# Correlações clinico-patológicas da marcação de C4d e sua influência na evolução de receptores de transplante renal

## Clinical and pathological correlations of C4d immunostaining and its influence on the outcome of kidney transplant recipients

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## Resumo

**Introdução:** A fração do complemento C4d é um marcador de rejeição mediada por anticorpos (RMA) em aloenxertos renais, embora na rejeição celular também se observem depósitos de C4d. **Objetivos:** Correlacionar a expressão de C4d com parâmetros clínicopatológicos e a evolução do enxerto renal em três anos. **Métodos:** Foram incluídos 146 receptores de transplante renal com biópsias por indicação. A marcação de C4d foi feita por imuno-histoquímica em parafina. Foram medidas a função e a sobrevida do enxerto e determinadas as variáveis preditivas de sua evolução por meio de modelo de regressão de Cox. **Resultados:** A marcação positiva para C4d foi detectada em 48 (31%) biópsias, das quais 23 (14,7%) tinham marcação difusa e 25 (16%), focal. A reatividade contra painel (%PRA) de classe I e II pré-transplante foi significativamente maior nos pacientes $C4d^+$ quando comparada aos $C4d^-$. Tanto glomerulite quanto pericapilarite foram associadas com C4d ($p = 0,002$ e $p < 0,001$, respectivamente). A presença de C4d em biópsias sem rejeição (SR), rejeição celular aguda (RCA) ou fibrose intersticial/atrofia tubular (FI/AT) não teve impacto na função ou na sobrevida do enxerto. Comparados a indivíduos com SR, RCA e FI/AT $C4d^-$, pacientes com RMA $C4d^+$ tiveram pior sobrevida do enxerto em 3 anos ($p = 0,034$), mas não houve diferença entre RMA *versus* SR, RCA e FI/AT $C4d^+$ ($p = 0,10$). Na regressão de Cox, função do enxerto no momento da biópsia e %PRA alto foram preditores de perda do enxerto. **Conclusões:** A pesquisa de C4d em biópsias do enxerto renal é útil para identificar RMA, com correlações clínicopatológicas bem definidas. O impacto do C4d em outros diagnósticos histológicos necessita de investigação adicional.

## Abstract

**Introduction:** C4d is a marker of antibody-mediated rejection (ABMR) in kidney allografts, although cellular rejection also have C4d deposits. **Objective:** To correlate C4d expression with clinico-pathological parameters and graft outcomes at three years. **Methods:** One hundred forty six renal transplantation recipients with graft biopsies by indication were included. C4d staining was performed by paraffin-immunohistochemistry. Graft function and survival were measured, and predictive variables of the outcome were determined by multivariate Cox regression. **Results:** C4d staining was detected in 48 (31%) biopsies, of which 23 (14.7%) had diffuse and 25 (16%) focal distribution. Pre-transplantation panel reactive antibodies (%PRA) class I and II were significantly higher in C4d positive patients as compared to those C4d negative. Both glomerulitis and pericapillaritis were associated to C4d ($p = 0.002$ and $p < 0.001$, respectively). The presence of C4d in biopsies diagnosed as no rejection (NR), acute cellular rejection (ACR) or interstitial fibrosis/tubular atrophy (IF/TA) did not impact graft function or survival. Compared to NR, ACR and IF/TA $C4d^-$, patients with ABMR $C4d^+$ had the worst graft survival over 3 years ($p = 0.034$), but there was no difference between ABMR *versus* NR, ACR and IF/TA that were C4d positive ($p = 0.10$). In Cox regression, graft function at biopsy and high %PRA levels were predictors of graft loss. **Conclusions:** This study confirmed that C4d staining in kidney graft biopsies is a clinically useful marker of ABMR, with well defined clinical and pathological correlations. The impact of C4d deposition in other histologic diagnoses deserves further investigation.

**Palavras-chave:** Transplante de rim. Complemento Cd4. Sobrevivência de enxerto. Rejeição de enxerto. Rejeição humoral.

