



# Frequency of acute antibody mediated rejection in renal allograft biopsies as detected by morphological findings and C4d immunostaining

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## ABSTRACT

**Introduction:** Acute antibody-mediated rejection (AMR) detection using C4d immunofluorescence in combination with histopathological examination in renal allograft biopsy is a gold standard for AMR. This study will be the first one in this context in Pakistan and will generate local data.

**Objectives:** To determine the frequency of AMR in renal allograft biopsies as detected by morphological findings and C4d immunostaining.

**Patients and Methods:** We performed a prospective cross-sectional study at histopathology department, Shifa international hospital, Islamabad. Allograft biopsies which were performed for diagnosis of graft dysfunction were included. Interpretation was performed, according to Banff 2013. All statistical analysis of the data was performed using statistical software SPSS 20.

**Results:** A total of 60 biopsies, including 59 (98.3%) males and 1 (1.7%) female were evaluated. Age range was 15-65 years (mean  $34.6 \pm 11.8$ ). Out of 60 biopsies, in the early post-transplant period, 14 (23.3%) out of 60 cases were diagnosed as AMR on morphology. One (1.6%) case had strong C4d positive in absence of morphological features. Out of these cases, 8 (57.1%) showed C4d positivity, 2 (14.3%) showed focal C4d positivity while 4 (28.6%) were C4d negative. In the late post-transplant period, chronic allograft nephropathy (CAN) cases were 19 (31.7%). Out of these cases, 13 (68.4%) showed morphological features of AMR. C4d was positive 11 (57.9%) and C4d was negative in 2 cases (10.5%).

**Conclusion:** C4d positivity favors AMR; however, it can be seen in absence of morphological features.

### Implication for health policy/practice/research/medical education:

Acute antibody-mediated rejection (AMR) has emerged as an important reason of allograft dysfunction. Histopathologic assessment of post-transplant renal biopsies was routinely performed in many centers for AMR. C4d has emerged as an easy and inexpensive means of detection of AMR. With its help in combination with histopathological findings, AMR can be timely diagnosed. These patients could be benefited from anti-humoral therapy and prevented from more intense rejection in future.

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## Introduction

In South Asia, true prevalence of end-stage renal disease (ESRD) patients in Pakistan and India are not established due to lack of national registries. Chronic glomerulonephritis and diabetic nephropathy are leading causes of ESRD patients in India. Patients are younger at the time of detection of ESRD (1,2).

Around 5% of all ESRD patients undergo transplant. In India, live related transplants constitute 30%-40% and cadaveric transplantation account for less than 2% (2). Life expectancy of these patients has prolonged due to availability of organ transplantation (3-5). Acute antibody-mediated rejection (AMR) has emerged as an important reason of allograft dysfunction. It usually occurs a few

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weeks post-transplant and is mediated by antibodies to donor epithelium (6). C4d detection was not possible for many years, as polyvalent anti-C4 reagent available at that time, reacted only with determinants of the C4c fragment, but not with the alpha-2 portion of C4 (7). Over last 2 decades, the incidence of acute rejection has reduced due to novel immunosuppressive drugs. The incidence of rejection episodes in whole series reported by Pallardó Mateu et al was 32.5% (8).

The incidence of acute AMR in un-sensitized patients is <5%, but it can reach 40%-90% among sensitized patients. AMR has a major impact on long-term graft survival (9). Allograft dysfunction in C4d-positive rejection episodes is often more pronounced. These patients benefit from intensive therapy, potentially preventing the previously reported high graft failure rate (10). Histopathologic assessment of post-transplant renal biopsies was routinely performed in many centers. Detection of C4d in renal allograft biopsy by IF in combination with routine histopathological examination is now the gold standard for AMR (6,11,12).

