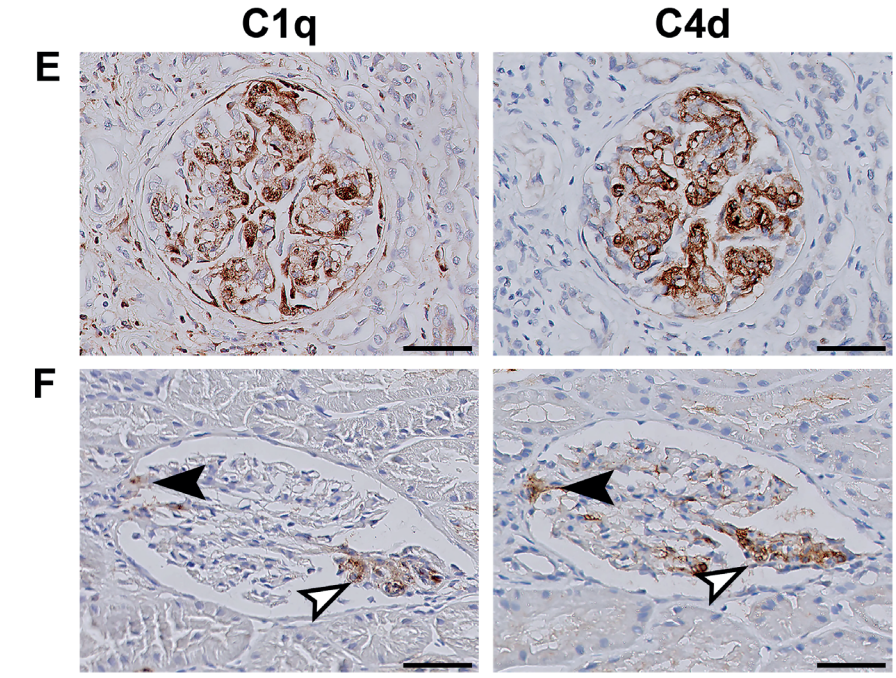
Figure 2. C4d positivity in relation to glomerulosclerosis

(A) The percentage of C4d+ cases and (B) the percentage of C4d+ glomeruli were significantly higher in patients with focal segmental glomerulosclerosis (FSGS) compared with both patients with minimal change disease (MCD) and healthy control subjects. (A) $^{**}p<0.01$ and $^{***}p<0.001$ (logistic regression); (B) $^{***}p<0.001$ (Kruskal-Wallis test). In contrast, when only considering C4d+ cases there was no significant difference in the percentage of C4d+ glomeruli between MCD and FSGS patients (data not shown). (C) The percentage of segmentally sclerotic glomeruli was directly correlated with the percentage of C4d+ glomeruli (R=0.63, $p<0.001$). (D) Summary of the mean percentage of segmentally sclerotic glomeruli in which C4d staining co-localized with sclerotic lesions and with nonsclerotic areas. (E) Summary of the mean percentage of C4d+ glomeruli in which segmental sclerosis was present or absent. (F) Distribution of FSGS variant (based on the Columbia classification) in C4d+ and C4d– cases. NOS, not otherwise specified.

Figure 3 (Right). Glomerular C1q staining in patients with native focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD)

(A) Summary of glomerular C1q positivity measured in patients with FSGS, patients with MCD, and healthy control subjects. $^{*}p<0.05$ and $^{**}p<0.001$ (logistic regression). (B) Venn diagram depicting the number of C4d+ and C1q+ patients with FSGS (top) or MCD (bottom). (C) C1q positivity within and outside segmental sclerotic areas of segmentally sclerotic glomeruli. (D) Summary of the mean percentage of C1q+ glomeruli in which segmental sclerosis was present or absent. (E, F) C1q and C4d deposits co-localized in the same staining pattern, global (E) or segmental (F). (F) White arrowheads indicate C4d and C1q co-localization with a tip lesion, and the black arrowheads indicate C4d and C1q co-localization with the vascular pole. Bars = 50 µm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.


A
C1q positive cases (%)
80
60
40
20
0
*
***
Controls (n=10)
MCD (n=80)
FSGS (n=40)
B
C4d+ FSGS
4
20
9
C1q+ FSGS
C4d+ MCD
15
6
10
C1q+ MCD
C
Localization of C1q in sclerotic glomeruli (%)
100
50
0
Sclerotic part
Non-sclerotic part
D
% of C1q positive glomeruli
100
50
0
Segmental sclerosis present
Segmental sclerosis absent


C1q
C4d
E
F

other hand, the probability of a C4d+ glomerulus being segmentally sclerotic was 53% (p=0.63), indicating that a C4d+ glomerulus is not more likely to be segmentally sclerotic than nonsclerotic. Finally, the distribution of FSGS variants based on the Columbia classification system was similar between C4d+ and C4d– patients, although all patients with collapsing FSGS were C4d+ (Figure 2F).

In addition, we examined C4d positivity in both global sclerosis and Bowman's capsule. Based on GEE analysis, the probability of a globally sclerotic glomerulus being C4d+ was 43% (p=0.50); moreover, in 40% of patients with FSGS, C4d deposition was observed in Bowman's capsule, and on average 14% ± 7% of glomeruli were affected (Figure 1F–H).

To determine whether C4d deposits co-localize with immune deposits, we analyzed both immunofluorescence and electron microscopy data (Supplementary Table S2). Our analysis showed that immune deposits were rarely present and were not correlated with C4d+ staining.

Complement pathway activation in FSGS

To determine whether C4d deposition is related to activation of the classical and/or lectin complement pathway, we stained the biopsy samples for C1q and MBL, respectively (Figure 3 and Supplementary Figure S1). The prevalence of C1q positivity in glomeruli was significantly higher in patients with FSGS (60%) compared with both MCD (26%; p<0.001) and control (20%; p<0.05) groups (Figure 3A). We found that 69% of C4d+ FSGS cases (20/29) were also C1q+ and 83% of C1q+ FSGS cases (20/24) were also C4d+ (Figure 3B). Moreover, we observed co-localization between C1q and C4d staining (Figure 3E and F). Although the association between C1q and C4d positivity was not statistically significant (p=0.06), C1q positivity was significantly associated with the percentage of C4d+ glomeruli (p=0.02). Finally, we found that 38% of C4d+ MCD cases were C1q+ and 29% of C1q+ MCD cases were C4d+ (Figure 3B). Glomerular MBL staining was observed infrequently in all patient groups, with no significant differences between the groups (Supplementary Figure S1).