**Keywords:** Kidney transplantation. Complement Cd4. Graft survival. Graft rejection. Humoral rejection.

## Introduction

The complement cleavage product C4d is a specific marker of antibody mediated rejection (ABMR) in kidney transplantation, and many studies have shown C4d as an independent predictor of renal graft outcome.[1-5] C4d represents one criterion to the diagnosis of acute and chronic ABMR according to Banff classification.[6] Staining for C4d in peritubular capillaries is present in other histological diagnosis of kidney graft biopsies, such as acute cellular rejection and/or acute graft injury,[3] interstitial fibrosis and tubular atrophy, calcineurin inhibitor toxicity, and even in grafts without dysfunction and with no morphological signs of rejection.[3,4,7] The influence of C4d on graft function and outcome over these different Banff categories is still unclear.

Another feature to consider about C4d staining is the accuracy and reproducibility of current techniques, which could affect its prevalence, clinical diagnosis and therapeutic decisions. In western countries, the prevalence of C4d positivity in renal allograft biopsies using different techniques varies from 17 to 60% among biopsies by indication.[3,8-13] In Brazil, Ludovico-Martins *et al.*[14] showed a C4d prevalence of 45% by immunofluorescence (IF). In this study, frozen-immunohistochemistry (IHC) and paraffin-IF had a good concordance rate to frozen-IF, but paraffin-IHC had a much lower accuracy. Few information is available about the reproducibility of C4d interpretation when this marker is detected by paraffin-IHC.[15]

The aims of this study were to determine the prevalence of C4d in graft biopsies according to Banff histopathology, and to correlate C4d expression with clinical and pathological parameters and graft function and survival over time. The reproducibility of C4d interpretation, performed by immunohistochemistry technique, was also measured.

## Materials and methods

This study included prospectively 113 renal transplant recipients who had allograft biopsies by indication for acute or chronic graft dysfunction at *Hospital de Clínicas de Porto Alegre* (HCPA) from January 2007 to July 2009. Other 33 patients who also had biopsies by indication at our centre from January 1991 to December 2006 were also included. In total, 156 biopsies of 146 patients were included. The study protocol was approved by the HCPA Research Ethics Committee.

### Clinical data

The following variables were evaluated: age, gender, type of donor, re-transplants, pre- transplant serum panel reactive antibodies (PRA) against HLA class I and II, HLA mismatches in loci A, B, and Dr, serum creatinine at biopsy, occurrence of delayed graft function (need for dialysis in the first week), and time between transplant and graft outcome (months). Time interval between transplant and biopsy was defined as early (≤ 6 months) or late (> 6 months). Renal graft function was determined by estimated glomerular filtration rate (eGFR) using the re-expressed Modification of Diet in Renal Disease (MDRD) equation.[16] Baseline immunosuppression consisted of prednisone, mycophenolate mofetil or sodium, and a calcineurin inhibitor (cyclosporine or tacrolimus). As induction therapy, Basiliximab, OKT3 or Thymoglobulin were used. Acute cellular rejection (ACR) was treated with intravenous methylprednisolone, and resistant cases received Thymoglobulin or OKT3. Patients with ABMR were treated with plasmapheresis and intravenous immunoglobulin.

### Histopathological diagnosis

Histopathologic diagnosis was performed by a renal pathologist who was blinded to clinical data, according to the Banff'2005 Meeting Report.[6] Patients were classified as: no rejection (NR), ACR, ABMR or interstitial fibrosis/tubular atrophy (IF/TA). No rejection included biopsies with normal tissue, acute tubular necrosis, calcineurin inhibitor toxicity, or borderline changes.

As we did not measure circulating anti-HLA antibodies, a "presumptive" diagnosis of ABMR was defined by C4d positivity and specific morphological features: margination of neutrophils in peritubular capillaries (PTC), fibrinoid necrosis, glomerulitis, thrombi in glomeruli and arteries, severe endarteritis (V3), and/or acute tubular injury. Patients who had a biopsy positive for C4d and morphological features suggestive of chronic ABMR – such as transplant glomerulopathy (glomerular basement membrane duplication or double contours, Banff score cg 1-3),

and/or fibrous intimal thickening in arteries without duplication of the internal elastic – were also included as ABMR. In ABMR group we included pure acute ABMR, ABMR with features of ACR, and chronic ABMR.

Graft function and survival were analyzed according to Banff category (SR, ACR, ABMR or IF/TA) and presence or absence of C4d, at a minimum follow-up of one year.