Banff group of nephrologists and transplant pathologists is leading body for updating classification of renal allograft rejection. C4d was incorporated in Banff classification in 2005 (13). In Banff 2007 peritubular capillaritis grading, C4d scoring, interpretation of C4d deposition without morphological evidence of active rejection and scoring of total interstitial inflammation (ti-score) were defined (14,15). Banff criteria for AMR were refined in June 2011 with inclusion of C4d negative AMR. At that time multicenter data was analyzed, however it was decided to collect more data and further research about it (16). Various studies from literature can be documented from 2007 onwards to support the role of C4d immunostaining for early detection of AMR. In this regard, a study conducted on 59 cases of acute renal rejection by Ranjan et al shows 55% of cases of acute rejection with C4d positivity. Out of these, 81% were of presumptive antibody mediated rejection P-AbAR/ AMR (6).

Analysis of C4d immunostaining shows agreement quite well with various histopathological findings for AMR (11). Comparing renal transplant recipients with and without AMR, patients exhibiting subclinical AMR (C4d positivity and capillaritis/glomerulitis) in their first biopsy were found to be at an increased risk of more intense chronic lesions in their follow-up biopsies (17).

C4d has emerged as an easy and inexpensive means of detection of AMR. With its help in combination with histopathological findings, AMR can be timely diagnosed. These patients could be benefited from anti-humoral therapy and prevented from more intense rejection in the future (2,18).

Despite the emergence of various molecular techniques, like panel reactive antibody (PRA) levels, flow cytometry cross-match and flow PRA bead assays, biopsy remains gold standard for detection of AMR (6,11-13,19).

Renal transplant is being performed in Shifa international hospital, since last 15 years. Previously only histopathological assessments of biopsies were done to look for rejection. Now C4d is introduced in routine practice and will be a great help in the early diagnosis and treatment of patients affected with AMR. This study will be the first study in this context in Pakistan and will generate local data.

### Objectives

To determine the frequency of AMR in renal allograft biopsies as detected by morphological findings and C4d immunostaining.

### Patients and Methods

#### Study and setting

We performed a prospective cross-sectional study at histopathology department, Shifa international hospital, Islamabad.

#### Definition of acute antibody mediated rejection

##### Clinical and laboratory data

Allograft biopsies which were performed for diagnosis of graft dysfunction were included. Non-probability, consecutive sampling was done. For histopathological assessment biopsy with more than 7 glomeruli and 1 or more arteries was included. Biopsy with no glomeruli or no arteries or with only medulla was considered non-diagnostic. For immunofluorescence, renal biopsy with or without glomeruli was included.

##### Preparation of biopsy sample

Using WHO sample size calculator, keeping confidence level 95%, anticipated population proportion 81% (6), and absolute precision required 10%, sample size is 60. Patient's medical record number age and gender patient were retrieved from patient record file or by telephone communication. Patient's confidentiality was maintained at each step of the research. Two transplant renal biopsy specimens were received from each patient, one in formalin for routine microscopy and other in transplant media (Zeus Scientific Inc., Branchburg, NJ, USA), for immunofluorescence.

##### Ethical issues

The research followed the tenets of the Declaration of Helsinki; informed consent was obtained; and the research was approved by the ethical committee of Shifa international hospital, Islamabad, Pakistan. All patients' information remained confidential.

##### Statistical analysis

Interpretation was performed according to Banff 2013 for assessment of AMR. All statistical analysis of the data was performed using SPSS version 20. Percentages, means and standard deviations were calculated.

## Results

A total of 60 biopsies, including 59 (98.3%) males and 1 (1.7%) female were evaluated (Figure 1). Age range was 15-65 years (mean  $34.6 \pm 11.8$ ) (Table 1). Out of 60 biopsies, in the early post-transplant period 14 (23.3%) out of 60 cases were diagnosed as AMR on morphology. Out of these cases 8 (57.1%) showed C4d positivity, 2 (14.3%) showed focal C4d positivity while 4 (28.6%) were C4d negative. One case had strong C4d positivity in absence of morphological features (Figure 2). In the late post-transplant period, chronic allograft nephropathy (CAN) cases were 19 (31.7%). Out of these cases, 13 (68.4%) showed morphological features of AMR. C4d was positive 11 (57.9%) and C4d was negative in 2 (10.5%) (Figure 3). Morphological and C4d patterns in AMR with and without CAN are illustrated in Figure 4A-H.