We also compared our findings regarding complement activation in FSGS with our findings in diabetic nephropathy patients with proteinuria in the nephrotic range. Our analysis revealed no difference with respect to the prevalence of C4d, C1q, or MBL deposits (Supplementary Table S3).

Glomerular C4d deposition in renal allografts preceding the development of recurrent FSGS

Next, we examined whether glomerular C4d deposition precedes the development of segmental sclerotic lesions in patients with FSGS by measuring C4d deposition in renal allograft biopsies without FSGS lesions obtained from patients with native primary FSGS (Supplementary Figure S2). The clinical characteristics of these transplant recipients are summarized in Table 2. We found that patients with recurrent FSGS had higher proteinuria levels (p<0.001) at an earlier onset (p<0.05) compared with both patients without recurrent FSGS and control subjects. Renal histologic changes (other than FSGS) were similar between patients without recurrent proteinuria, patients with nonrecurrent FSGS, and transplantation control subjects. As shown in Figure 4, the prevalence of C4d positivity was significantly higher among patients with recurrent FSGS (before the development of FSGS lesions) compared with transplantation control subjects (72% and 27%, respectively; p<0.01), indicating that in patients with FSGS, C4d deposition precedes the development of sclerotic lesions. Interestingly, however, C4d positivity was similar between patients with recurrent FSGS (72%) and patients who never developed recurrent FSGS (50%; p=0.10). Apart from peak proteinuria, we found no difference between C4d+ and C4d– patients with respect to their clinical characteristics (Supplementary Table S4).

Time course of glomerular C4d deposition in Munich Wistar Frömter rats

Our data obtained from biopsies taken before the development of FSGS lesions in patients who later developed recurrent FSGS indicate that glomerular C4d deposition can precede the onset of FSGS lesions. To investigate whether C4d deposition can also predict the subsequent development of FSGS, we measured glomerular C4d deposits in Munich Wistar Frömter (MWF) rats, an FSGS model; age-matched spontaneously hypertensive rats (SHRs) were used as a control group. Kidney sections were prepared from 4-week-old, 8-week-old, and 24-week-old SHR and MWF rats and stained for C4d (Figure 5). At 4, 8, and 24 weeks of age, MWF rats have no albuminuria, only albuminuria, and both albuminuria and glomerulosclerosis, respectively.[18] As shown in Figure 5A, we found a significantly higher percentage of C4d+ glomeruli in MWF rats compared with SHRs at both 8 weeks of age (31% ± 8.5% vs. 4% ± 4.7%, respectively; p<0.001) and 24 weeks of age (25% ± 11% vs. 2% ± 4%, respectively; p<0.01); in contrast, the percentage of C4d+ glomeruli did not differ significantly between MWF rats and SHRs at 4 weeks of age (18% ± 16% vs. 2.8% ± 3.0%, respectively; p=0.06). At 24 weeks of age, glomerular C4d deposition in MWF rats was significantly correlated with the occurrence of glomerulosclerosis (R^2=0.81; p<0.01); nevertheless, in 16% of glomeruli with FSGS lesions, C4d was present in nonsclerotic segments (Figure 5B), and 50% of all C4d+ glomeruli were completely nonsclerotic (Figure 5C). In total, 12% of all glomeruli at

Table 2. Characteristics of transplanted patients

	Native FSGS with recurrence in future allograft biopsy or recurrent proteinuria (n=18)	
	No recurrent proteinuria at time of biopsy (n=5)	Recurrent proteinuria[a] at time of biopsy (n=13)
Protein excretion rate at time of biopsy, g/24 h, median (IQR)	0.30 (0.15-0.76)	1.8 (1.3-2.5)
Time between Tx and recurrence of proteinuria, months, median (IQR)	8.3 (1.2-43)	0.33 (0.1-0.66)
Peak protein excretion rate <1 year of Tx, g/24h, median (IQR)	2.9 (1.9-15.8)	4.6 (3.6-11.5)
FPE, n (%)	NA	
Extensive focal		4 (31)
Extensive diffuse		9 (69)
FSGS variant in native Bx, n (%)		
Perihilar	1 (20)	1 (8)
NOS	2 (40)	4 (31)
Cellular	0 (0)	2 (15)
Tip	0 (0)	2 (15)
Collapsing	0 (0)	0 (0)
Unknown	2 (40)	4 (31)
FSGS variant in future allograft Bx, n (%)		
Perihilar	0 (0)	0 (0)
NOS	3 (75)	1 (33)
Cellular	0 (0)	0 (0)
Tip	0 (0)	0 (0)
Collapsing	1 (25)	2 (67)
Immunosuppressive regimen, n (%)		
Prednisone	5	13 (100)
Tacrolimus	3	10 (77)
Mycophenolate	4	11 (85)
Everolimus	0 (0)	1 (8)
Cyclosporine	1 (20)	5 (39)
Plasmapheresis, n (%)	0 (0)	9 (69)
Renal histological changes, n (%)	4 (80)	4 (30)

FPE, foot process effacement; IQR, interquartile range; NA, not applicable; NOS, not otherwise specified. [a] Recurrent proteinuria was defined as >1 g/24 h. [b] Kruskal-Wallis test. [c] Mann-Whitney *U* test. [d] χ^2 or Fisher's exact test.

	Native FSGS without recurrence in allograft biopsies or recurrent proteinuria (n=16)	Transplantation controls without FSGS (n=34)	*p* value
	0.20 (0.13-0.45)	0.27 (0.14-0.45)	<0.001[b]
	NA	NA	0.026[c]
	0.94 (0.64-1.5)	NA	<0.001[b]
	NA	NA	
		NA	0.27[d]
	0 (0)		
	5 (31)		
	0 (0)		
	0 (0)		
	2 (13)		
	9 (56)		
	NA	NA	0.49[d]
	16 (100)	33 (97)	1.00[d]
	11 (69)	32 (94)	0.031[d]
	12 (75)	27 (79)	0.93[d]
	1 (6)	13 (38)	0.018[d]
	5 (31)	2 (6)	0.017[d]
	1 (6)	1 (3)	<0.001[d]
	9 (56)	15 (45)	0.27[d]