### C4d immunostaining

C4d was detected by immunoperoxidase in formalin-fixed, paraffin-embedded sections. Slides were incubated overnight with a polyclonal anti-rabbit antibody (Abcam, American Research Products®, Palo Alto, CA, US), followed by incubation with Universal Link Biotinylated Secondary Antibody (Biocare Medical®, Concord, CA, US) and after streptavidine-horseradish peroxidase (Biocare Medical®, Concord, CA, US). Reaction was developed with Romulin AEC chromogen (Biocare Medical®, Concord, CA, US). Criteria for C4d positivity were a linear and circumferential staining in at least 25% of PTCs in cortex or medulla, excluding fibrotic and necrotic areas. Distribution of C4d was established as: 1) diffuse when > 50% of PTC were stained; 2) focal when 25 to 50% of PTC were stained; and 3) negative if less than 25% of PTC were stained.[11]

Two observers blinded to clinical data scored C4d staining independently, when inter-observer variability was calculated. Intra-observer variability was calculated based on two separated interpretations of the same observer, at least six months apart.

### Statistical analysis

Data was described as means and standard deviation or median and interquartile ranges. Comparisons between groups were performed by *t* independent test, Mann-Whitney test, one-way ANOVA, and $\chi^2$ test when appropriate. Intra-observer and inter-observer agreement rate was measured by Kappa statistic, in which values greater than 0.75 were considered as excellent concordance, between 0.40 and 0.75 fair to good, and less than 0.40 were considered as poor concordance.[17] Graft survival at three years was measured by Kaplan Meier. Multivariate Cox regression analysis was used to identify variables predicting graft loss at last follow-up, including variables with statistical significance in the univariate analysis. Data were processed and analyzed using SPSS for Windows, version 16.0 (Chicago, IL, US); p level < 0.05 was considered significant.

## Results

### Renal allograft biopsy findings and C4d staining

Median time (and interquartile ranges) between transplant and graft biopsy were 11 (8-22) days and 23 (11-68) months for patients biopsed in early and late periods, respectively. Histopathological diagnosis according to Banff classification are shown in Table 1. Median time to biopsy of IF/TA group was 20 (8-66) months and for the other categories together was 13 (8-29) days (p < 0.001). Median times from transplantation to last follow-up were 56 (30-99) *versus* 27 (16-34) months for these two groups, respectively (p < 0.001).

Peritubular capillary staining for C4d was detected in 48 (31%) biopsies, of which 23 (14.7%) had a diffuse staining and 25 (16%), a focal distribution. Positivity for C4d was found in 40 (83%) biopsies done early (< 6 months) post transplant as compared to 8(17%) in late (> 6 months) biopsies (p = 0.039).

### Clinical and demographic data according to C4d staining

Baseline clinical and demographic characteristics are presented in Table 2. Patients whose graft biopsies stained diffusely for C4d had significantly higher pre-

**Table 1** Histopathological diagnosis according to Banff 2005

| Histological diagnosis | n (%) |
|---|---|
| Normal tissue | 5 (3) |
| Borderline changes | 14 (9) |
| Acute cellular rejection | 62 (40) |
| Type IA | 37 (60) |
| Type IB | 3 (5) |
| Type IIA | 12 (19) |
| Type IIB | 8 (13) |
| Type III | 2 (3) |
| Humoral rejection | 17 (11) |
| Pure acute humoral rejection | 10 (59) |
| Humoral + cellular rejection | 3 (17) |
| Chronic humoral rejection | 4 (24) |
| Interstitial fibrosis/tubular atrophy | 34 (22) |
| Grade I | 11 (32) |
| Grade II | 20 (59) |
| Grade III | 3 (9) |
| Acute tubular necrosis | 19 (12) |
| Calcineurin inhibitor toxicity | 5 (3) |

n = 156.

transplant %PRA. No difference was found in %PRA levels comparing patients with diffuse and focal C4d. Comparing patients with ABMR rejection against the other Banff categories together, %PRA class I was 27 ± 34 and 4 ± 12 ($p < 0.001$) and class II was 37 ± 37 and 3 ± 12 ($p < 0.001$), respectively.

