

Glomerular C4d Staining Does Not Exclude a C3 Glomerulopathy



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Introduction: C4d, an early product in the classical/lectin complement pathway has shown potential in the evaluation of C3 glomerulopathy where its absence would support an alternative pathway abnormality. As autoimmune/genetic complement testing is not readily available to most parts of the world, glomerular C4d staining may serve as a useful additional step toward the diagnosis.

Methods: To test this hypothesis, C4d staining was performed on a large cohort of C3 glomerulopathy. Archival cases from 2011 to 2017 were reviewed and immunohistochemistry for C4d was performed, scored (scale of 0 to 3+), and correlated with the immunofluorescence and ultrastructural findings. Paraffin immunofluorescence was performed in cases of “discordant C4d” to unmask Igs.

Results: Twenty-seven cases of dense deposit disease (DDD) and 14 cases of C3 glomerulonephritis (C3GN) were retrieved. C4d demonstrated a range of staining intensities with negative/traces in only 22% of DDD and 64% of C3GN. Lower-intensity C4d staining (1 to 2+) was mostly concordant with similar amounts of Igs/C1q. Discordant 3+ staining was noted in approximately 50% of cases of DDD and 20% of cases of C3GN. Among them, paraffin immunofluorescence unmasked polyclonal Igs in 2 of 5 cases of DDD and 1 of 3 cases of C3GN.

Conclusion: This observational study suggests that the presence of glomerular C4d should not exclude a C3 glomerulopathy. In lower intensities, it appears to represent overlying classical/lectin pathway activation with concordant Ig/C1q deposits. A subset of cases, however, displays intense and discordant C4d staining, which raises the possibility of an associated lectin pathway abnormality, a potential future area of study.

Kidney Int Rep (2019) 4, 698–709; <https://doi.org/10.1016/j.ekir.2019.02.006>

KEYWORDS: C3 glomerulonephritis; C3 glomerulopathy; C4d; dense deposit disease; lectin pathway

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Direct immunofluorescence (DIF) remains the cornerstone in the evaluation of any proliferative glomerulonephritis (GN) and directs the pathologist to the underlying etiopathogenesis. Based on DIF studies, GN can be divided into pauci immune, immune complex-mediated, antiglomerular basement membrane antibody GN, monoclonal Ig-mediated GN, and C3 glomerulopathy.¹ C3 glomerulopathy, which is driven by abnormalities in the alternative complement pathway, is suspected when DIF demonstrates dominant C3c deposition. The definition of dominant was clarified

by the C3 glomerulopathy consensus report² as “C3c immunofluorescence 2 or more orders of magnitude above any Ig on a scale of 0 to 3+,” regardless of glomerular morphology, which may be membranoproliferative, diffuse endocapillary proliferative, necrotizing, and crescentic or mesangioproliferative in nature. Confirmation of alternate pathway complement abnormality requires search for autoantibodies and genetic evaluation of relevant complement factors with recommendations in the literature as to the minimum complement investigation.² However, for most parts of the world, these investigations are not available, and nephrologists and pathologists are mostly left with only a serum C3 and serum C4 to supplement biopsy findings, which commonly also lack ultrastructural evaluation. Accurate classification into the C3 glomerulopathy category can therefore be challenging.

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Received 5 November 2018; revised 28 January 2019; accepted 4 February 2019; published online 13 February 2019

Sethi *et al.*³ reported in a series of proliferative GN the utility of glomerular C4d staining in classification into immune complex-mediated and complement-mediated. This was based on the hypothesis that C4d deposition would be a feature of the classical pathway (with C1q deposition) or lectin pathway (without C1q deposition) complement activation, such as noted in lupus nephritis and IgA nephropathy, respectively, and its absence would suggest activation of the alternative pathway as expected in C3 glomerulopathy. Table 1 simplifies the concept. In the 30 cases of C3 glomerulopathy that they stained, none showed C4d more than 1+. Thus, C4d seemed to be a promising tissue marker that could potentially supplement routine DIF studies. We stained for C4d in a large cohort of C3 glomerulopathy with the objective to further investigate its utility toward the diagnosis.

METHODS

Case Selection and Review

Case files from 2011 to 2017 of the Department of Pathology, All India Institute of Medical Sciences, New Delhi, were searched for cases with the following key words: complement-mediated membranoproliferative glomerulonephritis (MPGN), C3 glomerulopathy, dense deposit disease, or C3 glomerulonephritis. Only cases with complete immunofluorescence profile, including IgG, IgA, IgM, C3, C1q, kappa and lambda, and electron microscopy were included for the purpose of this study.

The glomerular histology was classified as mesangio-proliferative, membranoproliferative, diffuse endocapillary proliferative, or necrotizing and crescentic. In all cases, digitally archived images of immunofluorescence and electron microscopy were reviewed together by 2 renal pathologists. On ultrastructural evaluation, cases were divided into DDD characterized by intensely osmiophilic intramembranous deposits and C3GN when the deposits were immune-type and subendothelial, mesangial, and subepithelial in location. Postinfectious GN representing the classical diffuse proliferative GN, with absence of capillary wall remodeling and only subepithelial humps on electron microscopy were excluded from our study cohort.

Other glomerular diseases were included as controls, including minimal change disease, diffuse proliferative

lupus nephritis, membranous nephropathy, fibrillary GN, and immune complex-mediated MPGN.

C4d Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were stained using the rabbit anti-human C4d monoclonal antibody (Clone SP91; Spring Biosciences, Pleasanton, CA) by the immuno-peroxidase method. Briefly, 3- μ m-thick sections were deparaffinized followed by heat-induced antigen retrieval in citrate buffer (pH 6) for 20 minutes. Primary antibody C4d was applied for 2 hours at a dilution of 1:100. DAB-based detection followed using Ultravision Quanto Detection system (ThermoScientific, Waltham, MA). Slides were counterstained with hematoxylin, dehydrated, cleared, and mounted with DPX.

Glomerular peripheral capillary wall C4d staining was scored semiquantitatively on a scale of 0 to 3+ as 0 (negative), traces, 1+, 2+, and 3+ on similar lines as the DIF evaluation at our center. When variability among glomeruli was noted, a range of staining intensity was reported, such as 1 to 2+, 2 to 3+, and so on. Purely mesangial staining was not considered positive in the scoring. Evaluation was performed by 2 renal pathologists (GS and SKS) and consensus was reached.

Paraffin Immunofluorescence

Paraffin immunofluorescence was performed in cases of C3 glomerulopathy with discordant C4d staining (i.e., not associated with any Ig deposition) to evaluate for masked Igs using a previously published protocol.⁴

Briefly, formalin-fixed paraffin-embedded tissue was cut at 3- to 4- μ m thickness on poly L lysine-coated slides, deparaffinized, and rehydrated. The slides were then immersed in tris EDTA, pH 9, for 30 minutes at room temperature. Enzymatic digestion was performed with proteinase K 1.25 mg/ml (Sigma Aldrich, St. Louis, MO) at room temperature for 15 to 30 minutes. Digestion was stopped by immersing in cold tris EDTA and the slides were left in the buffer for 40 minutes. After a 10-minute phosphate-buffered saline (PBS) rinse, the fluorescein isothiocyanate-labeled antibodies (BIOSSB, Santa Barbara, CA) were applied at standard dilutions (1:50). Following a 2-hour incubation and PBS rinse, the slides were mounted in glycerine and examined using an immunofluorescence microscope.

