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Original Research Paper

Diagnostic Status of C4d Among Histopathological Spectrum of Proliferative Glomerulonephritis

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ABSTRACT:

Introduction: C4d is widely used as a marker for antibody-mediated rejection in renal transplant biopsies, but the significance of C4d deposition in various glomerulonephritis (GNs) in native renal biopsies is not fully elucidated yet. Native renal biopsy is an advantageous tool in establishing a diagnosis, treatment and prognosis of glomerular and tubulointerstitial diseases and presence of C4d in native renal biopsies points towards activation of either classical or lectin pathway of complement fixation. Aim: To find the usefulness of C4d as a diagnostic tool in proliferative GN and to correlate C4d staining pattern with Immunoglobulins and complements on Immunofluorescence. Materials and Method: In this study, evaluation of glomerular C4d staining by Immunohistochemistry in 100 native kidney biopsies of proliferative GN over a period of 2 years at a tertiary care centre was done. Result: C4d staining was consistently present in cases of membranous nephropathy (n=24), IgA nephropathy (n=7), Lupus nephritis (n=23), Immune complex mediated GN (n=10), post infectious GN (n=7). One case each of membranoproliferative GN and IgM nephropathy were positive for C4d staining while negative staining was found in one case each of Anti-glomerular basement membrane disease and ANCA negative pauci-immune crescentic GN. 41 % cases of C3 glomerulopathy (n=5) also showed negative C4d staining. Conclusion: C4d is helpful for evaluating renal biopsies and acts as a supportive adjunct in understanding the pathogenesis of GNs. C4d positivity can be used as a diagnostic tool in native renal diseases and also in transplant biopsies' evaluation

KEYWORDS: C4d Immunohistochemistry, Native renal biopsy

INTRODUCTION:

C4d is a well-recognised biomarker of complement cascade. The usefulness of C4D in identification of antibody-mediated rejection has been well known, recently attention has been drawn to C4d deposition in native renal biopsies. The activation of complement through classical pathway (CP), lectin pathway (LP) or alternate pathway (AP) occurs in many glomerular diseases. Deposition of C4d along with presence of C1q points towards activation of the CP, whereas presence of C4d alone is a result of LP activation. On the contrary, deposits containing solely complement C3 and C5b-C9 without immunoglobulins or classical pathway components are indicative of alternative pathway (AP) activation. Activation of AP does not lead to formation of C4d and hence, there is no deposition of C4d in tissues. Data on the deposition of C4d in various proliferative GN's is sparse. Such data may help to delineate the pathogenic mechanisms underlying complement activation and may possibly identify targets against which therapies could be developed in future. In native renal biopsies in cases of proliferative glomerulonephritis the Immunohistochemical expression of C4d was studied with the aim of delineating the complement activation pathways in understanding the disease pathogenesis.

MATERIAL AND METHODS:

This crosssectional study was conducted on 100 kidney biopsies at a tertiary care centre over 2 years. All renal biopsy samples diagnosed as Proliferative Glomerulonephritis by histopathology during the study period were included. Sample size was calculated at 95% confidence level with α error 0.05, a total of 100 adequate native kidney biopsies with sufficient clinical information were included. Clinical data included demographics, renal function test, urine routine,
serum serologies for lupus and viral infections (hepatitis C and B, HIV) were recorded. Renal biopsy evaluation was based on current recommendations, including hematoxylin-eosin, Periodic acid–Schiff, Silver methenamine and Masson trichrome. In addition C4d by Immunohistochemical staining (IHC) was done in cases diagnosed as proliferative GN by histopathology. Direct Immunofluorescence staining was done in all cases and included Immunoglobulin G (IgG), Immunoglobulin A(IgA), immunoglobulin M(IgM), Complement C3, Complement C1q, kappa and lambda light chains and fibrinogen. Hundred cases of biopsy proven proliferative GN were subjected to IHC with ready-to-use anti – C4d antibody (clone Biocare Medical). Glomerular C4d was assessed regarding pattern and location (global, segmental, mesangial, capillary walls, granular, linear, globular, and smudgy). The C4d score varied from 0 to 3 (negative, minimal, focal and diffuse) depending on the percentage of area (capillary wall and mesangium) that stained positive: no staining = score 0, <10%= score 1 (minimal), 10–50%= score 2 (focal) and >50%= score 3 (diffuse) The data were transformed into variables, coded and entered in Microsoft excel. Data was analyzed and statistically evaluated using SPSS-PC-17version.