Delayed graft function occurred more times in patients who had focal C4d in biopsy than in diffuse and negative C4d groups ($p = 0.04$), and also in patients with ABMR as compared to the other Banff categories (87 *versus* 46%; $p = 0.003$). No statistical difference between the three groups was found for the other parameters (Table 2).

Positive staining for C4d was found in 83 and 17% of biopsies taken ≤ 6 months and > 6 months post transplantation, respectively ($p = 0.04$). Comparing type of maintenance immunosuppression and type of induction therapy, no difference was observed in C4d positivity in these graft biopsies.

### Association of C4d deposition with Banff diagnosis and morphologic features

The distribution of C4d in biopsies according to histopathology and morphologic Banff features are shown in Table 3. As the presence of C4d was a criterion to identify ABMR, this group was taken as the reference group. C4d deposits were detected in 9 (21%), 18 (29%), and 4 (12%) of the biopsies with NR, ACR, and IF/TA, respectively ($\chi^2 = 46.05$; $p = 0.001$). The majority of biopsies with histological criteria for acute cellular rejection (71%), histology other than rejection (79%) and chronic damage (88%) did not stain for C4d. Deposits of

**Table 2** Patient demographics and clinical characteristics according to C4d staining patterns

| | Diffuse C4d (n = 23) | Focal C4d (n = 25) | Negative C4d (n = 108) | p |
|---|---|---|---|---|
| Age (years) | 37 ± 12 | 44 ± 13 | 42 ± 11 | 0.08 |
| Gender, male (%) | 65 | 56 | 59 | 0.80 |
| Previous transplant | 4 (18) | 4 (16) | 8 (7) | 0.21 |
| PRA > 20% (n, %) | 8 (35) | 8 (32) | 12 (11) | 0.01 |
| PRA Class I (%) | 19 ± 29 | 11 ± 24 | 3 ± 11 | 0.002[a] |
| PRA Class II (%) | 13 ± 24 | 21 ± 34 | 4 ± 13 | 0.004[b] |
| Induction therapy (n, %) | 14 (61) | 18 (72) | 60 (56) | 0.30 |
| Donor, D/L (%) | 56/44 | 76/24 | 53/47 | 0.09 |
| HLA mismatches | 3.4 ± 1.3 | 3.7 ± 1.3 | 3.7 ± 1.4 | 0.50 |
| DGF (n, %) | 10 (44) | 18 (72) | 48 (44) | 0.04 |
| Creatinine at biopsy (mg/dL) | 4.4 ± 2.3 | 4.6 ± 2.7 | 3.7 ± 2.2 | 0.17 |
| Time to outcome in months (median, range) | 32 (16-46) | 30 (11-39) | 29 (19-41) | 0.80 |

PRA: panel reactive antibodies; D/L: deceased/living; DGF: delayed graft function; MM: mismatches loci A, B, Dr; [a]Diffuse C4d *versus* Negative C4d; [b]Focal C4d *versus* Negative C4d; one-way ANOVA and Tukey Post Hoc test, Kruskal-Wallis.

**Table 3** C4d staining, Banff histopathology and morphologic features of transplant biopsies

| | Diffuse C4d (n = 23) | Focal C4d (n = 25) | Negative C4d (n = 108) | p |
|---|---|---|---|---|
| Time to biopsy in days (median, range) | 15 (10-36) | 19 (11-64) | 19 (9-227) | 0.95 |
| No rejection (n, %) | 5 (22) | 4 (16) | 34 (32) | |
| Acute cellular rejection (n, %) | 8 (35) | 10 (40) | 44 (41) | < 0.001* |
| Humoral rejection (n, %) | 9 (39) | 8 (32) | 0 | |
| IF/TA (n, %) | 1 (4) | 3 (12) | 30 (28) | |
| Tubulitis (n, %) | 13 (15) | 14 (16) | 61 (69) | 0.99 |
| Glomerulitis (n, %) | 8 (35) | 4 (17) | 11 (48) | 0.02 |
| Arteritis (n, %) | 7 (26) | 7 (26) | 13 (48) | 0.04 |
| Neutrophils in PTC (n, %) | 7 (44) | 8 (50) | 1 (6) | < 0.001 |

IF/TA: interstitial fibrosis/tubular atrophy; PTC: peritubular capillaries; *$p < 0.001$ ($\chi^2$ and Fisher Exact test): ACR and HR *versus* NR and IF/TA.