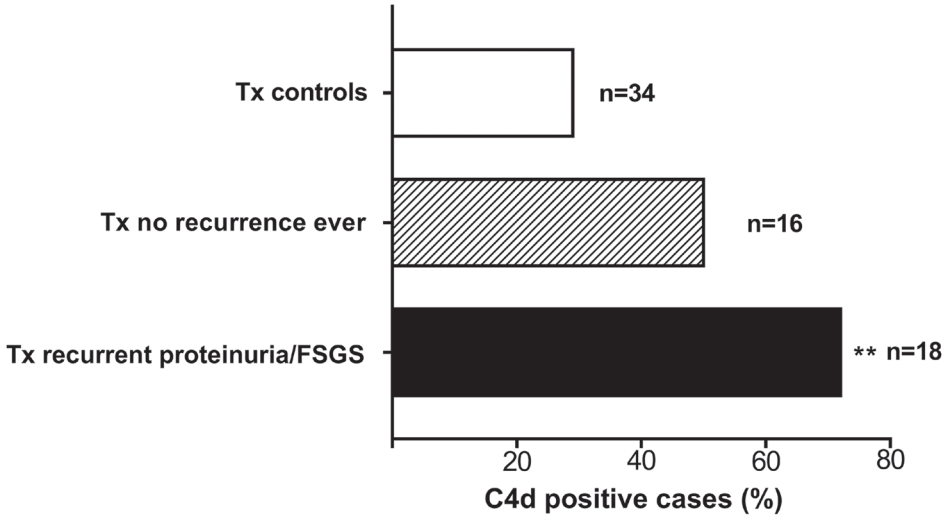
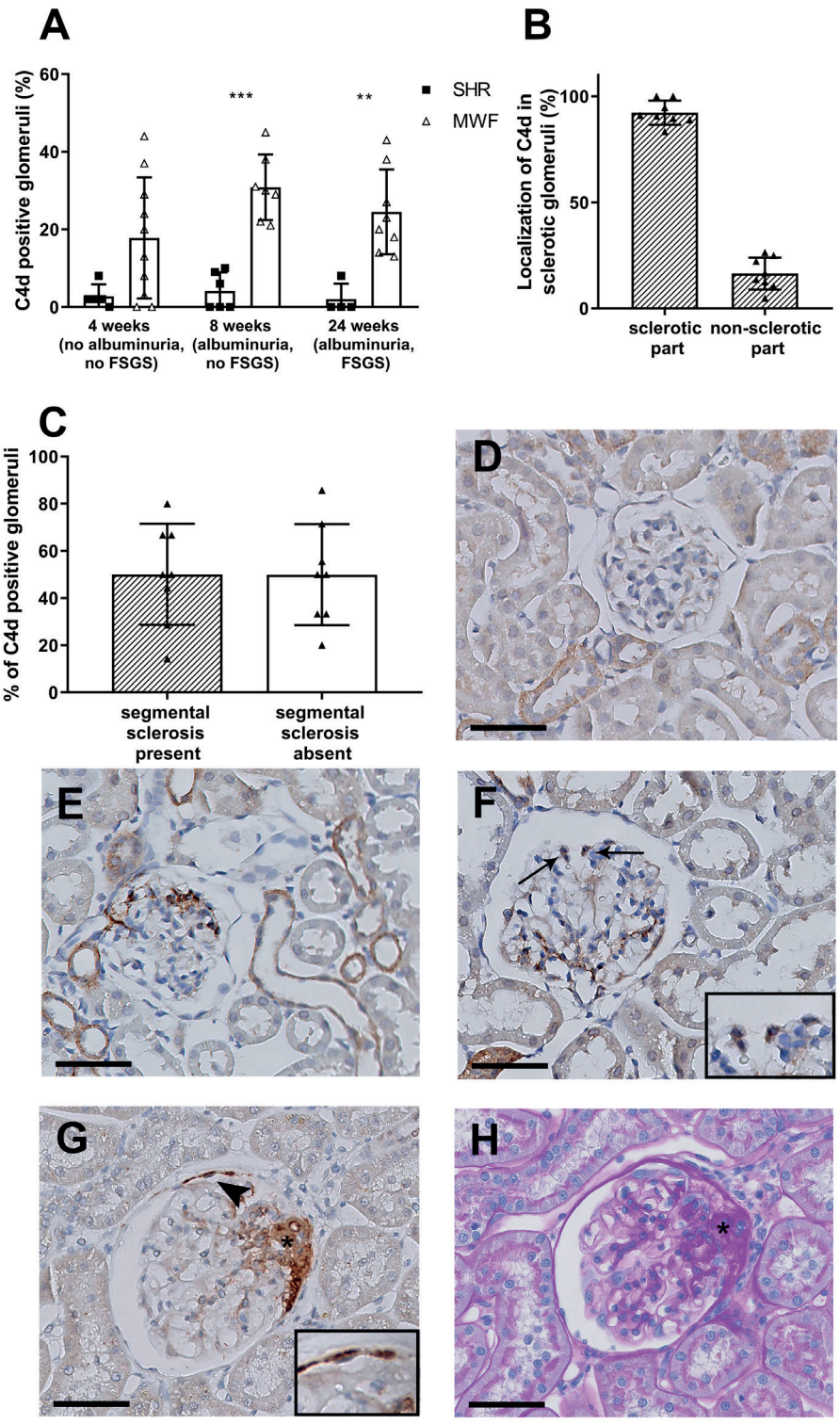


Figure 4. C4d deposition precedes the recurrence of focal segmental glomerulosclerosis (FSGS) after kidney transplantation.

Summary of C4d+ renal allograft biopsies, taken before the development of FSGS lesions, from patients who subsequently developed proteinuria/FSGS (n=18), from patients with native FSGS who never developed recurrent proteinuria/FSGS (n=16), and from transplantation control subjects without FSGS (n=34). ***p*<0.01 versus transplantation (Tx) control subjects (χ^2 test).

Figure 5 (Right). C4d positivity in the glomeruli of Munich Wistar Frömter (MWF) rats and spontaneously hypertensive rats (SHR).

(A) A summary of the percentage of C4d+ glomeruli in MWF rats and SHRs at 4, 8, and 24 weeks of age. ***p*<0.01 ****p*<0.001 (Student's *t*-test). (B) Summary of the mean percentage of segmental sclerotic glomeruli showing localization of C4d in the sclerotic and nonsclerotic regions. (C) Summary of the mean percentage of C4d+ glomeruli in which segmental sclerosis was absent or present. (D) Example image of a C4d– glomerulus. (E–G) Example images of C4d+ glomeruli in MWF rats showing a mesangial pattern (e), a podocyte-like pattern (F, arrows, enlarged in inset), and a segmental pattern (G, asterisk), which was often co-localized with segmental sclerotic lesions (H, asterisk). Note that C4d positivity is also visible at Bowman's capsule (G, arrowhead, enlarged in inset). Bars = 50 μm. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.