RESULTS:

In 100 cases of native renal biopsies, 56% of patients were female while 44% were male. Proliferative Glomerulonephritis was more common in age group of 0- 18 years which constituted 46% followed by 28% cases in age group of 31-50 years. The frequency distribution of various proliferative GN in our study had Lupus nephritis as the most frequent pathology (n=28) followed by membranous nephropathy (n=24). There were 12 cases each of C3 Glomerulopathy and Immune complex mediated GN. 11 cases of post infectious GN and 9 cases of IgA nephropathy .

Table 1 shows the number of cases that were positive for C4d deposition in various GN. Proliferative GN showed positive C4d staining in 80% cases. Table 2 shows the division of C4d positive cases according to the intensity of staining. 57% cases showed 2+ to 3 + intensity of C4d staining. The correlation of C4d staining intensity and various immune complexes and complement deposition in cases of proliferative GN was done. All cases (24/24) of membranous nephropathy showed uniform granular C4d staining demarcating all capillary loops. IgG deposition was present in all cases with 2 to 3+ intensity of staining (Figure 1). A significant correlation was found between positive C4d and IgG. In cases of post infectious GN, all biopsies showed 2+ to 3 + intensity of staining for C3. Seven biopsies showed 1to 2+ staining for C4d and 4 cases showed completely negative C4d staining. In IgA nephropathy, all cases showed IgA positivity with 2+ to 3+ intensity of staining on IF. Seven out of nine cases showed C4d staining with 1+ to 2+ intensity on IHC (Figure 2). In lupus nephritis glomerular deposition of Immune complex was present with varying degrees of staining intensity. Immunofluorescence staining of glomerular IgG was detected in 100%, IgA in 78.5%, IgM in 67.8%, C1q in 82% and C3 in 85.7% cases. C4d deposition was seen in 23/28 cases, 1+ in 4 cases, 2+ in 15 cases and 3+ in 4 cases. (Figure 3) C4d staining was positive in 10/12 cases of Immune complex mediated GN. A significant correlation was found between C4d deposition and IgA, IgM immunoglobulins in ICGN. In C3 glomerulopathy C4d staining was negative in 41.6% (5/12) cases (Figure 4) and 25% (3/12) showed 1+ positivity. No significant correlation was found between C4d deposition and various immunoglobulins and complements in C3GP.

Table1: Overall C4d positivity in various glomerulonephritis (n=100).

S. No	Diagnosis		C4d	
			Positive	Negative
1.	Lupus Nephritis	(n=28)	23	5
2.	Membranous Nephropathy	(n=24)	24	0
3.	IgA nephropathy	(n=9)	7	2
4.	Post infectious Glomerulonephritis	(n=11)	7	4
5.	Immune complex-mediated glomerulonephritis	(n=12)	10	2
6.	C3 glomerulopathy	(n=12)	7	5
7.	Anti-glomerular basement membrane disease	(n=1)	1	0
8.	IgM nephropathy	(n=1)	1	0
9.	Membranoproliferative glomerulonephritis	(n=1)	0	1
10.	ANCA negative Pauci-immune crescentic glomerulonephritis	(n=1)	0	1

Table 2 - The frequency distribution of C4d staining intensity in various Glomerulonephritis (GN) (n=100).