Rest of biopsies show additional pathological features. ACR was documented in 7 (11.6%) cases, CNI toxicity in 5 (8.3%) cases, TIN in 5 (8.3%) cases, tubular damage and necrosis 5 (8.3%) cases. In 2 of the above-mentioned cases of AMR, morphological features of both ACR and AMR were present. BK nephropathy was documented

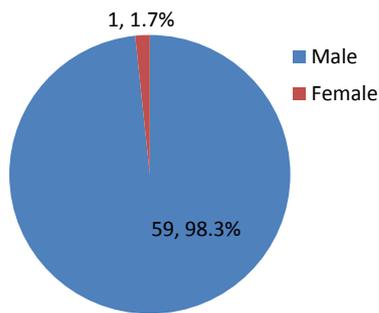


Figure 1. Gender distribution.

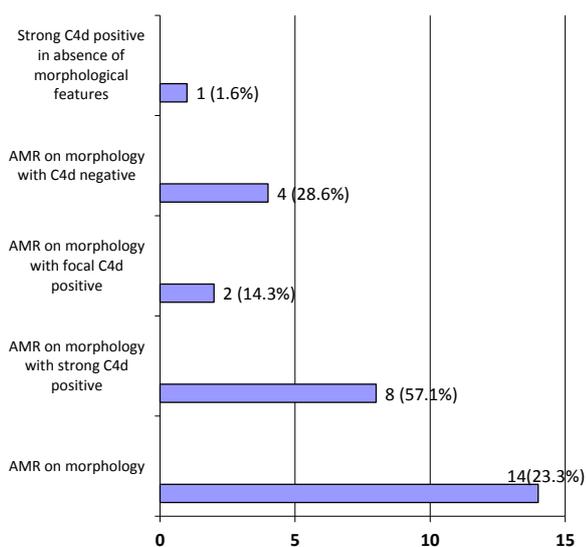


Figure 2. Distribution of acute antibody mediated rejection in early period of post transplantation.

Table 1. Range, mean and standard deviation of age and duration of post-transplant

Variables	Results
Age	15 - 65 y ( $34.6 \pm 11.8$ )
Duration Post-transplant	3 days - 180 months ( $33.8 \pm 46.8$ )

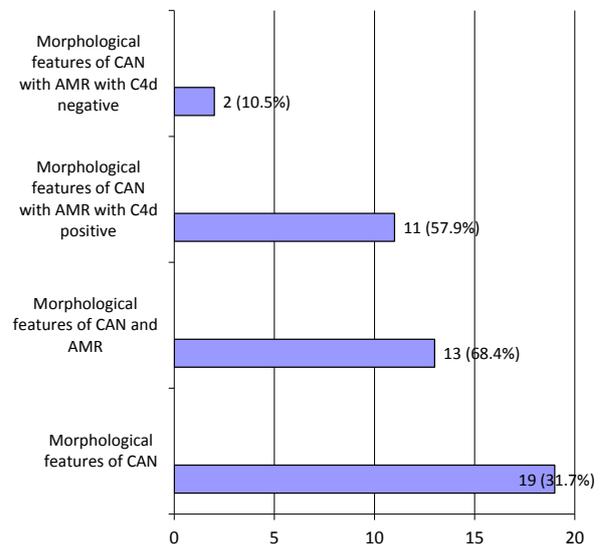


Figure 3. Distribution of acute antibody mediated rejection in late period of post transplantation in background of chronic allograft nephropathy.

in 3 (5%) cases. These cases were also C4d positive. IgA nephropathy, acute pyelonephritis and extensive oxalate crystal deposition each in one (1.7%) case.