RESULTS

Immunohistochemistry for C4d in Other Glomerular Diseases

Although we use C4d immunohistochemistry routinely in posttransplant biopsies, before proceeding with its evaluation in the study cohort of C3 glomerulopathy, we performed staining on a case each of membranous nephropathy, diffuse proliferative lupus nephritis,

Table 1. Simplistic scheme of evaluation of pathway of complement activation using immunofluorescence for C3c, C1q, Igs, and immunofluorescence/immunohistochemistry for C4d in the glomeruli

C3c	Igs	C1q	C4d	Pathway of activation
+	+	+	+	Classical pathway activation
+	+/-	-	+	Lectin pathway activation
+	-	-	-	Alternative pathway activation

+, present; -, absent; +/-, variable.

fibrillary GN, and minimal change disease and assessed staining patterns. In membranous nephropathy, a fine granular continuous capillary wall staining was observed diffusely corresponding to the subepithelial immune complex deposition (Figure 1b). A more irregular capillary wall and mesangial staining was observed in the case of diffuse proliferative lupus nephritis (Figure 1c), strong and diffuse smudgy predominantly mesangial staining was observed in fibrillary GN (Figure 1d), whereas the case of minimal change disease (Figure 1a) showed only a mesangial blush. Weak mesangial staining has been noted in our own experience in posttransplant kidney biopsies stained for C4d in the evaluation of antibody-mediated rejection and is considered nonspecific. We concluded that our immunostain for C4d specifically localized to the immune complexes in membranous nephropathy, fibrillary GN, and lupus nephritis and did not stain the capillary walls nonspecifically in the case of minimal change disease. It was therefore reliable for interpretation in the cohort of C3 glomerulopathy.

Immunohistochemistry for C4d in C3 Glomerulopathy

Based on the search criteria, 41 cases of C3 glomerulopathy were retrieved with complete immunofluorescence and electron microscopy details, which included 27 cases of DDD and 14 cases of C3GN.

Dense Deposit Disease

Twenty-seven cases, including 16 male and 11 female patients with an age range of 6 to 25 years, were retrieved (Table 2). All cases had a membranoproliferative pattern of injury except case 5, which had an endocapillary proliferative pattern of injury. On DIF, based on strict definitions as laid down by the C3 glomerulopathy consensus report, C3c was the dominant deposit in 18 cases (66.66%) with a characteristic coarse capillary wall granular and mesangial pattern of staining (Figure 2a). In the remaining 9 cases, significant Igs/C1q were noted, which included IgM only (2 cases), IgG and IgM (2 cases), IgM and C1q (2 cases), IgA/IgM and C1q (1 case), IgG/IgM and C1q (1 case), and IgA/IgG/IgM and C1q (1 case), which would theoretically preclude them from a diagnosis of C3 glomerulopathy. All deposits were polyclonal and were noted in both capillary wall and mesangial location.

On immunohistochemistry with C4d, a whole range of staining intensities was noted (Figures 2–6), including negative in 3 cases (11.1%), trace to 1+ in 4 cases (14.8%), 1 to 2+/2+ in 7 cases (25.9%), and 2 to 3+/3+ in 13 cases (48.1%).

All the 9 cases with significant Ig deposition had presence of glomerular C4d of comparable intensity, which could be attributed to overlying classical pathway activation in 5 cases (with C1q) and overlying lectin pathway activation in 4 cases (without C1q) (Figure 5).

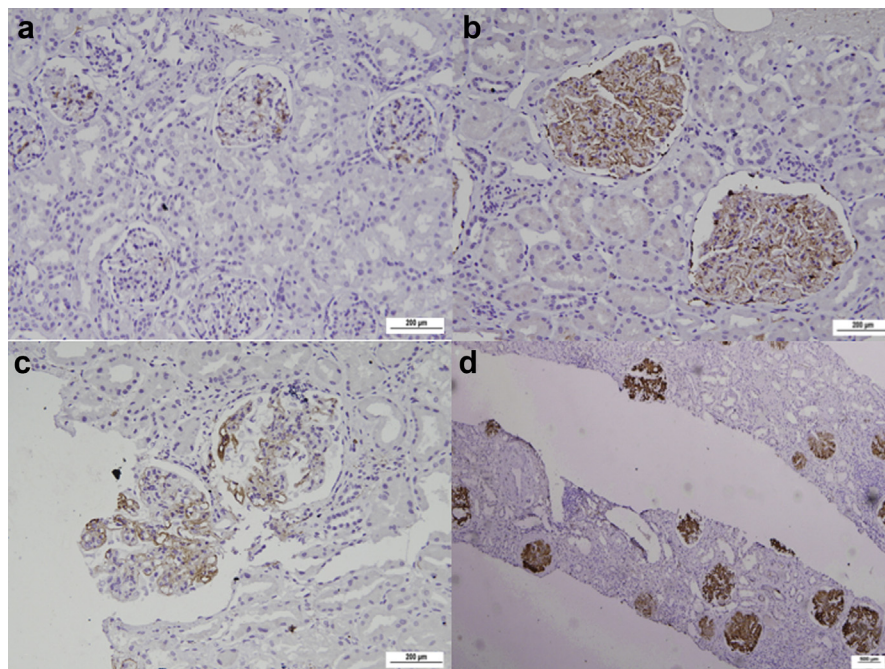


Figure 1. C4d immunohistochemistry in other glomerular diseases. (a) Minimal change disease demonstrates a focal mesangial blush (C4d immunohistochemistry, original magnification $\times 10$). (b) Membranous nephropathy demonstrates diffuse fine capillary wall granular staining (C4d immunohistochemistry, original magnification $\times 20$). (c) Diffuse proliferative lupus nephritis demonstrates variable mesangial and capillary wall granular staining (C4d immunohistochemistry, original magnification $\times 20$). (d) Fibrillary glomerulonephritis demonstrates diffuse “smudgy” mesangial staining (C4d immunohistochemistry, original magnification $\times 4$).