Diagnosis	**C4d intensity scoring**			
	0	**1+**	**2+**	**3+**
Lupus nephritis (n=28)	5	4	15	4
Membranous Nephropathy(n=24)	0	4	11	9
C3Glomerulopathy(n=12)	5	3	2	2
Infection related Glomerulonephritis (n=11)	4	3	4	0
Immune complex mediated Glomerulonephritis(n=12)	2	6	3	1
IgA Nephropathy(n=9)	2	2	5	0
Membranoproliferative Glomerulonephritis(n=1)	1	0	0	0
Anti-glomerular basement disease(n=1)	0	0	1	0
IgM Nephropathy(n=1)	0	1	0	0
Pauci-immune Glomerulonephritis(n=1)	1	0	0	0

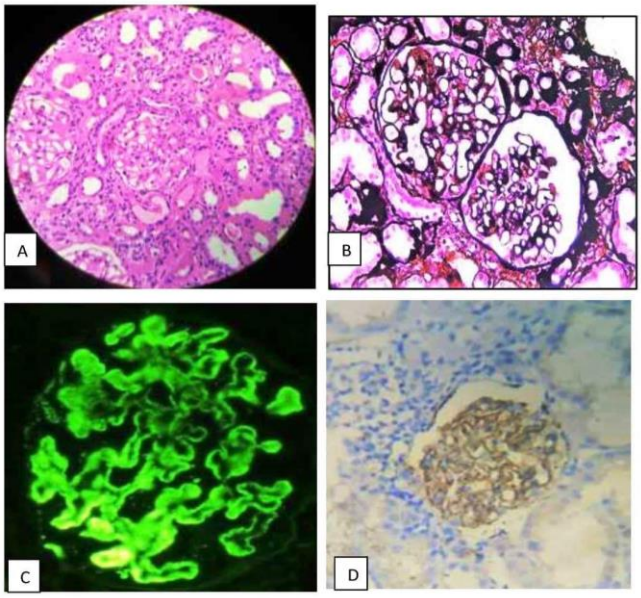

Figure-1: A. Membranous nephropathy showing diffuse thickening of glomerular basement membrane (Haematoxylin and Eosin 400x).
B: Membranous nephropathy- Jones methanamine silver stain highlights the spiking of glomerular basement membrane (400x)
C: Immunofluorescence 400x, showing 3+ intensity of staining of Ig G in the capillary walls.
D: Immunohistochemistry 400x, showing 3+ intensity of staining of C4d in glomerular capillary wall

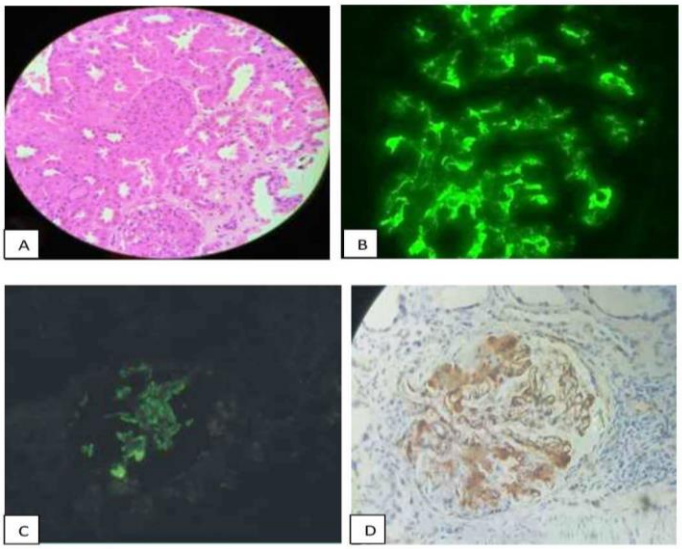

Figure 2: IgA Nephropathy
A: Haematoxylin and eosin (400X)
B: Immunofluorescence study IgA deposition
C: Immunofluorescence Study IgM
D: C4d staining on Immunohistochemistry