C4d in ACR types I, II and III were found in 25, 29 and 50% of the biopsies, respectively (p = 0.14).

Association between C4d positive staining and morphologic features of the Banff scheme was analyzed individually. Twenty-seven (56%) biopsies with positive C4d staining showed tubulitis as compared with 11 (57%) biopsies with negative C4d (p = 0.56). Glomerulitis was present in 12 (25%) of $C4d^+$ and in 11 (10%) of $C4d^-$ biopsies (p = 0.02), arteritis in 14 (29%) *versus* 13 (12%); p = 0.01, and margination of neutrophils in PTC in 15 (32%) *versus* 1 (0.9%) (p < 0.001), respectively. Grading C4d staining as diffuse, focal or negative yielded similar results (Table 3).

## C4d STAINING AND GRAFT FUNCTION

Follow-up graft function was determined at 12 months, 2 years and at last measured serum creatinine. Graft function (mL/min/1.73m$^2$) at those time points comparing $C4d^+$ and $C4d^-$ patients, in a median follow-up of 31 (17-42) months, were 47 ± 21 *versus* 54 ± 19 (p = 0.15), 50 ± 23 *versus* 53 ± 22 (p = 0.77) and 40 ± 26 *versus* 46 ± 28 (p = 0.30), respectively.

Analyzing graft function according to C4d grading, patients with focal C4d had the lowest eGFR at 12 months: 44 ± 21, 51 ± 21 (diffuse C4d) and 54 ± 19 (negative C4d) mL/min/1.73 m$^2$ (p = 0.19). At last follow-up, eGFR was also lower in patients with focal C4d in biopsy: 36 ± 28, 44 ± 24 and 46 ± 28 mL/min/1.73 m$^2$,respectively (p = 0.32). However, these differences did not reach statistical significance, which is probably related to the small number of patients in each C4d positive group. Overall, there was no difference in graft function between the three groups at the maximum follow-up period.

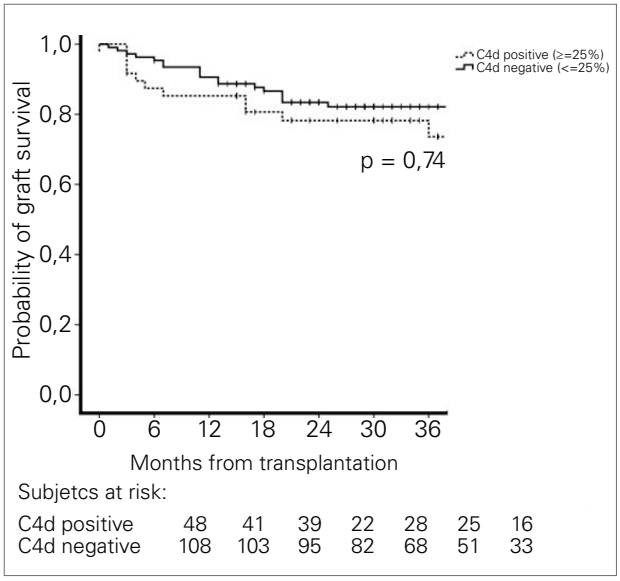
When we analyzed graft function according to Banff histology and C4d positivity, eGFR at last follow-up in patients with ACR was lower in the C4d positive group (43 ± 23 *versus* 50 ± 227 mL/min/1.73 m$^2$ in ACR C4d negative), but without statistical difference (p = 0.43). In patients with NR, eGFR was similar in all time periods comparing $C4d^+$ and $C4d^-$ groups. Patients with IF/TA and $C4d^+$ had the worst graft function at 2 years as compared to IF/TA $C4d^-$ (29 ± 7 *versus* 45 ± 23 mL/min/1.73 m$^2$; p = 0.046), but not at last follow-up (21 ± 12 *versus* 30 ± 24 mL/min/1.73 m$^2$, respectively; p = 0.49). However, the number of patients in the former subgroup was small (n = 4).