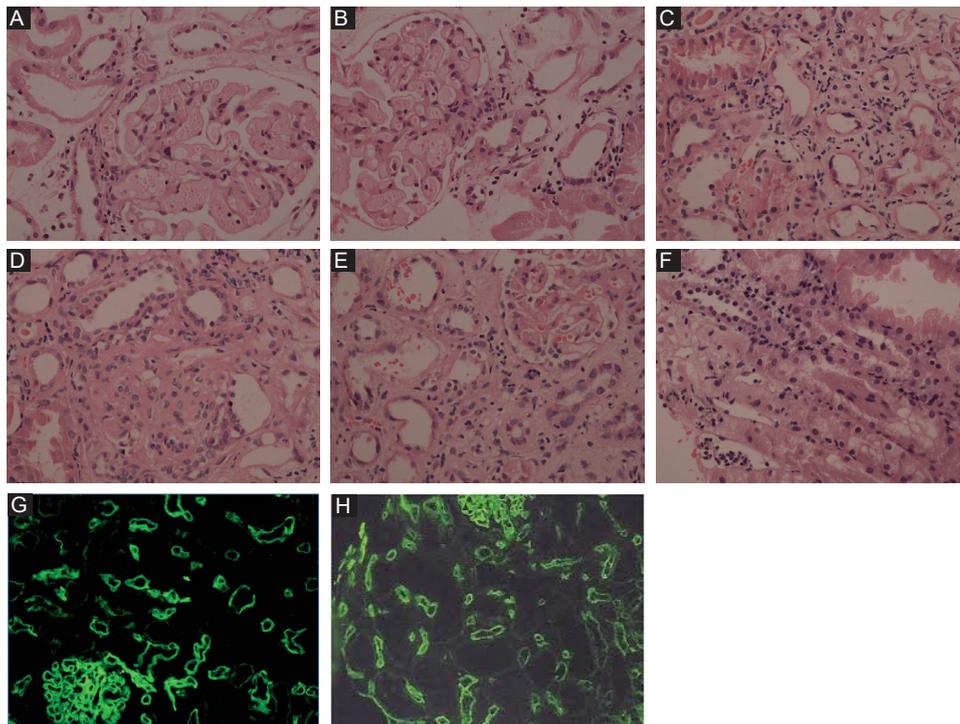
## Discussion

Long-term graft survival in renal transplantation strongly depends on events occurring early, particularly during first year after transplantation. A major event is an episode of acute rejection which has a major impact on long term performance of graft function. AMR should be considered in severe graft dysfunction setting, even when histopathologic features are equivocal (20).

Age range in our study was from 15 to 65 days with mean  $34.6 \pm 11.8$ . Patients in study conducted by Guduru et al had age range from 18 to 67 years (mean age 42.5 years) (11). The study by Ranjan et al had age ranges from 10 to 65 years with a mean of 34 years (6). Our cases had approximately similar age range.

In our study, 60 biopsies were evaluated with post-transplant period from 3 days to 5400 days (mean  $33.8 \pm 46.8$ ). Nickeleit et al analyzed 398 indication allograft post-transplant biopsies obtained during seven days to 7165 days (21). Results of our study had similar time post-transplant duration.

Biopsies included in our study were over period of 2 years. Ranjan et al, Carpio et al, Botermans et al, Nickeleit et al,



**Figure 4.** (A) Morphological features of acute antibody mediated rejection. Renal biopsy showing glomerulitis and mild degree of peritubular capillaritis (PC1). (B) Morphological features of acute antibody mediated rejection. Renal biopsy showing focal glomerulitis and moderate degree of peritubular capillaritis (PC2). (C) Morphological features of acute antibody mediated rejection. Renal biopsy showing marked degree of peritubular capillaritis (PC3). Tubular damage is also seen. (D) Morphological features of acute antibody mediated rejection, superimposed on chronic allograft nephropathy. Renal biopsy showing mild degree of peritubular capillaritis (PC1). (E) Morphological features of acute antibody mediated rejection, superimposed on chronic allograft nephropathy. Renal biopsy showing moderate degree of peritubular capillaritis (PC2) and focal glomerulitis. (F) Morphological features of acute antibody mediated rejection, superimposed on chronic allograft nephropathy. Renal biopsy showing marked degree of peritubular capillaritis (PC3). (G) C4d immunostaining. Glomerulus serves as a good internal control. Peritubular capillaries show diffuse circumferential deposits. (H) C4d immunostaining. Glomerulus serves as a good internal control. Peritubular capillaries show diffuse circumferential deposits.