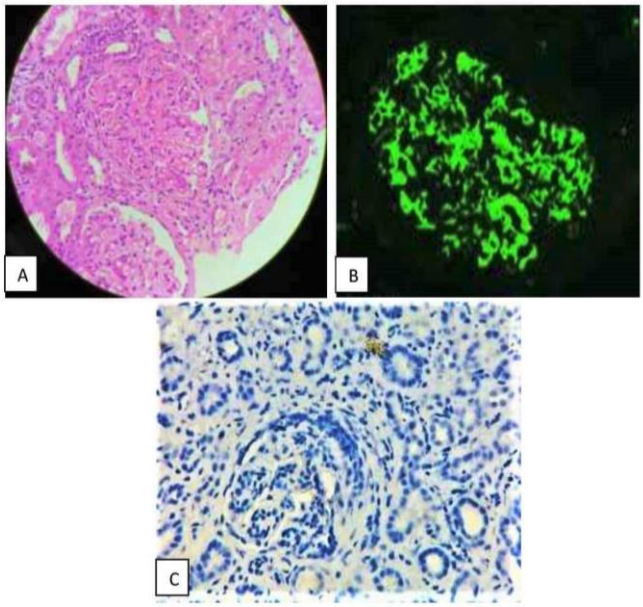

Figure 3: C3 Glomerulopathy
A: Haematoxylin and eosin (400X
B: Immunofluorescence study showing bright staining for C3 which was two order greater than any other immunoreactant.
C: C4d negative on Immunohistochemistry

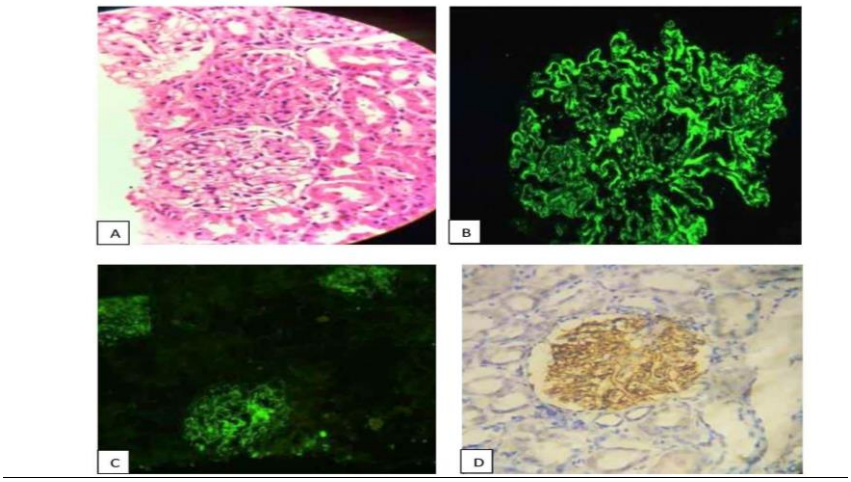

Figure 4: Lupus Nephritis:
A Haematoxylin and eosin (400x)
B: Immunofluorescence study IgG
C: Immunoflourescence Study C3
D: C4d staining on Immunohistochemistry.

DISCUSSION:

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in adults.[1] It is characterized clinically by proteinuria and morphologically by glomerular basement membrane (GBM) thickening due to sub epithelial deposits of IgG, C3, kappa and lambda light chains which are detected by immunofluorescence(IF). Subepithelial deposits and diffuse podocyte foot process effacement are visible on electron microscopy (EM). All cases (24/24) of membranous nephropathy presented clinically with nephrotic range proteinuria and showed uniform granular C4d distribution, demarcating all capillary loops, which was similar to the results found in earlier studies.[1,2] We found seven patients with positivity for C4d and C1q both and 17 patients had C4d positive but C1q negative, probably indicating the activation of the classical and lectin pathway respectively. Difficulty in diagnosis arises in early cases of Membranous nephropathy and also when the tissue is limited and available only for light microscopy making it difficult to differentiate from minimal change disease. Recently, various studies have analyzed the usefulness of C4d staining in the diagnosis of MN, [2,3] and differentiating early changes of MN from minimal change disease.[1] C4d is a reliable method to differentiate MN from other glomerular diseases if light microscopy and IF are of no help. It may also obviate the need of re-biopsy if tissue is not available for IF study. Systemic Lupus Erythematosus is the autoimmune disease characterized by production of autoantibodies to double-stranded DNA and nucleosomes. The tissue injury in Lupus nephritis (LN) is caused by the Immune complex-mediated complement activation via the classical pathway. C4d deposition in 28 patients of LN was studied. C4d positive staining was noted in 23 cases. 19 biopsies had C4d, C1q and C3 deposits in their glomeruli, indicative of the activation of the classical pathway, whereas four biopsies had C4d and C3 deposits without accompanying C1q deposits, indicating the activation of the lectin pathway. Remaining five cases showed negative C4d staining in the presence of C3 and C1q deposition, of which two were of Class II LN. In LN class II, relatively small numbers of stable immune complexes are present which prevents the mesangial clearing system from becoming overloaded and allows the complexes to be sequestered in the mesangium, which are later degraded and removed rather than remaining at the sites where they could initiate complement activation. This explains the absence of deposits of C4d in these cases.[4.5] Furthermore IHC staining for C4d on paraffin-embedded tissue is found to be less sensitive as compared to the Immunofluorescent staining on frozen tissue[6] which could also be the reason for negative staining of C4d. No significant correlation was found between C4d deposition and pathologic class, activity and chronicity index in lupus nephritis. Studies done by Kim SH et al [7] and Bemavathi et al[8] also did not find any significant correlation between C4d deposition and disease activity. Its presence however has been noted to be a sensitive marker for activation of classic pathway. IgA Nephropathy (IgAN) is an immune complex mediated glomerulonephritis. It has been noted that around 40% of cases of IgA Nephropathy progress towards end stage renal disease, it is important to identify such patients in order to treat them aggressively.[9] In our study of nine cases with IgA nephropathy, seven cases had C4d and C3 deposits without associated C1q deposits, that indicates activation of the lectin pathway. One had only C3 deposits in their glomeruli, without any C1q and C4d deposits, indicating the activation of the alternate pathway, and the other case showed negative staining for C4d, C3 and C1q. This observation was comparable with previous studies done by Rath A et al[10]. Sahin et al[11]observed positive C4d mesangial staining to be associated with worse glomerulosclerosis, mesangial hypercellularity, proteinuria and worse renal survival compared to C4d negative patients. Deposition of C4d in IgA nephropathy reflects activation of lectin pathway and several studies have found that this is linked with worse histopathological features, proteinuria and/or renal outcomes. The term C3 glomerulopathy (C3GP) is for glomerular diseases in which there is only deposition of C3 in glomeruli and no immunoglobulins of the classic pathway of complement activation. It is defined as C3 intensity two orders of magnitude more than any other immune reactant on a scale of 0 to 3 (including 0, trace, 1+, 2+, 3+). In our study of 12 cases of C3GP, C4d was negative in five biopsies. Remaining cases showed positive staining for C4d (1+ intensity in three cases and 2-3+ intensity in four cases). C3 and immunoglobulins also showed 1+ staining on IF. This raises the possibility that immune complexes act as a trigger for C3GP in the setting of remote post infectious GN. This also confirms CP/LP