Table 2. Immunofluorescence profile and C4d immunohistochemistry in cases of dense deposit disease

Case	Age/Sex	IgA	IgG	IgM	C3	C1q	Kappa	Lambda	C4d
1	9/F	0	0	0	3+	Traces	0	0	0
2	10/M	Traces	0	1+	3+	0	0	0	0
3	17/M	0	0	1+	3+	0	0	0	0
4	15/F	0	0	2+	3+	2+	2+	2+	1+
5 ^a	9/F	0	0	Traces	3+	0	0	0	Traces to 1+
6	9/F	0	Traces	1+	3+	0	Traces	Traces	Traces to 1+
7	23/M	2+	0	2+	3+	1+	Traces	Traces	Traces to 1+
8 ^a	12/M	0	Traces	1–2+	3+	Traces	0	0	1–2+
9	14/M	0	0	0	2+	0	0	0	1–2+ ^b
		<i>0</i>	<i>1–2+</i>	<i>2+</i>	<i>2–3+</i>	<i>Traces</i>	<i>Traces</i>	<i>Traces</i>	
10	12/M	0	0	1+	3+	Traces	1+	1+	1–2+
11	14/F	0	1+	2+	3+	0	1+	1+	1–2+
12	6/F	0	0	1+	3+	Traces	Traces	Traces	1–2+
13	8/M	0	0	0	3+	0	0	0	1–2+ ^b
14	19/F	Traces	0	2+	3+	Traces	Traces	Traces	2+
15	11/M	0	0	1+	3+	0	Traces	Traces	2–3+ ^b
		<i>0</i>	<i>0</i>	<i>2+</i>	<i>2+</i>	<i>0</i>	<i>0</i>	<i>0</i>	
16	11/F	Traces	Traces	1+	3+	0	1–2+	1+	2–3+ ^b
17	10/M	0	0	1+	3+	2+	0	Traces	3+
18	9/F	0	0	0	2+	0	0	0	2–3+ ^b
		<i>0</i>	<i>0</i>	<i>0</i>	<i>2+</i>	<i>0</i>	<i>0</i>	<i>0</i>	
19	25/M	Traces	1+	1+	3+	0	1+	1+	3+ ^b
20	12/M	0	0	Traces	3+	Traces	0	0	3+ ^b
		<i>0</i>	<i>Traces</i>	<i>Traces</i>	<i>2+</i>	<i>Traces</i>	<i>0</i>	<i>0</i>	
21	10/F	0	0	Traces	3+	1+	Traces	Traces	2–3+ ^b
22	19/M	1+	1+	1–2+	3+	1+	1+	1+	2–3+ ^b
23	21/M	0	0+	2+	3+	2+	2+	2+	2–3+
24	24/M	Tr	1–2+	3+	2–3+	1+	3+	3+	3+
25	13/M	0	0	Traces	3+	1+	Traces	0	2–3+ ^b
		<i>0</i>	<i>0</i>	<i>Traces</i>	<i>3+</i>	<i>-</i>	<i>-</i>	<i>-</i>	
26	16/M	0	0	0	3+	0	0	0	3+ ^b
		<i>0</i>	<i>3+</i>	<i>3+</i>	<i>3+</i>	<i>Traces</i>	<i>3+</i>	<i>3+</i>	
27	16/F	0	2+	2+	3+	0	2+	2+	3+

Capillary wall staining was scored on a semiquantitative scale of 0 to 3+ and variable staining was reported as a range (e.g., 1–2+). Results of paraffin immunofluorescence performed in cases with discordant C4d staining are in italics.

F, female; M, male.

^aResults of paraffin immunofluorescence as fresh frozen tissue was non representative.

^bDiscordant C4d staining.

Of the remaining 18 cases (which fulfilled C3 glomerulopathy consensus criteria), 3 had a negative C4d. Two had traces to 1+ C4d, which was concordant with trace Igs on the DIF without C1q and suggested minor overlying lectin pathway activation (Figure 3). Four had 1 to 2+ C4d, of which 2 cases had 1+ IgM and traces of C1q, suggesting minor overlying classical pathway activation, and 2 cases had no accompanying Igs and were labeled “discordant C4d.” Interestingly, 9 cases had 2 to 3+/3+ C4d, all of which were “discordant” and lacked associated significant Ig deposits (Figure 4).

Thus, a total of 11 cases had a discordant C4d. Paraffin immunofluorescence was performed on 5 of these cases in which sufficient tissue was available to evaluate for masked Igs, and in 2 cases polyclonal IgG/IgM was unmasked with trace C1q, suggesting overlying classical pathway activation (Figure 6). However, in 3 cases, the strong C4d staining remained unexplained.

On electron microscopy, all cases had intensely osmiophilic intramembranous deposits with associated variable-sized mesangial deposits (Figure 2c). In addition, case 13 had subepithelial humps.

Clinical details at presentation were available in 23 patients and infectious triggers or history of febrile illness were sought. Case 5 and case 20 had a history of fever before presentation. Case 5 also had a history of cola-colored urine. Case 3 had a history of mediastinal tuberculosis. The rest did not have any apparent history of infection at presentation.

C3 Glomerulonephritis

A total of 14 cases of C3GN were retrieved from case files with age ranging from 10 to 64 years, including 10 male and 4 female patients (Table 3). All cases displayed immune-type subendothelial and mesangial electron-dense deposits with evidence of capillary wall remodeling on electron microscopy (Figure 2d) and a membranoproliferative pattern of injury on light microscopy.

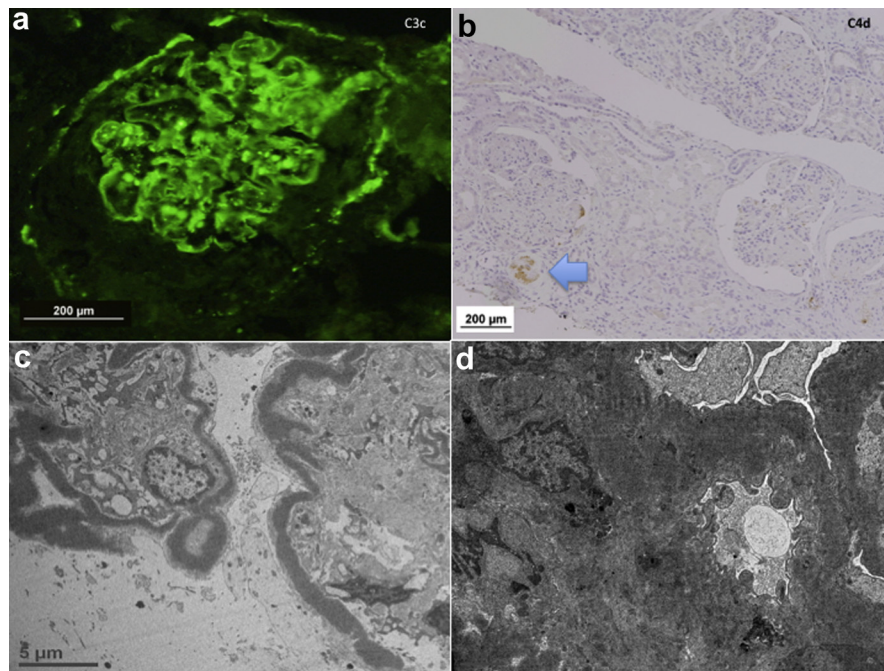


Figure 2. A representative case of C3 glomerulopathy. (a) Intense glomerular capillary wall granular staining with large mesangial granular deposits of C3c are noted (fluorescein isothiocyanate–labeled C3c, original magnification $\times 20$). (b) C4d is negative in the glomeruli. Nonspecific entrapment is noted in a globally sclerotic glomerulus (arrow; C4d immunohistochemistry, original magnification $\times 10$). (c) Transmission electron microscopy in cases of C3 glomerulopathy may show intensely osmiophilic intramembranous deposits and are labeled dense deposit disease (uranyl acetate–lead citrate, original magnification $\times 570$). (d) They may show subendothelial, transmembranous, and mesangial immune-type electron-dense deposits and are labeled C3 glomerulonephritis (uranyl acetate–lead citrate, original magnification $\times 1550$).