A
C4d positive glomeruli (%)
60
40
20
0
***
**
SHR
MWF
4 weeks (no albuminuria, no FSGS)
8 weeks (albuminuria, no FSGS)
24 weeks (albuminuria, FSGS)
B
Localization of C4d in sclerotic glomeruli (%)
100
50
0
sclerotic part
non-sclerotic part
C
% of C4d positive glomeruli
100
80
60
40
20
0
segmental sclerosis present
segmental sclerosis absent
D
E
F
G
H

24 weeks of age were C4d+ without segmental sclerosis. Overall, the probability of a segmentally sclerotic glomerulus being C4d+ was 94% (GEE; $p<0.001$), whereas the probability of a C4d+ glomerulus being segmentally sclerotic was only 57% ($p=0.40$), indicating that a segmental sclerotic glomerulus is more likely to be C4d+ than C4d–, whereas a C4d+ glomerulus is not more likely to be segmentally sclerotic than nonsclerotic. Similarly, the probability that a globally sclerotic glomerulus is C4d+ was 82% ($p<0.01$). Representative examples of C4d staining in MWF rats are shown in Figure 5D through G. Finally, we observed C4d positivity in Bowman's capsule in 24-week-old MWF rats (Figure 5G, arrowhead); this positivity was found in 7% ± 3.5% of all glomeruli in MWF rats but was absent in the glomeruli of SHRs.

Discussion

Here, we determined glomerular deposition of C4d in native biopsies obtained from patients with primary FSGS, in allograft biopsies collected before the recurrence of FSGS, and in MWF rats, an established model for studying FSGS. We found that FSGS lesions are associated with glomerular C4d deposition. Moreover, our analysis revealed that C4d accumulates in the glomeruli before the development of FSGS, in FSGS patients who received a kidney transplant, and in MWF rats, indicating that glomerular C4d deposition precedes the development of FSGS lesions.

The deposition of complement proteins in sclerotic lesions in FSGS is often considered to be the result of nonspecific entrapment of macromolecules.[14] However, we found that segmental sclerosis—but not global sclerosis—was associated with C4d deposits, suggesting that sclerosis in itself is not sufficient to lead to the entrapment of complement proteins. Differences in C4d deposition between global and segmental sclerosis may be due to differences in how these lesions develop; for example, global sclerosis can develop because of age-dependent physiologic processes.[19,20] In addition, we found C4d deposits in the unaffected glomeruli of patients with FSGS and in glomeruli with adhesion lesions, which may indicate early FSGS lesions that are not yet identifiable using light microscopy.

The clinical relevance of glomerular complement deposits in FSGS is currently a topic of study. One of the first studies to investigate this relevance was reported 3 decades ago by Habib *et al.*,[21] who found no difference in clinical outcome between patients with complement deposits and patients without complement deposits. However, a 2016 study by Zhang *et al.*[22] showed that the combination of IgM and C3 deposits can predict poor renal survival in patients with FSGS. Moreover, Thurman *et al.*[7] reported that plasma levels of complement activation fragments in patients with

FSGS are correlated with disease severity. Interestingly, mouse models of FSGS have been used to show that complement activation contributes to glomerulosclerosis; specifically, loss of complement factor C3 and complement factor D, as well as depletion of IgM, reduces the degree of glomerulosclerosis, whereas loss of complement-inhibiting factor H increases the degree of glomerulosclerosis.[4-6]

We found that C1q positivity was more prevalent in patients with FSGS compared with both patients with MCD and healthy control subjects; in contrast, MBL+ staining was relatively rare, suggesting that the classical complement pathway likely plays a predominant role in the pathogenesis of FSGS. This finding is consistent with previous results showing that IgM, which activates the classical complement pathway, is required for the development of adriamycin-induced FSGS.[5,8,22] Although 83% of C1q+ biopsies were C4d+ and although C1q and C4d staining were frequently co-localized, C4d and C1q did not always co-localize. A possible explanation may be that C1q deposits are less stable than C4d deposits, which would also explain that a relatively high percentage (i.e., 30%) of C4d+ samples were C1q–.

In FSGS, complement activation is likely a downstream mechanism that contributes to the formation of sclerotic lesions, and studies support the notion that complement activation is a second "hit" after podocyte damage in the development of FSGS. For example, in the above-mentioned animal models of induced FSGS, although the development of glomerulosclerosis is reduced in mice lacking specific complement factors, proteinuria and podocyte damage still developed, indicating that complement activation is a secondary process in the development of FSGS.[4,5] Similarly, our data obtained with MWF rats show that compared with SHR controls, C4d deposition is significantly increased at the onset of albuminuria, indicating that podocyte injury was present before complement activation.[23] Interestingly, at 24 weeks of age nearly all sclerotic glomeruli in the MWF rats were C4d+, although the total number of C4d+ glomeruli did not increase compared with rats at 8 weeks of age. This finding suggests that the C4d+ glomeruli at 8 weeks of age are likely the glomeruli that will develop segmental glomerulosclerosis by 24 weeks of age.

There are several possible mechanisms by which podocyte damage can trigger complement activation. First, Strassheim *et al.*[5] proposed that activation of the classical complement pathway in FSGS is caused by the binding of naturally occurring IgM antibodies to podocytes via neo-epitopes generated during the initial podocyte injury. Two C4d+ FSGS patients had lesions reminiscent of electron-dense deposits on electron microscopy. One of these patients met the criteria for C1q nephropathy, and the presence of C4d deposits could be related to this. Nevertheless, our results do not necessarily suggest that immune complexes are a common cause of C4d

deposits in FSGS, because immune deposits were present only rarely and were not significantly correlated with C4d deposits. It is still possible that the binding of natural IgM antibodies to cell-surface proteins causes complement activation,[24] because this binding does not necessarily lead to stable, detectable immune complexes; alternatively, the classical complement pathway could be activated by an antibody-independent process.[25] Second, severe podocyte damage and/or podocyte loss could lead to increased complement activation via a loss of complement-regulating factors; consistent with this hypothesis, Barisoni *et al.*[26] reported the selective loss of the C3b and C4b receptor CR1 in podocytes of patients with collapsing FSGS but not in patients with MCD. Loss of—and/or damage to—podocytes also causes endothelial injury, which can lead to complement activation.[27] In contrast, complement-mediated endothelial injury could also lead to podocyte injury. We previously reported that C4d is often present in the kidneys of patients with extensive endothelial injury,[28-31] and others have shown that complement activation is involved in endothelial injury in a variety of kidney diseases.[32-35] Experimental models have shown that podocyte damage can follow primary endothelial cell injury,[36] and subsequent podocyte depletion has been suggested as the final common pathway resulting in proteinuria and reduced estimated glomerular filtration rate.[37-39] Similarly, complement-mediated endothelial injury may lead to podocyte injury in FSGS as well, a notion supported by a growing body of experimental and clinical data suggesting that endothelial injury is involved in FSGS.[40-45] Finally, complement activation in FSGS could be due to increased susceptibility at the genetic level. In support of this notion, mutations in complement-regulating genes have been described in patients with FSGS,[46] and it is possible that the mutation burden in complement-related genes is under-recognized in patients with FSGS, similar to a study showing that genetic abnormalities in complement genes have been under-recognized in patients with malignant hypertension.[47]