of complement activation and points to autoimmune disease as a likely contributing factor. Two cases had C3 without accompanying C1q and C4d deposits, indicating the activation of the alternate pathway, which could be atypical PIGN. We also studied 12 biopsies of immune complex mediated GN representing three main categories (i.e., autoimmune diseases, chronic infections and monoclonal gammopathy). Ten out of 12 cases (83%) showed positive C4d staining of which eight had 2–3+ staining intensity of immunoglobulins on direct immunofluorescence. Seven out of 12 biopsies also showed bright 2–3+ intensity of staining for C3. C1q was noted in six biopsies (50%) suggestive of immune complex-mediated GN. In Immune complex mediated GN, C4d was a marker of activation of classical pathway or lectin pathway. Recognition of C3GP, and additional information of C4d staining in renal biopsies alert the clinician to investigate the complement system and these cases may be amenable to targeted inhibition of the complement pathway at the level of C3 activation. Hence, positive C4d staining serves as a marker for immune complex-mediated GN, whereas a negative result of C4d in a dominant C3 staining may suggest the possibility of C3glomerulopathy. Hence, result of C4d stain helps to determine whether the proliferative GN is mediated by the activation of the AP or by CP/LP. The sensitivity and specificity for negative or trace C4d for diagnosis of C3GP (compared with immune complex mediated GN) was 93% and 100% vs 97% and 63% for negative C1q and 87% and 100% for negative IgG respectively.[12] It is proposed that C4d is a valuable marker to distinguish Immune complex mediated GN from C3GP. The characteristic features of post infectious GN on light microscopy are proliferative GN, bright C3 staining with or without immunoglobulins on immunofluorescence, and subepithelial humps on electron microscopy. Therefore, kidney biopsy findings of post infectious GN and C3GN share common features. 11 cases of post infectious GN were also studied. Four biopsies of post infectious GN, which were negative for C4d and C1q staining, showed C3 staining of 2-3+ intensity. It is possible that these patients represent a subset of post infectious GN in which the AP is activated (atypical post infectious GN). Further evaluation of the AP may be justified in these patients to determine whether an underlying abnormality is present. In the remaining seven biopsies, there was mild (1–2+) staining for C4d with mild staining intensity for Immunoglobins (1–2+) in five and bright staining of Igs (3+) in two biopsies, suggesting that C4d staining in these patients is likely caused by CP activation induced by immune complexes. Alternatively, LP activation by the microbial pathogens may contribute to C4d staining in these patients. C4d staining helps in differentiating between C3GP and atypical PIGN and could suggest the underlying pathogenesis of these glomerulopathies, such that it alerts the clinician to look for genetic mutation causing dysregulation of the complement pathway system. In almost every pathology laboratory C4d staining is routinely available thus it may become an important tool to identify the underlying etiology in proliferative GN. One case of ANCA-negative pauci-immune Crescentic GN showed negative staining for IgG, IgA, IgM, C3 and C1q on IF. Immunostaining for C4d was also negative. Xing GQ et al [13]in their study had 7 patients with MPO-ANCA positive GN, that showed positive glomerular and small blood vessel staining for C3 (indicating alternate pathway activation) and no staining for C4d. The same group reviewed renal biopsies from 12 patients with ANCA negative pauci immune GN and found 8 out of 12 were C4d positive and 6 of these 8 were mannose binding lectin positive indicating activation of Lectin pathway with resultant deposition of C4d. Single case of anti-glomerular basement membrane (anti-GBM) glomerulonephritis in our study, with positive anti-GBM antibodies, showed strong (3+) linear IgG staining accompanied by negative IgA and IgM and mild (1+) staining intensity of C3 and C1q on direct immunofluorescence assessment. C4d immunostain was performed and showed (2+) staining intensity along capillary wall indicating activation of classical complement pathway. Presence of C4d deposition in anti-GBM glomerulonephritis has been associated with recurrence in transplanted kidneys.[14] Aggressive therapy in such cases can possibly prevent graft loss C4d determines the complement pathway involved in various glomerulonephritis. This would potentially enable us to identify pathologic lesions that correlate with response to certain therapeutic agents, such as anti C5 therapy and emerging therapies to target C3 convertase activity in renal disorders.C4d may be helpful tool in evaluation of renal biopsies and may act as a supportive adjunct in understanding the pathogenesis of GNs. C4d staining may help to recognize patients with worse renal prognosis and thus may help guide treatment decisions[15] Many newer drugs affecting complement pathways are now available so recognizing key complementary pathways

in different glomerular diseases will be crucial. Paraffin embedded renal biopsies can be studied for C4d staining by IHC in most labs and is valuable when tissue is not available for IF and/or EM studies. The potential use of IHC C4d staining in renal allograft biopsies is equally important for diagnosis of immune complex mediated GN, such as early recurrent membranous GN.

CONCLUSION:

C4D can be considered as a novel diagnostic biomarker for native renal diseases, especially those with a complement activity in the etiological background. Staining of C4d along with immunoglobulin markers like IgG and IgM and complement proteins can be advantageous in demarcating different complement activation pathways in glomerular diseases[16]This not only provides an insight into the pathogenesis of the glomerular diseases but also explains the possibility of using targeted therapy identifying key complement pathways in such diseases.