Again on DIF, based on strict definitions as laid down by the C3 glomerulopathy consensus report, C3c was the dominant deposit in 11 cases. In 3 cases (cases 6, 10, and 11) the associated Ig was polyclonal IgM of 2+ intensity with variable C1q; however, these cases were still deemed C3 glomerulopathy based on the classical appearance of C3c on DIF (Figure 2a).

Similar to DDD, a range of C4d staining intensities was noted, including negative in 4 cases (28.57%) (Figure 2b), traces in 5 cases (35.71%), 1 to 2/2+ in 2 cases (14.28%), and 2 to 3+/3+ in 3 cases (21.42%). The 2 cases with 1 to 2+/2+ C4d had concomitant IgM/C1q as described previously and could therefore be attributed to mild overlying classical pathway activation.

In 3 cases, however, similar to DDD, “discordant” C4d was noted and therefore paraffin immunofluorescence was performed to exclude masked Igs. In 2 of the 3 cases, no Igs were unmasked and the C4d again remained unexplained. In 1 case, 2 to 3+ IgM was unmasked.

In 12 cases, clinical details were available. Case 2 had a history of fever and cola-colored urine, and case 7 had chronic liver disease, further unclassified. The rest did not have any apparent history of infection.

Immunohistochemistry for C4d in Immune Complex MPGN

To compare with the other form of MPGN, which is immune complex–mediated, we stained 16 cases from

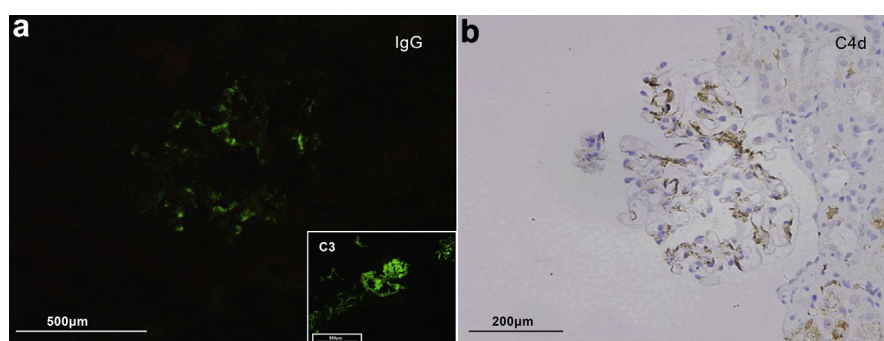


Figure 3. Case 11, Table 2. (a) A case of dense deposit disease with 1+ staining for IgG noted in the mesangium and capillary walls (fluorescein isothiocyanate–labeled IgG, original magnification $\times 20$). Inset: Note the intense glomerular C3c staining (fluorescein isothiocyanate–labeled C3c, original magnification $\times 20$). (b) Similar staining pattern and intensity is noted on C4d (C4d immunohistochemistry, original magnification $\times 20$).

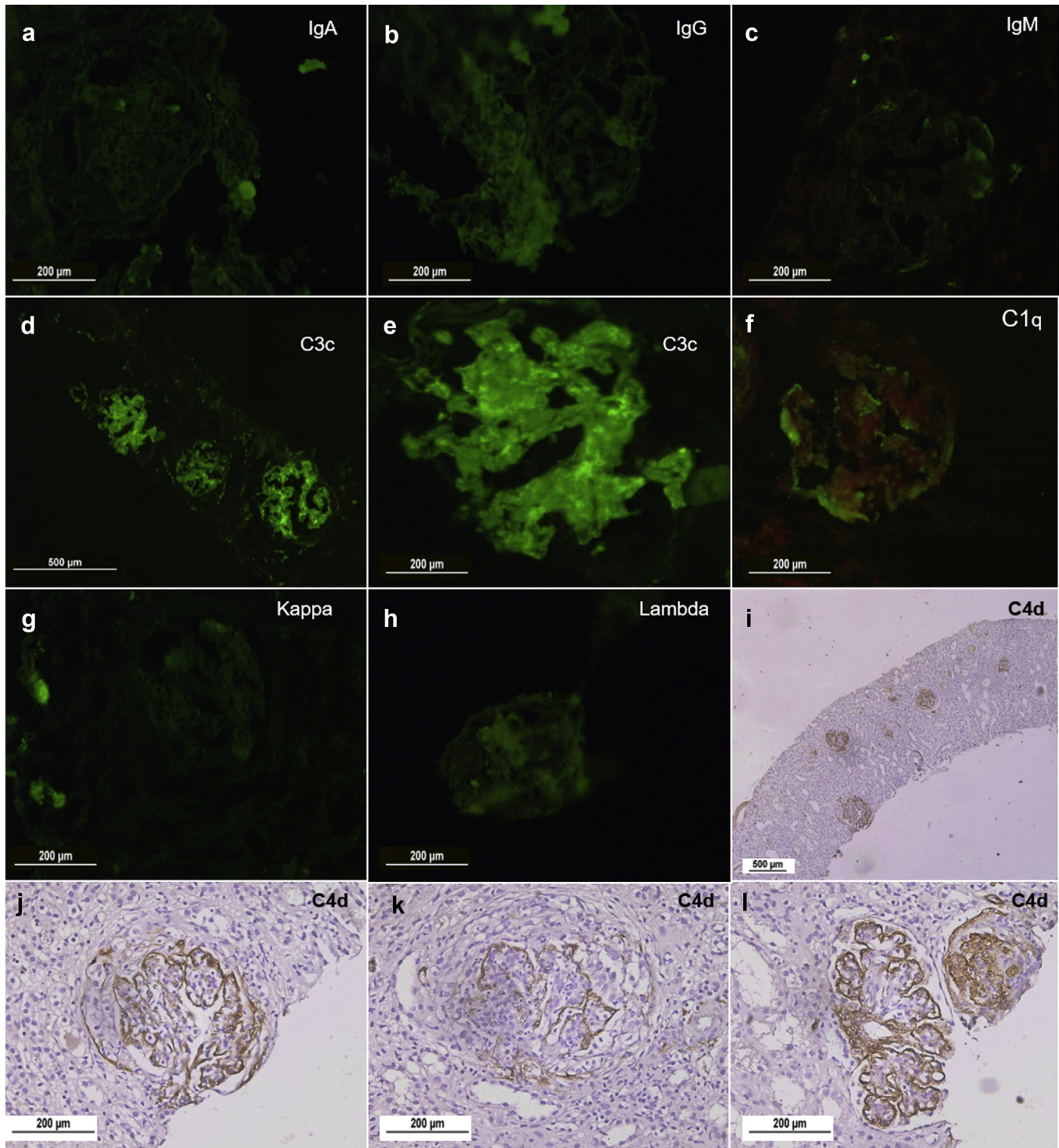


Figure 4. Case 20, Table 2. (a–h) A case of dense deposit disease with isolated C3 immunofluorescence staining (fluorescein isothiocyanate–labeled antibodies, original magnification $\times 10$ and $\times 20$). (i–l) C4d immunohistochemistry shows diffuse staining of the glomeruli with variable coarse capillary wall granular deposits (C4d immunohistochemistry, original magnification $\times 4$ to $\times 20$).

our case files. All cases had shown significant polyclonal IgG deposition along with C3. All 16 cases stained for C4d diffusely with a coarse granular capillary wall and mesangial staining of 2 to 3+ intensity (Figure 7).