Our finding of C4d deposits in Bowman's capsule is particularly interesting, because the activation of parietal epithelial cells lining Bowman's capsule—and their migration to the glomerular tuft—is believed to be an early step in the formation of FSGS lesions.[48] We observed C4d positivity in Bowman's capsule in both sclerotic and nonsclerotic glomeruli in both MWF rats and FSGS patients. Morigi *et al.*[6] found that glomerular complement activation contributes to the activation of parietal epithelial cells and that parietal epithelial cell markers co-localize with complement factor C3. We sporadically observed C4d positivity in the synechiae and/or adhesion lesions in both FSGS patients and MCD patients and hypothesize that these reflect activated parietal epithelial cells migrating to the glomerular tuft.[48]

Making a distinction between MCD and (early) FSGS can be difficult because of sampling error of focal lesions. Our findings that C4d deposits are present (i) in nonsclerotic glomeruli of patients with FSGS, (ii) in the (early) development of FSGS, and (iii) at the site of small adhesions suggest that the presence of C4d deposits in an otherwise normal biopsy might be indicative of (early) FSGS. However, given that some cases with MCD also had C4d deposits, our data do not necessarily suggest that C4d staining alone is sufficient for discriminating between FSGS and MCD. C4d is used as a diagnostic marker at various institutions worldwide. However, there is considerable variability in C4d staining procedures across institutions,[49] and C4d deposition should be validated in other centers before applying the staining in the clinic.

Our data obtained in patients with recurrent FSGS in the allograft kidney suggest that C4d deposition precedes the development of FSGS lesions. Post-transplantation C4d deposition in FSGS has been described previously but only in the context of case reports or relatively small studies, which often analyzed C4d staining only in peritubular capillaries.[50-56] In our cohort of patients with recurrent FSGS, we observed a high percentage of cases in which glomerular C4d staining preceded the development of FSGS lesions. The deposition of C4d cannot be explained by the presence of antibody-mediated rejection or transplant glomerulopathy, because these cases were excluded from our analysis. Although the pathogenic process underlying recurrent FSGS is currently unknown, its rapid onset suggests the involvement of a circulating factor. Future studies investigating recurrent FSGS should take into account the possible role of the complement system.

In addition to our finding of complement activation in patients with FSGS, we also observed evidence of complement activation in patients with diabetic nephropathy and overt proteinuria, which is consistent with previous reports.[28,57-64] Complement activation has been associated with a variety of proteinuric glomerular diseases that have a significant risk of developing glomerulosclerosis and end-stage renal disease.[58,65-67] However, the pathogenesis of these glomerular diseases differs widely, and the complement system may be activated because of a variety of causes. Thus, future studies should investigate whether complement activation is a common mechanism leading to glomerulosclerosis and end-stage renal disease.
Although we attempted to minimize heterogeneity among the patients with FSGS by excluding causes of secondary FSGS, our patient group was still relatively heterogeneous, including various levels of proteinuria and FSGS variants. To address this issue we used the MWF rat model, which has a highly homogenous phenotype, yielding data similar to our patient data. Future research should be designed to determine whether—and at what point in the complement system

pathway—inhibitors might be beneficial in patients with FSGS. For example, a subgroup of patients with steroid-resistant FSGS is already being treated with the B cell–depleting antibody rituximab.[68] Given our evidence supporting a role for the classical complement pathway in FSGS, rituximab may serve to protect these patients from complement-mediated injury by depleting B cells.

In conclusion, we provide evidence suggesting that glomerular C4d deposition precedes the development of FSGS lesions, arguing against the hypothesis that nonspecific entrapment in sclerotic lesions is the sole mechanism underlying glomerular complement deposition in these patients. Given that C4d is a stable biomarker of complement activation, our data provide compelling evidence that complement activation plays a role in the pathogenesis of FSGS.

Methods

Biopsy samples

Biopsies from patients with native FSGS and patients with MCD were obtained from the archives of the Department of Pathology at Leiden University Medical Center; the biopsies were originally obtained from 2001 through 2017 (FSGS) and from 1985 through 2017 (MCD). FSGS and MCD were confirmed based on pathology reports. Exclusion criteria for FSGS included secondary FSGS (defined by renal comorbidity other than MCD, virus-induced FSGS, or medication-induced FSGS). We included patients with hypertension, atherosclerosis, and/or obesity in which it was unclear whether these clinical findings caused FSGS. Patients were not selected based on genetic background, because genetic testing was performed infrequently. We also included 10 pretransplantation biopsies from living donors as healthy control subjects and 8 biopsies from patients with diabetic nephropathy and proteinuria in the nephrotic range (but without FSGS) as a control group of patients with proteinuria and a high risk of developing end-stage renal disease.

In addition, we obtained renal allograft biopsies collected from patients who received a kidney transplant due to primary FSGS. Patients with the following features were considered to have recurrent FSGS: the recurrence of continuously unexplained proteinuria (>1 g/day) within 1 year of renal transplantation and/or histologic evidence of FSGS in any subsequent biopsy of the allograft kidney measured through the end of the study period (December 31, 2017). Patients who did not develop unexplained, continuous proteinuria within 1 year of transplantation and with an absence of FSGS lesions in follow-up transplant biopsies were defined as recurrence-free patients. From patients with recurrent FSGS, we included biopsies taken before

the onset of recurrent FSGS lesions; as control samples, we used transplant biopsies taken within 1 year after transplantation from patients without FSGS in the native and allograft kidneys. Detailed inclusion and exclusion criteria are included in the Supplementary Material (Supplementary Methods and Supplementary Figure S2). After applying the inclusion and exclusion criteria, 20 biopsies from 16 patients with nonrecurrent FSGS, 24 biopsies from 18 patients with recurrent FSGS, and 39 biopsies from 34 transplantation control subjects were included in the final analysis.