and Larpparisuth et al performed studies over one year, 15 years, 9 years, 8 years and 8 years, respectively (6,20-23). In this study, we evaluated 60 indication biopsies and demonstrated that a substantial fraction (26.7%) of all biopsy-proven acute allograft rejection had a component of AMR as judged by morphology and C4d deposition in PTC. However, we also found that 6.25% of AMR cases would not have been recognized without C4d stain. These cases showed acute tubular injury. C4d staining of renal allograft biopsies is therefore essential in the recognition of AMR especially when features of only tubular injury are present. Similar findings are reported by Mauyyindi et al, who had analyzed 232 renal allograft biopsies and demonstrated 25% of C4d AMR cases without morphological features (24).

Our study showed 26.7% cases of C4d positive AMR, which fall in the spectrum of previous studies results. In Western countries, C4d varies from 17% to 60% among the indication biopsies. Regarding Asian population, only few prevalence studies had been conducted so far. In a Thai study, the prevalence of C4d was 16.4%. Lower prevalence of C4d has been reported in indication biopsies in studies by Mengel et al (16) in 20.7% and Cheunsuchon

et al in 16.4%. Out of these biopsies, 10.4% showed diffuse positivity and 5.9% had focal positivity (25). The reason of this variation includes type of biopsy, immunological risks for AMR, percentages of PTC positivity, time since transplant, case selection and techniques applied for immunostaining. In Harvard study, about 30% of all acute renal allograft rejections had a humoral component (7). In the study conducted in Brazil by Carpio et al, they showed 31% prevalence of C4d (22). C4d deposition had varied from 30% to 45% in other studies (6,10,21,26,27). In the largest study to date, Nicleleit et al investigated a total of 398 diagnostic biopsies performed and found C4d positivity in 12 specimens (30%) (21). Although our result is similar to this study, there is a significant difference in sample size.

We had 18 cases of acute rejection. Among them, 16 (26.7%) cases had morphological features of AMR while 2 (11%) cases of ACR. Out of 16 cases, 10 (62.5%) showed C4d positivity, with further subdivision as 8 (80%) diffuse and 2 (20%) focal C4d positivity. Rests of cases were negative for C4d immunostaining. Botermans et al evaluated 151 patients which had a clinical suspicion of acute rejection. Among them, 128 (84.8%) patients

showed histological evidence of AMR, and only 12.5% (n=16) showed C4d diffuse positivity by IF (20). In another study, conducted by Mauiyyedi et al, they had a sample size of 232 with 67 patients showing AMR (24). Out of them 75% had morphological evidence of AMR. Ludovico-Martins et al analyzed 20 cases with features of AMR. C4d deposition was found in 45% of their cases (28). In our study, stronger C4d positivity was associated with diffuse morphological features including peritubular capillaritis and glomerulitis. We found that focal versus diffuse C4d pattern was related to presence or absence of morphological features of AMR, respectively. Ranjan et al evaluated 132 core biopsies and C4d was performed in 126 of cases. AMR was diagnosed in 81% of total 45% of cases of rejection. Diffuse C4d was positive in 36.7% of total cases (6). Our results were similar to results of Ludovico-Martins et al and Mauiyyedi et al, but showed variation from Boterman et al and Ranjan et al's results. This difference may be due to a significant difference in sample size.

AMR is commonly accompanied by ACR i.e. these cases are classified as mixed ACR and AMR but AMR can occur in isolation. In literature, approximately 20% to 40% of ACR cases also have concurrent AMR. In our two cases, morphological features of both ACR and AMR were present. These cases were also C4d positive. Matignon et al conducted a study on 87 patients and found AMR in 29 and AMR with ACR in 18 (29). A study in Massachusetts general hospital and Harvard medical school found, ACR with AMR in 45% of cases (26). Concurrent presence of both AMR and ACR is associated with more severe graft dysfunction and treatment of both are different (30). Hence, it is important to look for both in a renal biopsy and report them separately.