DISCUSSION

Although C4d was initially used only in the assessment of transplant biopsies for evidence of antibody interaction with the endothelium, its utility as a diagnostic

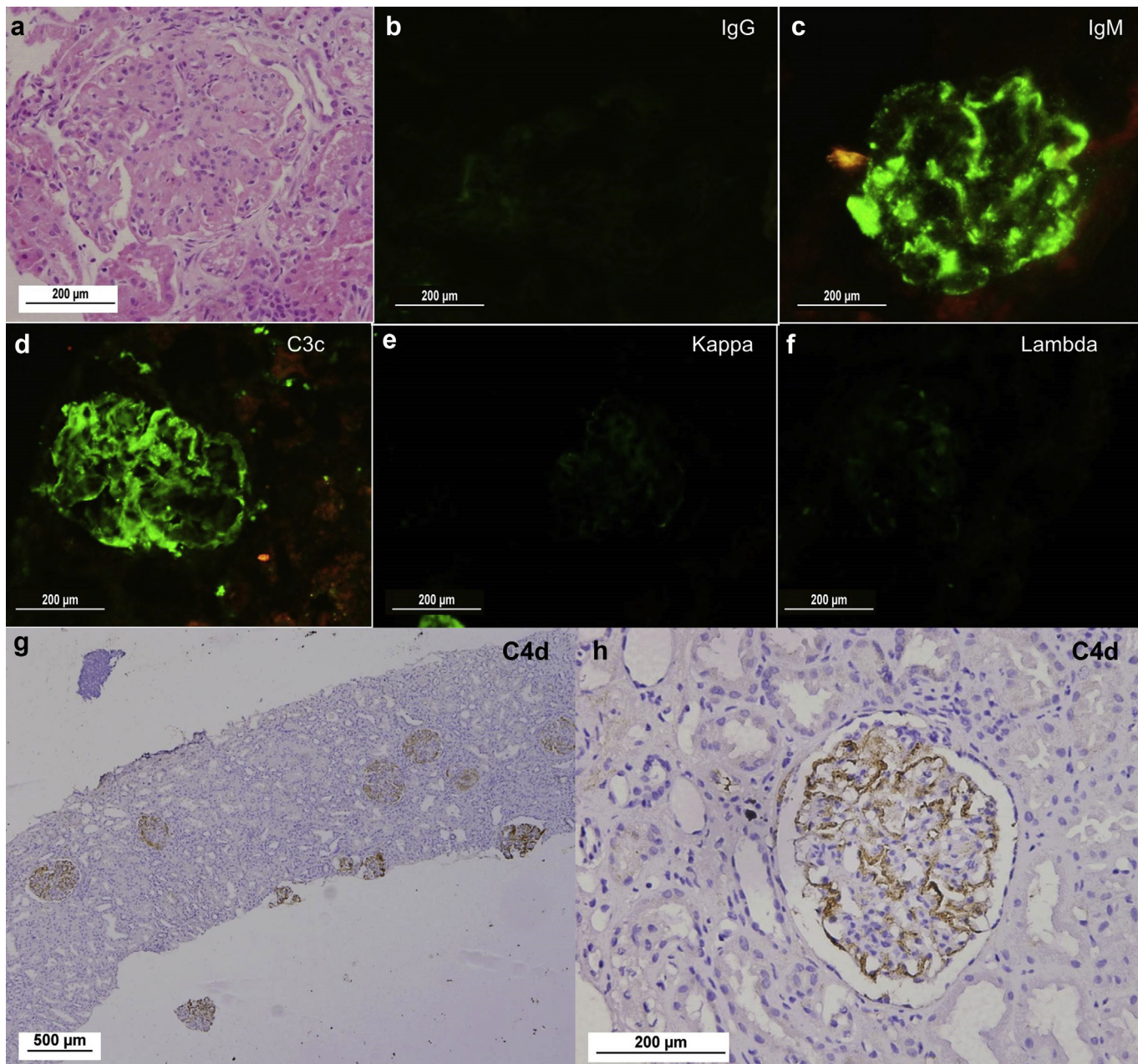


Figure 5. Case 15, Table 2. (a) A case of dense deposit disease with membranoproliferative pattern of glomerular injury (hematoxylin-eosin, original magnification $\times 20$). (b–f) Paraffin immunofluorescence on this case showed coarse capillary wall granular and mesangial staining for IgM (3+) and C3c (3+) and was negative for IgG, kappa, and lambda (fluorescein isothiocyanate–labeled antibodies, original magnification $\times 20$). (g,h) C4d immunohistochemistry showed diffuse but variable glomerular capillary wall staining ranging from 2+ to 3+ concomitant with the IgM staining pattern and intensity (C4d immunohistochemistry, original magnification $\times 10$ to $\times 20$).

marker in the setting of native GN became evident in conditions such as membranous nephropathy, where immune complex formation results in activation of the classical complement pathway.⁵ C4d had also recently been evaluated in a broader study cohort of proliferative GN. Sethi *et al.*³ divided cases into *immune complex–mediated*, which included MPGN, IgA nephropathy, lupus nephritis, fibrillary GN, and membranous nephropathy, and *complement–mediated*, C3 glomerulopathy and studied the utility of immunofluorescence for C4d. In the first group, bright (2 to 3+) C4d staining was noted in

all cases except 2 cases of IgA nephropathy. In the 30 cases of C3 glomerulopathy, which included 25 cases of C3GN and 5 cases of DDD, negative staining was noted in 24 specimens (80%) and trace to 1+ staining was noted in 6 specimens (20%). None of the biopsies of C3 glomerulopathy showed the bright 2 to 3+ staining noted in immune complex–mediated GN. The authors concluded that “C4d serves as a positive marker for immune complex–mediated GN but is absent or minimally detected in C3 glomerulopathy” and “a negative C4d serves as a marker for DDD.”

