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# ROLE OF C4d IMMUNOSTAINING IN DIAGNOSIS AND PROGNOSIS OF ACUTE AND CHRONIC RENAL ALLOGRAFT REJECTION

Pathology

**Pallavi Arvind Patil** Assistant Professor, Department of Pathology , Smt. Kashibai Navale Medical college and general hospital, Narhe Pune

**Swapnil Karnik*** Chief Histopathologist ,Ruby hall Clinic ,Pune. *Corresponding Author

## ABSTRACT

Renal allograft biopsy is the gold standard for diagnosis of rejection. Incorporation of C4d as a marker for humoral rejection is a major addition for Banff Schema, 2005. We evaluated the pattern of C4d staining in indicated renal allograft biopsies from January 2009 to March 2012. There were total 108 renal allograft biopsies received in the department; out of which 59 biopsies had features consistent with rejection.

Although C4d staining was performed in all the 108 biopsies; only those with histological suspicion of rejection were included for deriving the actual percentage of C4d positivity. Morphologic features like peritubular capillary dilatation, tubulitis and interstitial inflammation were seen more frequently in C4d-positive biopsies . Cases with features of ATN, cellular rejection, Transplant glomerulopathy were included. Among the 59 cases, 24 (40.67%) cases were C4d positive.

90% of C4d positive cases eventually lost their grafts and the remaining 10 % became dialysis dependent.

Thus from our study we can conclude that C4d positivity is an indicator of Antibody mediated rejection and indicates grave prognosis.

## KEYWORDS

Renal Allograft , rejection ,C4d,immunostaining.

## INTRODUCTION

Transplant rejection is a complex process in which both cell-mediated immunity and circulating antibodies play a role. Renal biopsy is the gold standard for diagnosis of acute rejection in renal transplant recipients. For the diagnosis of cellular rejection, well-defined histological criteria were laid down under the Banff system in 1993 and were further revised in 1997. In view of these observations, the Banff (1997) classification was revised in 2003 incorporating morphological criteria for acute antibody mediated rejection, supported by immunopathological criteria, and serological evidence for acute humoral rejection. The diagnostic criteria of antibody mediated rejection in renal allograft biopsy include:

**Type 1:** ATN like- C4d positivity in peritubular capillaries

**Type 2:** Capillary glomerulitis, polymorphonuclear and/or mononuclear leucocytes in peritubular capillaries (with C4d positivity)

**Type 3:** Arterial-transmural inflammation/ fibrinoid change with C4d positivity 1

Acute antibody mediated rejection is mediated by antibodies to the donor HLA that activate the classical complement pathway. This leads to a number of split products of complement (C3a, C3b, C3d, C4b). C4d is a fragment of C4 released during activation of the classic complement pathway by the antigen–antibody complex. Detection of C4d is regarded as an indirect sign, a 'footprint' of an antibody response.C4d is the degradation product of the activated complement factor C4, a component of the classical complement cascade which is typically initiated by binding of antibodies to specific target molecules1-5.

## AIMS AND OBJECTIVES

1. To study the prevalence of C4d positivity in renal allograft biopsies in patients of kidney transplant suspected to have acute and chronic rejection.

2. To study the prognostic importance of C4d positivity in renal allograft biopsies suspected to have acute rejection

## MATERIALS AND METHODS

Tissue processing of the renal biopsies was carried out for 22 hours in LEICA automated tissue processor. After tissue processing is embedded in molten paraffin wax (60-650C). It is done with the help of LEICA tissue embedding machine. Sections were taken and stained by hematoxylin and eosin (H & E) stain. Three micron sections were studied under light microscopy and the findings were noted. Special stains such as Periodic Acid Schiff (PAS), Jones Silver, Masson's trichrome and C4d immunostaining were done by standard techniques in all cases to substantiate the diagnosis.

## OBSERVATIONS

This study included retrospective and prospective case analysis of 108 renal biopsies. There were 25 females and 83 males in the age range 16 to 70 years (mean age - 39 years). The mean serum creatinine at the time of biopsy was 4.4 mg/dl.