Animals

Male MWF rats and SHRs were obtained from the colony at the Charité-Universitätsmedizin Berlin.[18] The kidneys were collected from MWF rats and SHRs at ages 4 (10 and 5 rats, respectively), 8 (7 and 6 rats, respectively), and 24 weeks (8 and 4 rats, respectively). MWF rats were used as a model for FSGS. At 4 weeks of age MWF rats do not have albuminuria or glomerulosclerosis. At 8 weeks of age they have significantly increased urinary albumin excretion but no FSGS lesions, and at 24 weeks of age they have both progressive proteinuria and FSGS.[69] SHRs were used as hypertensive controls without albuminuria or FSGS.[70]

Immunohistochemistry

Adjacent 4-µm-thick sections of paraffin-embedded kidney or renal biopsy samples were stained for C4d, C1q, MBL, or periodic acid–Schiff. C4d staining was performed using a rabbit anti-human C4d primary antibody (1:75; Biomedica, Vienna, Austria) or rabbit anti-rat C4d primary antibody (1:100; Hycult Biotech, Uden, the Netherlands). C1q staining was performed using a rabbit anti-human C1q primary antibody (1:1200; Dako, Glostrup, Denmark) and MBL staining using a rabbit anti-human primary antibody (1:450; Atlas antibodies, Bromma, Sweden). The appropriate negative control fraction at the same concentration as the primary antibody was used as negative control. As positive controls for C4d, C1q, and MBL staining, we used a patient with antibody-mediated rejection, a patient with lupus nephritis, and a liver resection sample, respectively. An EnVision horseradish peroxidase–conjugated antibody (Dako) was used as the secondary antibody. Sections were counterstained with hematoxylin. C4d–, C1q–, MBL-, and periodic acid–Schiff-stained sections were scanned using an ultra-fast scanner (Philips Biotechnology, Amsterdam, the Netherlands).

Scoring

For patient biopsies, all scorable glomeruli within a section were scored as C4d-positive (C4d+) or C4d-negative (C4d–). If at least 1 nonglobally sclerosed glomerulus contained C4d in any part of the glomerular tuft, the patient was considered to be C4d+. C1q and MBL were scored similarly to C4d. C4d positivity in glomeruli

in SHR and MWF rats at 4, 8, and 24 weeks of age was measured by scoring 30 to 40 glomeruli in randomly chosen microscopy fields. The localization of C4d with respect to glomerular lesions was determined by examining sequential periodic acid–Schiff-stained sections. A detailed description of the scoring of C4d and C1q can be found in the Supplementary Methods.

Statistical analysis

Data were analyzed using IBM SPSS Statistics, version 23.0 (IBM Corp., Armonk, NY). Dichotomous data were analyzed using logistic regression or the χ^2/Fisher's exact test. Continuous data were analyzed using the Student's *t*-test, Mann-Whitney U test, or Kruskal-Wallis test. Correlations were analyzed using the Pearson's or Spearman's correlations. Summary data are presented as the mean ± SD, median with interquartile range, or as a number with percentage. Binary outcomes for individual glomeruli were used to determine the probability of C4d positivity for sclerotic glomeruli and the probability of sclerosis for C4d+ glomeruli. To take into account the correlation among glomeruli in the same patient or rat, we used logistic regression within GEE analysis. Alpha was set to 0.05.

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Supplementary materials

Supplementary Methods

Inclusion criteria of allograft biopsies from patients with primary focal segmental glomerulosclerosis (FSGS):

- Renal allograft biopsies collected from patients who received a kidney transplant due to primary FSGS in the native kidney.

Exclusion criteria for allograft biopsies from patients with primary FSGS:

- Renal co-morbidity related to secondary FSGS in the native kidney, renal viral infection, or drug-induced toxicity;
- confounding factors underlying C4d positivity (e.g., thrombotic microangiopathy, antibody-mediated rejection, or transplant glomerulopathy);[S1]
- inadequate tissue for evaluation.

Inclusion criteria for allograft control biopsies:

- Transplant biopsies taken within one year after transplantation from patients without FSGS lesions in the native and allograft kidneys.

Exclusion criteria for allograft control biopsies:

- Confounding factors for C4d positivity in the native and/or transplant biopsy, or
- unexplained proteinuria within one year of transplantation.

Defining positivity in all biopsy samples:

- In a human biopsy section, all glomeruli within a section were analysed.
- If at least one non-globally sclerosed glomerulus contained C4d in any part of the glomerular tuft, the patient was considered to be C4d+.
- The percentage of C4d+ glomeruli was calculated based on the total number of non-globally sclerosed glomeruli per section. In separate analyses, C4d deposition was studied in relation to global sclerosis.
- The localization of C4d deposits within segmental and global sclerosis, within Bowman's capsule, and within adhesion lesions was determined by examining sequential PAS-stained sections.
- In spontaneously hypertensive rats (SHR) and Munich Wistar Frömter (MWF) rats at 4, 8, and 24 weeks of age, positivity was analyzed by scoring 30-40 glomeruli in randomly chosen microscopy fields.
- In addition, at 24 weeks of age the co-localization of C4d deposits with segmental sclerosis and/or Bowman's capsule was determined for all glomeruli within the section.

Clinical and histological data collection:
The following clinical data were collected at the time of biopsy:

- protein-to-creatinine ratio,
- protein excretion rate, and/or;
- dipstick.
- eGFR.
- medication used.

The following histological data were collected:

- FSGS variant in the allograft kidney.
- FSGS variant in the native kidney. FSGS variant in the native biopsy was unavailable for 15 kidney transplant recipients, who were diagnosed with FSGS at another medical center.

Immune complex deposition

- IgM, IgG, and IgA immunofluorescence data were obtained from the original pathology reports for patients with FSGS and patients with Minimal change disease.
- Electron microscopy (EM) data regarding electron-dense deposits were obtained from the original pathology reports and/or from 10,000x images taken from patients for whom tissue was available for EM.

[S1]Haas M, Loupy A, Lefaucheur C, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am J Transplant*. 2018;18(2):293-307.

Supplementary Table S1. Proteinuria levels in patients with focal segmental glomerulosclerosis (FSGS) and minimal change disease (MCD) stratified by C4d positivity.