According to Banff classification, extent of peritubular capillaritis should be mentioned in the report along with C4d result because the rate of C4d positive graft dysfunction was significantly higher if present along with peritubular capillaritis. Peritubular capillaritis is an important indicator of AMR in renal biopsies (31). In our study, 15 (25%) cases had diffuse peritubular capillaritis and 8 (13.3%) cases showed focal peritubular capillaritis. Glomerulitis was also present focally in 6 (10%) cases and in diffuse pattern in 9 (15%). Total of 16 cases had a good morphological evidence of AMR but only 10 showed diffuse C4d positivity. Kozakowski et al, studied 1322 renal biopsies with 329 showing peritubular capillaritis. Focal peritubular capillaritis was found in 10.5% of cases and diffuse peritubular capillaritis was in 14.4% of cases (32). Different studies reported the prevalence of peritubular capillaritis between 17%-46% (33,34). Results of diffuse peritubular capillaritis are quite close to comparison study but those of focal peritubular capillaritis were almost double of our results. All these studies had variation in total number of cases.

Out of 16 AMR cases, only 10 (62.5%) showed C4d

positivity. Eight cases showed diffuse PTC positivity for C4d and 2 (12.5%) were focal C4d positive. Diffuse C4d deposition in renal allografts biopsies is associated with graft failure. A study done by Kedainis et al from Washington University, reviewed 368 indication biopsies from 301 transplant patients (35). In study by Kayler et al, 106 biopsies were included. C4d was positive in 40 and negative in 66 (36). These studies proposed that not only diffuse but also focal C4d positivity is a poor prognostic factor for AMR and graft survival. The impact of focal C4d positive biopsies should be considered in further treatment strategies.

We had one case in which C4d was positive with focal evidence of tubular damage but peritubular capillaritis or glomerulitis were absent. In their study, two patients had widespread C4d immunostaining in PTC. The study performed by Taheri et al, 17% of total 44 renal specimens, showed diffuse positivity for C4d, with no microscopic features of AMR (24). They concluded that C4d immunostaining should be performed in every renal graft biopsy as features of AMR can be focal or even masked by other microscopic features. These cases can be missed if C4d staining was not performed. In Pakistan, C4d immunostaining was not available at every institution performing and evaluating renal allograft biopsies.

In our study we demonstrated 6.25% of AMR cases had diffuse C4d positivity with no morphological features of AMR. Similar findings were reported by various other studies, including a study Taheri et al; They reported 17% of 44 renal specimens with diffuse C4d positivity with no histological features of AMR (36). They concluded that C4d staining should be performed in all renal allograft biopsies and possibility of AMR should be considered even if its features are masked by other microscopic changes. In a study of Boston, it was mentioned that 25% of cases would have been missed if C4d immunostaining had not been performed (37). Therefore, it had been recommended in Banff criteria of AMR, to combine morphological features with C4d immunostaining in every renal biopsy. Even focal morphological features must be given weight age and mentioned in the final report.

Initially CAN was used which was a nonspecific term, as it can be the result of various conditions. In Banff 2005 schema it was replaced by interstitial fibrosis and tubular atrophy (IF/TA). If features of AMR are present on background of CAN, then these cases are categorized as chronic active antibody mediated rejection (38). It is reported that C4d serves as a reliable marker for detection of AMR in such cases (39). In our study, 29 cases were diagnosed as CAN. Out of these 10 had only features of CAN, two had features of CAN and morphological features of AMR, 11 had CAN, with morphological features of AMR and diffuse C4d positivity, one case of CAN, with morphological features of AMR and focal C4d positive. In studies performed during the early 21st century, a frequency of AMR in background of CAN was

reported in the range of 4.4% to 61% (21,24,40).