**Table 8:** Age distribution of the renal allograft biopsy patients included in the study.

| Age | 15-25 Yrs | 26-35 Yrs | 36-45 Yrs | 46-55 Yrs | 56-65 Yrs | 66-75 Yrs |
|---|---|---|---|---|---|---|
| No. of Patients | 11 | 39 | 30 | 18 | 8 | 2 |

**Light microscopic features:**

**Table 9-** C4d immunostaining in 108 renal allograft protocol biopsies.

| Diagnosis | No. of Cases | No. of C4d positive cases | percentage |
|---|---|---|---|
| ATN | 15 | 2 | 13.33 % |
| ATN with CNI Toxicity | 7 | 0 | 0% |
| ATN with Tubulointerstitial nephritis | 1 | 0 | 0% |
| CNI Toxicity | 12 | 0 | 0% |
| P-AbMR | 8 | 8 | 100% |
| CAN +P-AbMR | 11 | 7 | 63.63% |
| ACR Type 1A | 16 | 0 | 0% |
| ACR Type 1B | 14 | 0 | 0% |
| ACR Type 2 | 0 | 0 | 0% |
| BK virus Nephropathy | 1 | 0 | 0% |
| ACR Type I B +BK Nephropathy | 1 | 0 | 0% |
| Mild Tubulitis | 1 | 0 | 0% |
| Mild Tubulitis with ATN | 1 | 1 | 100% |
| Transplant Glomerulopathy | 1 | 0 | 0% |
| ACR Type I A with Transplant Glomerulitis | 2 | 2 | 100% |
| ACR Type I B, Severe Tubulitis with early vascular rejection. | 1 | 1 | 100% |
| ACR Type I B + Transplant glomerulopathy | 1 | 1 | 100% |
| ACR Type I A + CAN+ P-AbMR | 2 | 2 | 100% |
| Recurrence of disease | 8 | 0 | 0% |
| Cortical Necrosis | 3 | 0 | 0% |
| Diabetic nephropathy | 1 | 0 | 0% |
| Acute interstitial nephritis | 1 | 0 | 0% |
| Total | 108 | 24 | 22.22% |

**ATN- Acute tubular necrosis; CNI- Calcineurin inhibitor; ACR- Acute cellular rejection; P-AbMR- Presumed antibody mediated rejection**

The light microscopic findings of all the biopsies are given in Table 9.

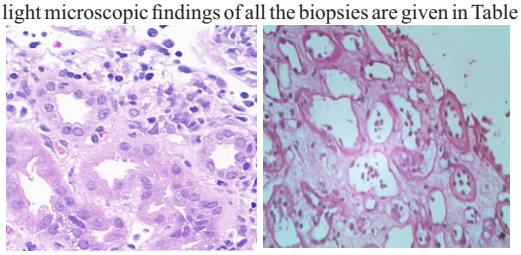

**Figure 12 - Peritubular capillary (PTC) margination (neutrophils in PTC) and PTC dilatation (PTC-D)**

**C4d staining** was done in all these biopsies, and the prevalence of C4d positivity was calculated to be **22.22%**; the cases with CNI toxicity, viral infection, interstitial nephritis, Recurrence of native kidney disease, cortical necrosis and diabetic nephropathy were excluded from the data of kidney biopsies with suspected rejection, on the basis of histology alone. Hence remaining **59** cases of graft rejection were considered for evaluating the prevalence of C4d positivity; out of which **24 cases were C4d Positive.** Thus the overall C4d positivity seen in the cases of graft rejection is **40.67%.**

**Table 10 - C4d immunostaining in 59 renal allograft biopsies**

| Histological Diagnosis | No. Of Cases | No.of C4d Positive Cases | Percentage |
|---|---|---|---|
| P- AbMR | 8 | 8 | 100% |
| CAN +P-AbMR | 11 | 7 | 63.63% |
| ACR Type I A | 16 | 0 | 0% |
| ACR Type I B | 14 | 0 | 0% |
| ACR Type I B + BK nephropathy | 1 | 0 | 0% |
| Mild Tubulitis with ATN | 1 | 1 | 100% |
| ACR Type I A withTransplant glomerulitis | 2 | 2 | 100% |
| ACR Type IB ,severe tubulitis with early vascular rejection | 1 | 1 | 100% |
| ACR Type I B with Transplant glomarulopathy | 1 | 1 | 100% |
| ACR Type I A +CAN+P-AbMR | 2 | 2 | 100% |
| ATN + P- AbMR | 2 | 2 | 100% |
| Total | 59 | 24 | 40.67% |

After extrapolating the cases with presumed antibody mediated rejection and cellular rejection from the data of all the renal biopsies with graft dysfunction, the prevalence of C4d positivity was calculated. Thus the overall C4d positivity was calculated to be 40.67%.

**C4d immunostaining:**

C4d immunostaining was positive in 24 out of 59 biopsies (40.67 %). The details of C4d positivite cases are shown in Table 2.