	FSGS		*p* value	MCD		*p* value
	C4d+	C4d-		C4d+	C4d-	
Protein-creatinine-ratio (mg/mmol), median (IQR)a	856 (206-1210)	556 (218-887)	0.43	672 (515-1418)	221 (43-810)	0.019
Protein excretion rate (g/24h), median (IQR)[b]	5.8 (3.4-8.8)	7.7 (2.6-8.4)	0.75	13.3 (5.3-14.9)	8.6 (3.5-13.2)	0.29
KDIGO category[c]			0.46			0.13
Normal to mildly increased	1 (4)	0 (0)		0 (0)	1 (2)	
Moderately increased	0 (0)	1 (10)		0 (0)	10 (18)	
Severely increased	27 (96)	9 (90)		16 (100)	43 (80)	

FSGS: focal segmental glomerulosclerosis; MCD: minimal change disease; IQR: interquartile range; KDIGO: Kidney Disease: Improving Global Outcomes
[a] Protein-creatinine ratio was calculated for 25 patients with FSGS and 28 patients with MCD.
[b] Protein excretion rate was present for 20 patients with FSGS and 43 patients with MCD.
[c] KDIGO category (combined Protein-creatinine ratio, protein excretion rate and dipstick was present for 38 patients with FSGS and 70 patients with MCD.

Supplementary Table S2. immunofluorescence and electron microscopy data patients with FSGS or MCD

	FSGS		*p* value	MCD		*p* value
	C4d+	C4d-		C4d+	C4d-	
IgM positive, n (%)[ab]	4 (15)	1 (10)	0.70	3 (18)	10 (15)	0.82
IgG positive, n (%)	1 (4)	0 (0)	0.54	0 (0)	3 (5)	0.37
IgA positive, n (%)	0 (0)	0 (0)	NA	1 (6)	2 (3)	0.51
Electron dense deposits, n (%)[c]	2 (14)	0 (0)	0.37	2 (13)	1 (2)	0.11

FSGS: focal segmental glomerulosclerosis; MCD: minimal change disease
[a] n=37 for patients with FSGS, n=82 for patients with MCD.
[b] One patient with FSGS and two patients with MCD showed 2+ IgM. All other positive cases were 1+.
[c] n=21 for FSGS and n=72 for patients with MCD.

Supplementary Table S3. Clinical characteristics and complement deposition in patients with FSGS and patients with diabetic nephropathy.

	FSGS (n=40)	DN (n=8)	*p* value
Age, years, median (IQR)	45 (16-55)	57 (44-64)	0.09
Sex, male, n (%)	20 (50)	53 (63)	0.26
Proteinuria, g/24h, median (IQR)	5.9 (3.0-8.4)	7.0 (4.6-8.5)	0.49
eGFR, ml/min/1.73 m^2, mean ± SD	72 ± 40	63 ± 37	0.54
C4d positive, n (%)	29 (73)	6 (75)	1.00
C1q positive, n (%)	24 (60)	6 (75)	0.69
MBL positive, n (%)	1 (3)	0 (0)	1.00

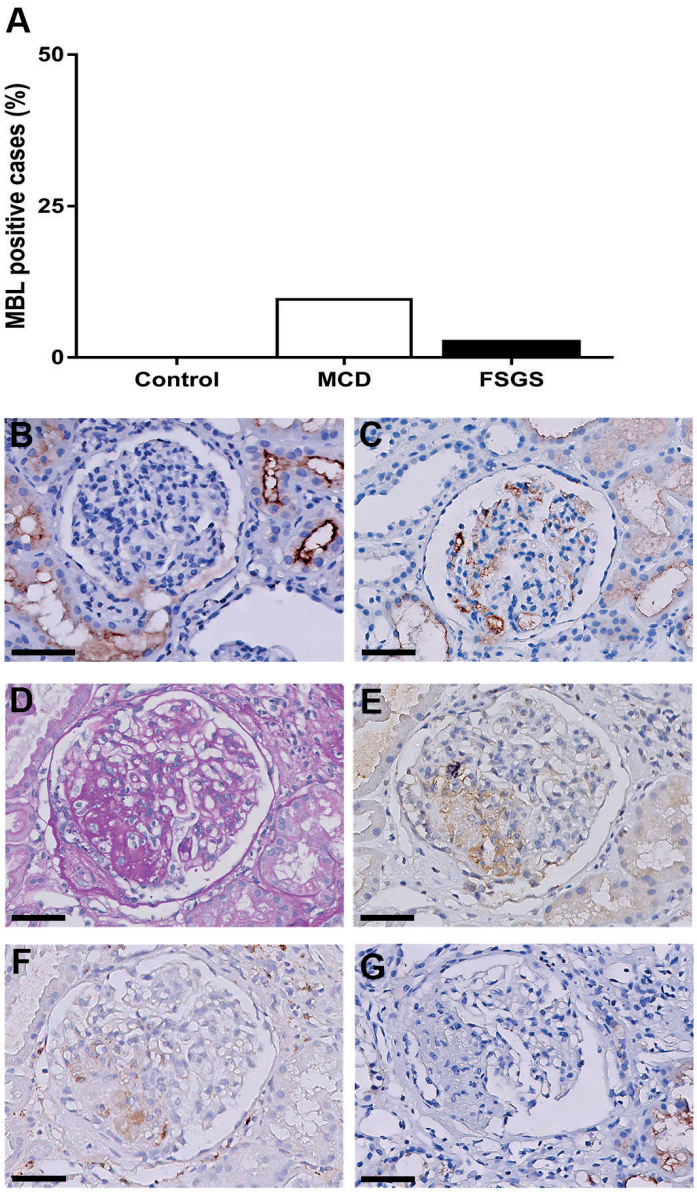
FSGS: focal segmental glomerulosclerosis; DN: diabetic nephropathy; IQR: interquartile range; SD: standard deviation; eGFR: estimated glomerular filtration rate; MBL: mannose-binding lectin

Supplementary Table S4. Characteristics of patients with a C4d-positive or C4d-negative transplant biopsy with a native diagnosis of FSGS