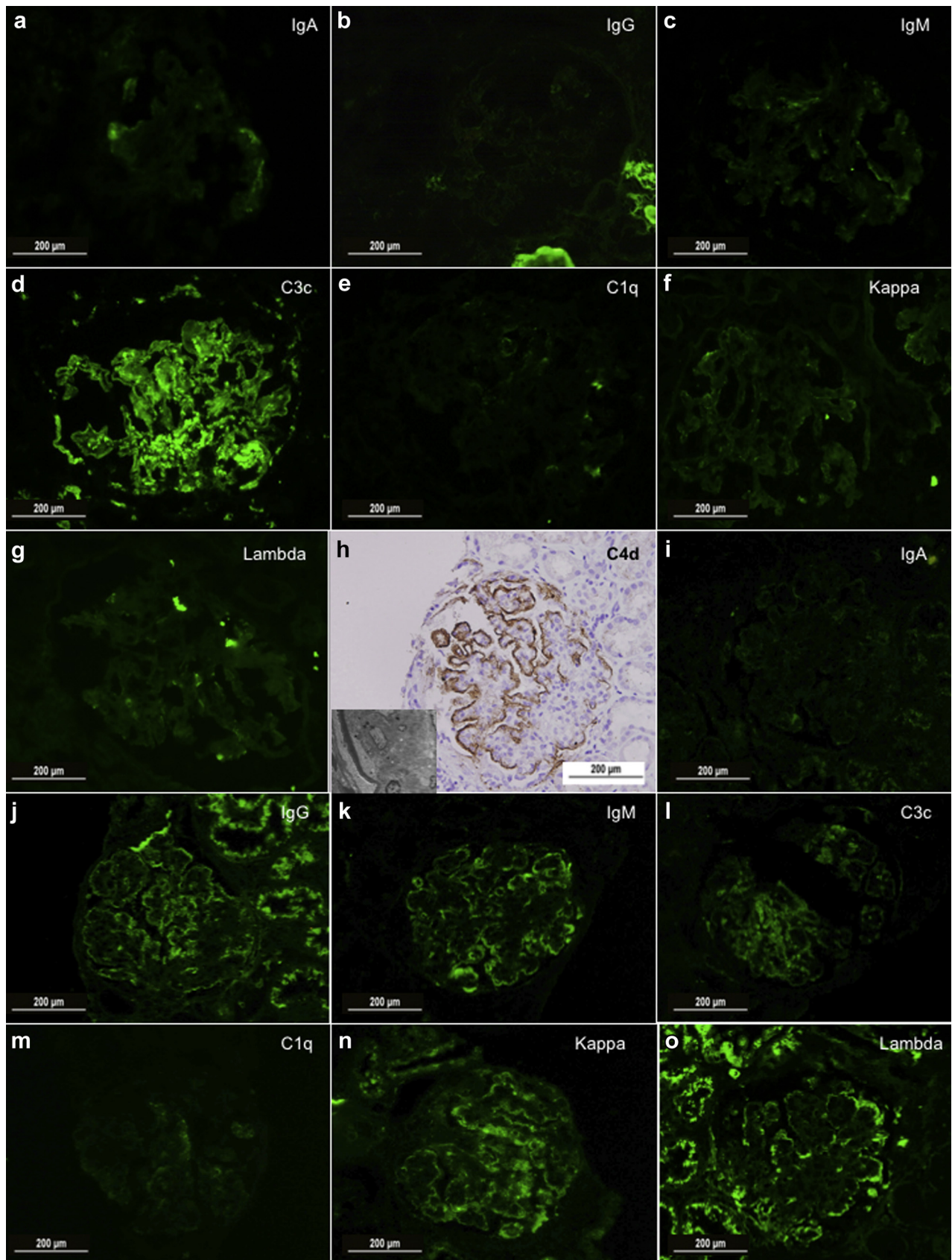


Figure 6. Case 26, Table 2. A case of dense deposit disease. (a–g) Intense isolated C3c staining on immunofluorescence (fluorescein isothiocyanate–labeled antibodies, original magnification $\times 20$). (h) Discrepant C4d results with 3+ glomerular capillary wall staining (C4d immunohistochemistry, original magnification $\times 20$). Inset: Intensely osmiophilic intramembranous deposits noted on electron microscopy. (i–o) Paraffin immunofluorescence unmasked IgG (3+), IgM (3+), kappa (3+), and lambda (3+) with trace C1q in a capillary wall location. IgA was negative. C3c staining was observed and was slightly dim in comparison to the frozen immunofluorescence (fluorescein isothiocyanate–labeled antibodies, original magnification $\times 20$).

Table 3. Immunofluorescence profile and C4d immunohistochemistry results in cases of C3 glomerulonephritis

Case	Age/Sex	IgA	IgG	IgM	C3	C1q	Kappa	Lambda	C4d
1 ^a	14/F	0	0	Traces	3+	0	Traces	0	0
2 ^a	14/M	0	0	Traces	3+	0	Traces	Traces – 1+	0
3	27/F	0	0	1+	3+	0	Traces	Traces	0
4	15/F	0	0	1+	3+	Traces	0	0	0
5	64/M	0	Traces	1+	3+	1+	1+	2+	Traces
6	12/M	0	0	2+	3+	0	0	1+	0 to Traces
7	50/M	0	0	Traces	3+	2+	Traces	0	Traces
8	28/M	Traces	0	Traces	3+	Traces	Traces	Traces	Traces
9 ^a	17/M	0	0	0	2–3+	0	0	0	Traces
10	13/M	0	0	2+	3+	Traces	2+	Traces	1–2+
11	10/M	Traces	0	2+	3+	1+	1+	1+	2+
12	23/M	0	0	0	3+	0	–	–	2–3+ ^b
		<i>Traces</i>	<i>Traces</i>	<i>1+</i>	<i>3+</i>	<i>Traces</i>	<i>Traces</i>	<i>1+</i>	
13	10/M	0	0	0	2+	0	0	0	3+ ^b
		<i>0</i>	<i>0</i>	<i>Traces</i>	<i>0</i>	<i>–</i>	<i>–</i>	<i>–</i>	
14 ^a	25/F	0	0	0	3+	0	0	0	3+ ^b
		<i>0</i>	<i>0</i>	<i>2–3+</i>	<i>3</i>	<i>0</i>	<i>0</i>	<i>0</i>	

Results of paraffin immunofluorescence performed in cases with discordant C4d staining are in italics.

F, female; M, male; –, not performed.

^aResults of paraffin immunofluorescence as fresh frozen tissue was non representative.

^bDiscordant C4d staining.

This conclusion was further tested in more specific cohorts of C3-dominant glomerulopathy and MPGN with variable results. In a small cohort of 15 cases of MPGN, Gupta *et al.*⁶ assessed the utility of C4d immunohistochemical staining in differentiating C3 glomerulopathy from MPGN (immune complex-mediated). In the 6 cases diagnosed as C3 glomerulopathy based on consensus criteria, 3 showed moderate to strong intensity C4d staining with a negative C1q, suggesting the role of lectin pathway, and 3 cases were negative for C4d. Bouatou *et al.*⁷ on a series of 35 MPGN concluded that C4d staining is of no value to discriminate between C3 glomerulopathy and immune complex-mediated MPGN due to overlap in staining patterns. This is concordant with our own observations of presence of variable amounts of C4d in cases of C3 glomerulopathy (Figure 8).