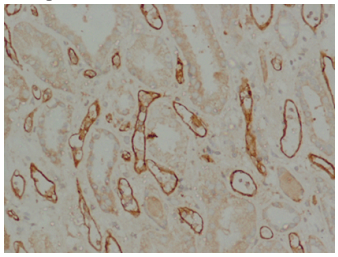

**Figure 14-** C4d was typically detected along peritubular capillaries (PTC) with a strong linear staining pattern

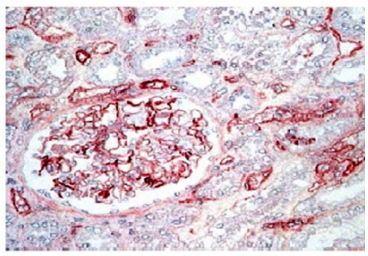

**Figure 15 -** Glomerular C4d staining

**Table 11** – Grading of C4d staining by Immunohistochemistry.

| BANFF Grade | Interpretation | % Biopsy area |
|---|---|---|
| C4d0 | Negative | 0% |
| C4d1 | Minimal | 1-10% |
| C4d2 | Focal | 10-50% |
| C4d3 | Diffuse | >50% |

**Figure 16 -**Intensity of C4d staining in peritubular capillaries AR = Acute rejection; CR = Chronic rejection.

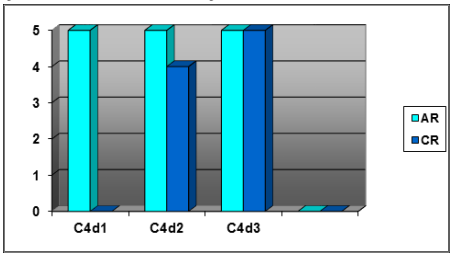


**Table 16:-** BANFF grading and subsequent clinical outcome in patients diagnosed of C4d positive antibody mediated acute/chronic allograft rejection.

| Case Reg. no. | BANFF Grade | Follow-up Creatinine | Clinical Outcome |
|---|---|---|---|
| 605/09 | C4d1 | 12.2 | Lost Graft |
| 115/09 | C4d1 | 8.8 | Lost Graft |
| 630/09 | C4d1 | 8.2 | Lost Graft |
| 1165/09 | C4d3 | 9.8 | Lost Graft |
| 417/09 | C4d1 | 11 | Lost Graft |
| 2208/09 | C4d1 | 10 | Lost Graft |
| 2744/09 | C4d2 | 18.2 | Lost Graft |
| 2954/09 | C4d2 | 9 | Lost Graft |
| 3042/09 | C4d2 | 22 | Lost Graft |
| 4250/09 | C4d2 | 4.3 | Hemodialysis dependent |
| 2360/10 | C4d3 | 15.2 | Lost Graft |
| 4500/10 | C4d3 | 14 | Lost Graft |
| 561/11 | C4d2 | 9.9 | Lost Graft |
| 683/11 | C4d3 | 15 | Lost Graft |
| 581/11 | C4d3 | 9 | Lost Graft |
| 1722/11 | C4d3 | 12 | Lost Graft |
| 2969/11 | C4d2 | 3.3 | Hemodialysis dependent |
| 3220/11 | C4d3 | 13 | Lost Graft |
| 4428/11 | C4d2 | 12.5 | Lost Graft |
| 4723/11 | C4d3 | 14 | Lost Graft |
| 1420/12 | C4d2 | 10 | Lost Graft |
| 1480/12 | C4d2 | 12.3 | Lost Graft |
| 1551/12 | C4d3 | 18.3 | Lost Graft |
| 1664/12 | C4d3 | 13.2 | Lost Graft |

## DISCUSSION

This study evaluates the significance of the detection of C4d in the interpretation of diagnostic renal allograft biopsies.

The gold standard for the diagnosis of rejection and for guiding patient management of renal transplant recipients is the histological evaluation of a renal allograft biopsy . Over the past decades, no well-defined criteria for the proper identification of humoral rejection episodes in the early or late post-transplantation period have been defined. Hence, antibody-mediated rejection episodes frequently remained undiagnosed and unclassified. Consequently, nearly all acute rejection episodes have been classified as 'cell mediated'. Currently, C4d is regarded as an immunohistochemical marker for a humoral-mediated allo-response, not only by the pioneers in this field but also by many other centres which have included the use of C4d immunostaining during the work-up of allograft dysfunction6.

In the present study, all biopsies performed due to graft dysfunction during the period of January 2009 to March 2012 were selected and C4d immunostaining was performed, irrespective of histological diagnosis. The overall C4d positivity in histologically indicated renal biopsies was 40.67% (24/59), whereas it was comparatively low, i.e 22.22% (24/108) in all the protocol renal allograft biopsies. These figures are compatible with other studies having C4d positivity varying from 15% to 62%3. In the present study, 100 % of the biopsies demonstrating the features of acute rejection on histology were C4d positive. The actual percentage of acute rejection in the different studies varies markedly between 10 and 60% due to selection of patients, time since graft and clinical indication of biopsies. Similar observations have been made by other studies. Herman et al7 studied the deposition of C4d in paediatric renal allograft biopsies and analysed morphological criteria of humoral rejection described by Banff (2003) and showed an overall C4d positivity to be 52%.