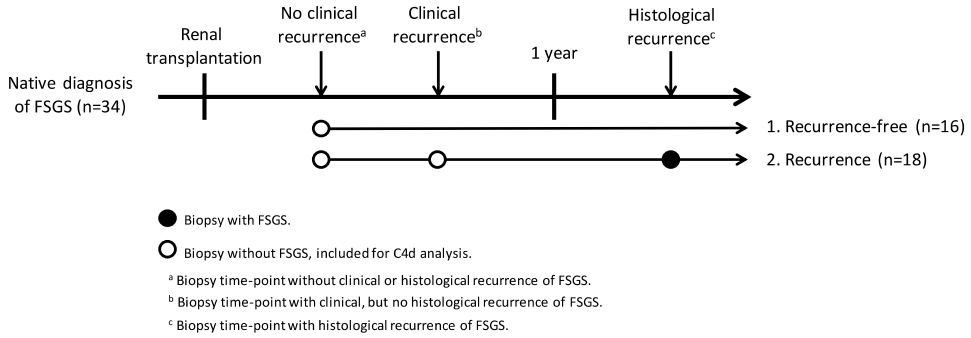
	Transplant biopsies of native FSGS patients without segmental sclerotic lesions (n=34)		*p* value
	C4d-positive n=21	C4d-negative n=13	
Protein excretion rate at time of biopsy, g/24 h, median (IQR)	1.0 (0.24-1.6)	0.32 (0.14-2.0)	0.51[a]
Time between Tx and recurrence of proteinuria, months, median (IQR)	0.66 (0.29-2.5)	0.33 (0.1-13)	0.75[a]
Peak protein excretion rate <1 year of Tx, g/24h, median (IQR)	2.7 (1.0-4.5)	1.6 (0.57-10.1)	0.80
FPE, n (%)	NA	NA	
Extensive focal			
Extensive diffuse			
FSGS in native Bx, n (%)			0.73[c]
Perihilar	2 (10)	0 (0)	
NOS	7 (33)	4 (31)	
Cellular	2 (10)	0 (0)	
Tip	1 (4)	1 (8)	
Collapsing	2 (10)	0 (0)	
Unknown	7 (33)	8 (61)	
FSGS in future allograft Bx, n (%)			0.49
Perihilar	0 (0)	0 (0)	
NOS	3 (75)	1 (33)	
Cellular	0 (0)	0 (0)	
Tip	0 (0)	0 (0)	
Collapsing	1 (25)	2 (67)	
Immunosuppressive regimen, n (%)			
Prednisone	21 (100)	13 (100)	NA
Tacrolimus	16 (76)	8 (61)	0.45[c]
Mycophenolate	17 (81)	10 (77)	1.00[c]
Everolimus	2 (10)	0 (0)	0.51[c]
Cyclosporine	5 (24)	6 (46)	0.26[c]
Plasmapheresis, n (%)	8 (38)	2 (15)	0.25[c]
Renal histological changes, n (%)	5 (39)	12 (57)	0.29[c]

FSGS: focal segmental glomerulosclerosis; IQR: interquartile range; FPE: foot process effacement; Bx: biopsy; eGFR: estimated glomerular filtration rate; NA: not applicable; NOS: not otherwise specified.
[a] Mann-Whitney U test. [b] n=1 and n=2 for C4d+ and C4d-, respectively. [c] χ^2 test or Fisher's exact test.

Transplant biopsies of patients with recurrent proteinuria or recurrent FSGS in future biopsies (n=18)		*p* value	Native FSGS without recurrence in allograft biopsies or recurrent proteinuria (n=16)		*p* value
C4d-positive n=13	C4d-negative n=5		C4d-positive n=8	C4d-negative n=8	
1.2 (0.76-2.4)	1.8 (0.95-2.25)	0.59[a]	0.27 (0.11-0.81)	0.19 (0.12-0.33)	0.71[a]
0.66 (0.27-2.5)	0.1 (0.05-6.4)	0.12[a]	2[b]	108[b]	0.22[a]
4 (2.8-6.0)	11 (10.6-12.8)	0.03	1 (0.74-1.5)	0.77 (0.42-1.6)	0.34
		0.76	NA	NA	
3 (33)	1 (25)				
6 (67)	3 (75)				
		0.77[c]			0.43[c]
2 (20)	0 (0)		0 (0)	0 (0)	
5 (50)	1 (20)		2 (20)	3 (38)	
2 (20)	0 (0)		0 (0)	0 (0)	
1 (10)	1 (20)		0 (0)	0 (0)	
0 (0)	0 (0)		2 (20)	0 (0)	
0 (0)	3 (60)		4 (50)	5 (62)	
		0.49	NA	NA	
0 (0)	0 (0)				
3 (75)	1 (33)				
0 (0)	0 (0)				
0 (0)	0 (0)				
1 (25)	2 (67)				
13 (100)	5 (100)	NA	8 (100)	8 (100)	NA
10 (77)	3 (60)	0.58[c]	6 (75)	5 (63)	1.00[c]
11 (85)	4 (80)	1.00[c]	6 (75)	6 (75)	1.00[c]
1 (8)	0 (0)	1.00[c]	1 (12.5)	0 (0)	1.00[c]
3 (23)	3 (60)	0.27[c]	2 (25)	3 (38)	1.00[c]
7 (54)	2 (40)	1.00[c]	1 (12.5)	0 (0)	1.00[c]
6 (46)	2 (40)	1.00[c]	6 (75)	3 (38)	0.32[c]



Supplementary Figure S1. Glomerular MBL staining in patients with native FSGS and MCD. (A) Summary of the prevalence of MBL+ glomerular staining in patients with FSGS, patients with MCD, and healthy controls. The three groups did not differ significantly (p=0.50). (B) Example image of an MBL- glomerulus in a patient with FSGS. Note the MBL+ staining at the brush border in the tubules, as described previously (Hirano M, Ma BY, Kawasaki N, et al. J Immunol. 2005;175(5):3177-3185), which serves as an internal positive control. (C) Example image of an MBL+ glomerulus in a patient with MCD. (D-G) Adjacent sections of a segmentally sclerotic glomerulus in a patient with FSGS. Segmental sclerotic lesions (D; Periodic acid-Schiff) were positive for C4d (E) and C1q (F), but negative for MBL (G), suggesting involvement of the classical pathway in FSGS. The scale bars represent 50 µm.



Supplementary Figure S1: Glomerular MBL staining in patients with native FSGS and MCD.

(A) Summary of the prevalence of MBL+ glomerular staining in patients with FSGS, patients with MCD, and healthy controls. The three groups did not differ significantly (p=0.50). (B) Example image of an MBL- glomerulus in a patient with FSGS. Note the MBL+ staining at the brush border in the tubules, as described previously (Hirano M, Ma BY, Kawasaki N, et al. *J Immunol.* 2005;175(5):3177-3185), which serves as an internal positive control. (C) Example image of an MBL+ glomerulus in a patient with MCD. (D-G) Adjacent sections of a segmentally sclerotic glomerulus in a patient with FSGS. Segmental sclerotic lesions (D; Periodic acid-Schiff) were positive for C4d (E) and C1q (F), but negative for MBL (G), suggesting involvement of the classical pathway in FSGS. The scale bars represent 50 µm.