In 2005 and onwards, AMR in background of CAN was observed in 5.1% to 50% of cases (6,13,41-43). Yoon et al reported 5.1% of biopsies, and this difference is probably due to the inclusion of only protocol (42). Issa et al found C4d deposition in 24% of biopsies (43). Previous studies by the same group had reported C4d immunodeposition in 25% and 36% of cases (44). In upcoming literature, complement independent i.e. C4d negative type of CAN has been supported (45). It had been emphasized to perform graft biopsy even in late post-transplant period to reach diagnosis. Currently in addition to therapy of AMR, antifibrinogenic agents had been developed and there is a hope that these can reverse fibrosis (46,47).

In renal allografts, findings other than rejection can be seen and can also be a cause of graft dysfunction. In our 60 cases, ACR was documented in 7 (11.6%) cases, cyclosporine drug toxicity in 5 (8.3%) cases, tubulointerstitial nephritis in 5 (8.3%) cases, tubular damage and necrosis 5 (8.3%) cases. BK nephropathy was documented in 3 (5%) cases. Each of IgA nephropathy, acute pyelonephritis and extensive oxalate crystal deposition were present in one (1.7%) case. Taheri et al had acute tubular necrosis (ATN) in 27%, CNI toxicity in 12.1% and 14.6% of cases had other findings including recurrence of primary glomerulonephritis, BK virus nephropathy and TIN and cortical infarction (37). Kulkarni et al had seven cases of ATN, CNI toxicity in 5 and BK virus nephropathy (13). As AMR can be the cause of graft dysfunction even many years of renal transplant, thus in every case, a biopsy must be submitted for C4d staining. This is important as morphological features can be masked due to any other concurrent etiology.

Donor specific antibodies level is a must in current Banff criteria of AMR in addition to morphological evidence and C4d positivity. In many previous cases DSA levels were available and results were compiled along with morphological features and C4d immunostaining pattern. We had C4d staining in 36.7% of total cases. In different studies 30%, 31%, 46%, 50% and 39.3% cases had C4d positivity (10,22,48-50). DSA levels were not available in any of our cases. Our results were close to results of all these studies results, however, parameters which differed were sample size, lack of DSA levels, shorter duration post-transplantation and difference in C4d immunostaining techniques.

We analyzed C4d staining only by IF technique which is superior to IHC technique. Out of our 60 cases, C4d positivity was in 36.7% of cases including diffuse positivity in 43 and focal positivity in 2 cases. Carpio et al evaluated 146 renal biopsies. C4d staining was performed by IHC. Among them 31% had C4d staining, with further sub-distribution as diffuse positivity in 14.7% and focal positivity in 16% (22). Worthington et al studied 92 cases and C4d was performed by immunohistochemistry. Among them 15% and 24% cases showed diffuse and focal C4d immunopositivity (51). Although total C4d positivity

results are close, further subdivision into diffuse positivity and focal positivity show variation. Even though IHC had documented to provide appropriate results in renal transplant setting for C4d, IF is still considered as a gold standard (52). Hence, cases with focal C4d positivity by IHC should be evaluated by IF technique for definitive characterization.

### Conclusion

C4d positivity favors AMR, however, it can be seen in absence of morphological features and results should be interpreted by taking in account other findings like donor specific antibody and endothelial activation markers. With the increasing use of C4d by transplant pathologists worldwide, several shortcomings of C4d have been identified and C4d appears to be a less sensitive marker than initially thought. Furthermore, molecular studies have provided insight suggestive of a complement-independent form of AMR.

### Limitations of the study

Our study had few deficiencies including low sample count and lack of DSA levels. Hence, variability in results of our study while comparing it to other studies was seen. Previous studies had repeatedly proven that C4d staining is strongly related to DSA levels. However, emerging data show that AMR can be C4d negative and still relates to DSA levels. So additional work is required in this particular topic to see the actual trend of AMR in our region.

### Authors' contribution

HN conceived the study and interpreted the light microscopy and immunofluorescent findings. HT collected, analyzed the data and prepared the manuscript. Both authors read, revised and approved the final manuscript.

### Conflicts of interest

The authors declared no competing interests.

### Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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