This C4d appeared to be evidence of overlying classical or lectin pathway activation and was accompanied by concomitant Igs in most cases. In cases with a discordant C4d, the possibility of masked Igs was considered and paraffin immunofluorescence was performed. Messias *et al.*⁸ recommend paraffin immunofluorescence on all cases of C3 glomerulopathy to check for masked Igs and to avoid unnecessary investigations into the complement pathway. In their study, 14 cases of C3 glomerulopathy were reclassified as membranous-like GN with masked IgG-kappa deposits, MPGN with monoclonal Igs, and MPGN consistent with mixed essential cryoglobulinemia after paraffin immunofluorescence. Sethi *et al.*⁹ reported the utility of C4d as a “marker of masked immunoglobulins” in 2 cases, one a membranous-like nephropathy and the other an MPGN. In both cases, immunofluorescence for IgG was negative but C4d was strongly positive

and paraffin immunofluorescence performed unmasked the IgG-kappa deposits. In our experience, masked polyclonal Igs explained discordant C4d in 2 of 5 cases of DDD and 1 of 3 cases of C3GN.

However, strong intensity C4d staining remained unexplained in the remaining cases even after paraffin immunofluorescence. Although not documented in their cohort, Sethi *et al.*³ discussed the possibility of a small minority of C3 glomerulopathy with an Ig 0/1+, C4d of 1+/2+ or 3+ with or without C1q. They state that this could be attributed theoretically to an overlying lectin pathway or remote classical pathway activation triggered by infections, autoimmune disease or monoclonal Ig. This is different from the entity of C4 DDD¹⁰ in which the C3 is negative on immunofluorescence with a strong C4d staining. Evidence of infectious trigger in the form of febrile illness was documented in only 1 case of DDD with 3+ C4d staining. Although this study is limited by lack of in-depth evaluation for infectious triggers, it is reflective of the usual clinical practice.

In view of a general higher frequency of intercurrent infections, in our patient group overlying classical or lectin pathway activation was not surprising; however, in many cases it confounded the definition of C3 glomerulopathy based on current guidelines of the C3 glomerulopathy consensus report.² Movement from a 0 to 3+ defining scale to a 0 to 4+ scale would help in accommodating up to 2+ Igs/C1q and still maintain a 2 order of magnitude difference from a 4+ C3c staining. This also, however, raises the question of how to differentiate a “C3GN with overlying classical pathway/lectin pathway activation” from an “immune complex-mediated MPGN,” as both would look similar

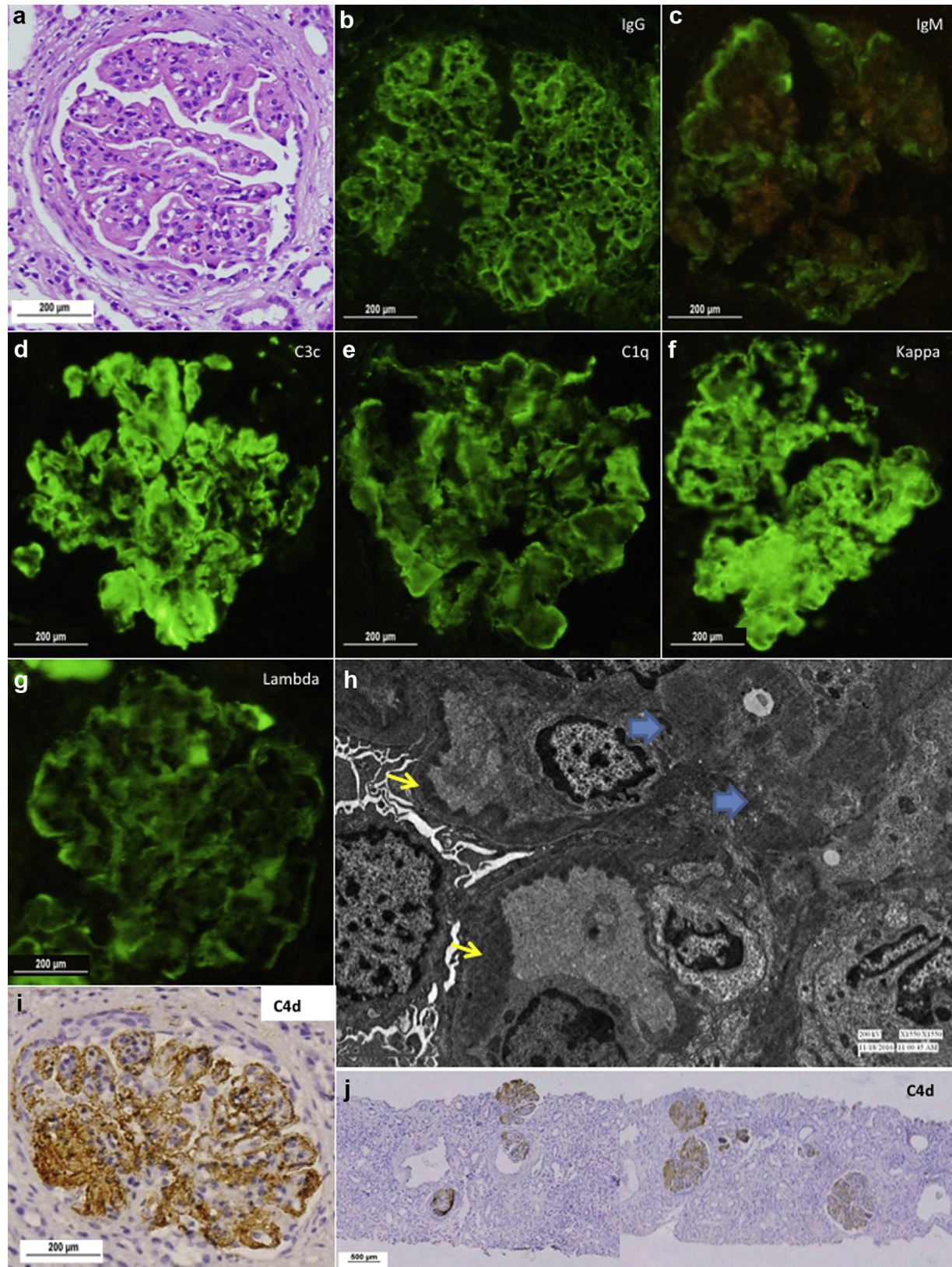


Figure 7. A representative case of immune complex–mediated membranoproliferative glomerulonephritis. (a) Membranoproliferative pattern of injury on light microscopy (hematoxylin–eosin, original magnification $\times 20$). (b–g) Direct immunofluorescence demonstrates coarse capillary wall granular and mesangial granular staining of IgG (2+), IgM (1+), C3c (3+), C1q (2+), kappa (3+), and lambda (2+) on a scale of 0 to 3+ (fluorescein isothiocyanate–labeled antibodies, original magnification $\times 20$). (h) Transmission electron microscopic images show subendothelial (yellow arrows) and large mesangial deposits (blue arrows) with widespread podocyte effacement (uranyl acetate–lead citrate, original magnification $\times 1550$). (i) Immunohistochemistry for C4d displays intense coarse granular capillary wall staining along with mesangial staining (C4d immunohistochemistry, original magnification $\times 20$). (j) At low-power examination, the diffuse staining of the glomeruli is noted (C4d immunohistochemistry, original magnification $\times 4$).