Analysis of C4d immunostaining with various histological changes of renal allograft biopsies correlates well with presumptive diagnosis of antibody-mediated rejection. In the present study, distinguishing morphological features of the C4d-positive rejection versus C4d-negative rejection included capillary margination by polymorphs in PTC (65% versus 4.54%), dilatation of PTC (100 % versus 0%). However, statistically significant associations of C4d positivity were seen only with capillary margination of neutrophils in the PTC and dilatation of PTC with C4d-positive rejection. Mauiyyedi et al8 also noted more neutrophils in PTC (65% versus 9%), neutrophilic glomerulitis (55% versus 4%), neutrophilic tubulitis (55% versus 9%), severe necrosis in glomeruli (20% versus 0%) or arteries (25% versus 0%) in C4d-positive rejection. Nickeleit et al9 reported 57% glomerulitis and 45% end-arteritis in C4d-positive biopsies. In the study of Herman et al10, C4d positivity in PTC was associated with accumulation of polymorphonuclear cells in PTC.

The biopsies from AbAR+ patients had a higher incidence of severe vasculitis and glomerulitis. Fibrin thrombi in the glomeruli and vessels, fibrinoid necrosis and dilatation of PTCs were also more frequent in the AbAR+ group. The biopsy specimens more often had polymorphonuclear leukocytes and monocytes/macrophage in the PTCs and glomeruli. Graft loss was higher in AbAR+ patients.

In our study the overall C4d positivity in biopsies showing features of CAN was 63.63%. No associations of C4d were found with any other features of CAN, although transplant arteriopathy is found more frequently in the C4d-positive group.

Our study shows that,C4d-positive cases had a higher mean value of serum creatinine at the time of biopsies. But no correlation of C4d positivity and serum creatinine at the time of maximum follow-up was found.This is one of the first Indian prospective study to document C4d immunostaining in acute rejection as well as chronic allograft nephropathy.

Individual histological features like capillary margination of polymorphs and dilatation of peritubular capillaries (PTC-D), glomerulitis, fibrin thrombi, acute tubular necrosis and fibrinoid necrosis were more common in the C4d positive group. These morphological features are helpful to differentiate acute antibody-mediated rejection from acute cellular rejection.

In the end, it can be stated that C4d immunostaining is a useful marker, not only in the diagnosis of acute antibody-mediated rejection episodes, but also in biopsies with CAN that fail to correlate the degree of graft dysfunction with morphological features. It appears that the humoral limb of rejection has a role not only in the early post-transplant period, but also in late cases of chronic allograft nephropathy. C4d immunostaining deserves a place in the biopsy diagnosis of graft dysfunction.

**Table 17: -** Comparison of C4d positivity in indicated renal allograft biopsies of the present study with literature.

| | Troxell et al 11 (n = 60) | Ranjan et al12 (n =126) | Present Study (n = 82 ) |
|---|---|---|---|
| C4d Positivity | 15% (9) | 45% (57) | 40.67%(24) |
| Focal | - | 40% (23/57) | 37.5%(9/24) |
| Diffuse | 100% (9/9) | 60% (34/57) | 29.16%(7/24) |
| AbMR | - | 81% (13/17) | 100%(8/8) |
| ACR 1A | 33.3%(6/18) | 20%(2/10) | 0%(0/16) |
| ACR 1B | 43%(3/7) | - | 0%(0/14) |
| ACR2A | 40%(2/5) | - | - |
| CAN | - | 30%(7/24) | - |
| ATN | - | 25%(2/8) | 13.33%(2/15) |
| BK Virus | - | 100%(1/1) | 0%(0/2) |
| Normal Biopsy | 12% | 45%(10/18) | - |
| CAN + AbMR | - | - | 63.63%(7/11) |
| ACR Type I A + CAN + AbMR | - | - | 100%(2/2) |

AbMR- Antibody mediated rejection; ACR – Acute cellular rejection; CAN- Chronic allograft nephropathy; BK virus- polyoma virus; ATN- Acute tubular necrosis.

## CONCLUSION

1. Although C4d staining was performed in all the 108 biopsies; only those with histological suspicion of rejection were included for deriving the actual percentage of C4d positivity.

2. Cases with features of ATN, cellular rejection, Transplant glomerulopathy were included. Among the 59 cases, 24 (40.67%) cases were C4d positive.

3. Follow up of C4d positive cases with serum creatinine levels 2 weeks after antirejection therapy was done. The creatinine levels were 2 to 3 times higher than at the time of biopsy.

4. 90% of C4d positive cases eventually lost their grafts and the remaining 10 % became dialysis dependent.
   Thus from our study we can conclude that C4d positivity is an indicator of Antibody mediated rejection and indicates grave prognosis.

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