REFERENCES:

1. Espinosa-Hernández M, Ortega-Salas R, López-Andreu M, Gómez-Carrasco JM, Pérez-Sáez MJ, Pérez-Seoane C, Aljama-García P. C4d as a diagnostic tool in membranous nephropathy. Nefrologia. 2012 May 14;32(3):295-9.
2. Hui M, Uppin MS, Prayaga AK, Raju SB, Rajasekhar L. C4d immunohistochemistry in membranous nephropathy. J Lab Physicians. 2014 Jul;6(2):76-9. doi: 10.4103/0974-2727.141500. PMID: 25328330; PMCID: PMC4196367.
3. Custódio FB, Silva CAD, Helmo FR, Machado JR, Reis MAD. Complement System and C4d expression in cases of Membranous nephropathy. J Bras Nefrol. 2017 Oct-Dec;39(4):370-375.
4. Kim MK, Maeng YI, Lee SJ, et al. Pathogenesis and significance of glomerular C4d deposition in lupus nephritis: activation of classical and lectin pathways. *Int. J. Clin. Exp. Pathol.* 2013;6(10):2157-67.
5. Sahin OZ, Gurses S, Taslı F, et al. Glomerular C4d Staining Can Be an Indicator of Disease Activity in Lupus Nephritis. *Ren. Fail.* 2012;35(2):222-5.
6. Chantranuwat C, Qiao J-H, Kobashigawa J, Hong L, Shintaku P, Fishbein MC. Immunoperoxidase Staining for C4d on Paraffin-embedded Tissue in Cardiac Allograft Endomyocardial Biopsies. *Appl. Immunohistochem. Mol. Morphol.* 2004:166-171.
7. Kim SH, Jeong HJ. Glomerular C4d Deposition Indicates in situ Classic Complement Pathway Activation, but is not a Marker for Lupus Nephritis Activity. *Yonsei Med. J.* 2003;44(1):75-80
8. Bheemavathi.A, Durga K, Gowrishankar S. Correlation of Glomerular C4d Staining with Disease Activity in Lupus Nephritis-A prospective study. *International Journal of Scientific and Research Publications.2014:*4(2).
9. Nasri H, Ahmadi A, Rafieian-kopaei M, Bashardoust B, Nasri P, Mubarak M. Association of glomerular C4d deposition with various demographic data in IgA nephropathy patients; a preliminary study. *J Nephropathol.* 2015;4(1):19-23.
10. Rath A, Tewari R, Mendonca S, Badwal S, Nijhawan VS. Oxford classification of IgA nephropathy and C4d deposition; correlation and its implication. *J Nephropharmacol.* 2016;5(2):75-79.
11. Sahin OZ, Yavas H, Taslı F, et al. Prognostic value of glomerular C4d staining in patients with IgA nephritis. *Int. J. Clin. Exp. Pathol.* 2014;7(6):3299-3304..
12. Sethi S, Nasr SH, De Vriese AS, Fervenza FC. C4d as a Diagnostic Tool in Proliferative GN. *J. Am. Soc. Nephrol.* 2015;26(11):2852-2859.
13. Xing GQ, Chen M, Liu G, Heeringa P, Zhang JJ, Zheng X, E J, Kallenberg CG, Zhao MH. Complement activation is involved in renal damage in human antineutrophil cytoplasmic autoantibody associated pauci-immune vasculitis. J Clin Immunol. 2009 May;29(3):282-91. doi: 10.1007/s10875-008-9268-2. Epub 2008 Dec 10. PMID: 19067130.
14. Batal I, Chalasani G, Wu C, Shapiro R, Bastacky S, Randhawa P. Deposition of Complement Product C4d in Anti–Glomerular Basement Membrane

Glomerulonephritis. *Am. J. Kidney Dis.* 2009;53(6):1098-1101.

15. Chandra P. C4d in Native Glomerular Diseases. Am J Nephrol. 2019;49(1):81-92. doi: 10.1159/000496059. Epub 2019 Jan 4. PMID: 30612132.

16. Raman S, Mishra P, Panigrahi A, Rout NK, Dash K. Glomerular C4d deposition in proliferative glomerular diseases. Indian J Pathol Microbiol. 2021 Jan-Mar;64(1):69-77. doi: 10.4103/IJPM.IJPM_364_20. PMID: 33433412.