C3 glomerulopathy

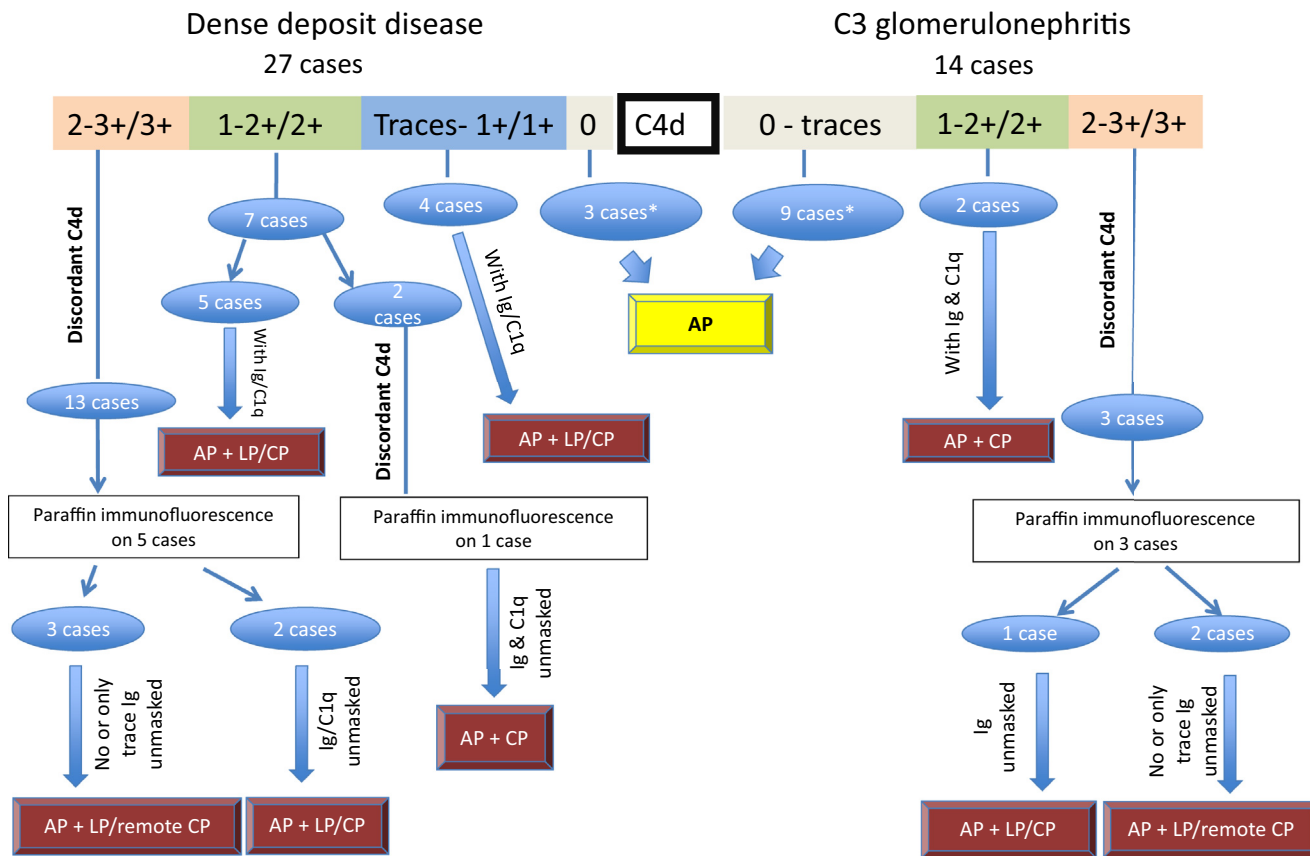


Figure 8. In C3 glomerulopathy, summary of C4d immunohistochemistry with direct immunofluorescence correlation including paraffin immunofluorescence with possible mechanisms of complement activation. There are likely complex overlying activations of the classical and/or lectin pathway that result in deposition of Igs, C1q, and C4d in addition to the C3. *Negative to trace Igs/C1q were noted even with negative to trace C4d. AP, alternative pathway; CP, classical pathway; LP, lectin pathway.

on transmission electron microscopy. We feel that the quality of C3c staining with classical garland pattern and mesangial rings is a soft pointer to a C3GN; however, this was not tested adequately in this study. This difficulty in accurate classification in the scenario of subendothelial and mesangial deposits may explain the finding of markers of alternate pathway abnormalities such as C3Nef in 53% of cases classified as immune complex-mediated MPGN.¹¹

Another confounding factor in accurate classification is the questionable significance of IgM, an issue that was briefly touched on in the C3 glomerulopathy consensus report.² In this study, purely mesangial IgM was considered entrapment and therefore not significant. Capillary wall IgM staining was semiquantified and in many cases correlated with similar amounts of C4d. The possibility that the capillary wall C4d and IgM was evidence of nonspecific entrapment in remodeled capillary walls as noted in cases of transplant glomerulopathy and chronic thrombotic microangiopathy was also considered.¹² However, the fact that a proportion of cases of C3 glomerulopathy with well-developed membranoproliferative

pattern of injury was negative for all Igs and C4d points away from this possibility, and “to ignore or not to ignore” only capillary wall IgM and its accompanying C4d is a question that remains.

This study is obviously limited by the absence of detailed genetic/autoimmune workup for alternative pathway abnormality; however within these limitations, important conclusions could still be drawn. The first conclusion: It is not unusual to find C4d deposition in cases of C3 glomerulopathy and therefore its presence should not exclude the diagnosis. We felt that it should trigger a closer look at the DIF studies, as the lower-intensity C4d staining in most cases correlated with concomitant Ig deposition. On the other hand, a negative C4d adds credence to a diagnosis of C3 glomerulopathy.

Second, this study documents a cohort of C3 glomerulopathy with intense C4d staining without accompanying C1q or Ig deposition even after paraffin immunofluorescence. This may be evidence of lectin pathway abnormality in this cohort and represents a future area of study.

CONCLUSION

This study highlights for the practicing renal pathologist that the presence of C4d does not exclude the possibility of a C3 glomerulopathy.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by the Department of Biotechnology, Ministry of Science and Technology, Government of India grant BT/PR11030/MED/97/1644/2016 (GS, PH). We acknowledge the transmission electron microscopy facility, Department of Anatomy, All India Institute of Medical Sciences, New Delhi for facilitating viewing and diagnosis in the cohort.

AUTHOR CONTRIBUTIONS

Research idea and study design: GS; pathology data acquisition, pathology data analysis/interpretation: GS, SKS, AN, LS, IP, AB; clinical data acquisition: PH, AS, AB, SB, SKA; mentorship: AKD. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

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