## C4d STAINING AND GRAFT SURVIVAL

Analysis of graft loss according to each Banff category showed that significantly more patients with ABMR (7/17, 41%) and IF/TA (15/34, 44%) lost their grafts ($\chi^2$ = 13.32; p = 0.004) as compared to patients with NR (5/43, 12%) and ACR (13/62, 21%). Comparing $C4d^+$ and $C4d^-$ groups, graft survival at nearly three years of follow-up was lower for patients with $C4d^+$ but without statistical significance (Figure 1). Graft survival according to C4d grading was not statistically different either, but patients with focal C4d had a lower survival rate than the group with diffuse C4d (67 *versus* 78%, log-rank = 1.398; p = 0.23) or negative C4d (67 *versus* 83%, log-rank = 1.113; p = 0.29). Comparing graft survival at three years by type of donor and positivity for C4d in biopsy, we found that grafts from deceased donors with C4d survived less than those from deceased donors without C4d (63 *versus* 79%, respectively; p = 0.17) but this difference did not reach statistical significance due to sample size. Irrespective of C4d, grafts from living donors survived the same (87 *versus* 85% for $C4d^+$ and $C4d^-$, respectively; p = 0.51).

The effect of C4d deposits in PTC at the time of biopsy on graft survival was evaluated for each Banff category. No difference in graft survival rate was detected for ACR $C4d^+$ as compared to ACR $C4d^-$ cases, 77 *versus* 83% (log-rank = 0.005; p = 0.94), as well as for NR $C4d^+$ *versus* $C4d^-$ (log-rank = 1.519; p = 0.21) and IF/TA $C4d^+$ *versus* $C4d^-$ (log-rank = 0.411; p = 0.41) groups. Additionally, we grouped patients with NR, ACR and FI/AT as one category and stratified them according to C4d positivity, and those

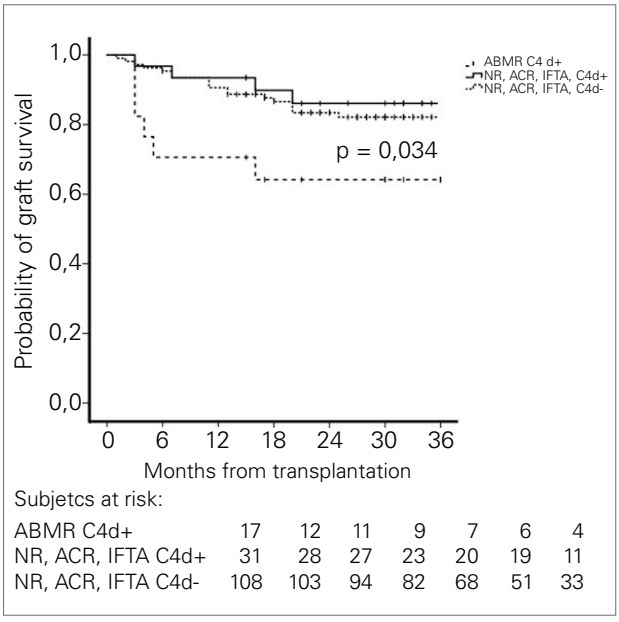
**Figure 1**. Graft survival rates according to C4d positivity in biopsy. No difference was found between $C4d^+$ and $C4d^-$ patients at last follow-up (74 *versus* 83%, log-rank=0.11; p=0.74).



patients were compared to patients with ABMR. Graft survival was significantly lower in ABMR as compared to grouped categories C4d$^-$, but the difference between ABMR and grouped categories C4d$^+$ was not statistically significant (Figure 2).

Multivariate Cox regression showed eGFR at the time of graft biopsy and PRA > 20% as significant predictors of graft failure (Table 4). C4d deposition did not predict graft loss, but ABMR rejection was the Banff category associated with a trend towards graft failure. After adjustment, ABMR did not remain a significant predictor of graft loss.

**Figure 2**. Kaplan-Meier curves of graft survival rates showing a significantly lower survival for patients with antibody-mediated rejection (ABMR) as compared to patients grouped as no rejection (NR), acute cellular rejection (ACR) or interstitial fibrosis/tubular atrophy (IF/TA) negative for C4d (64 versus 82%; p = 0.034, log-rank = 4.48). When grouped NR, ACR or IF/TA positive for C4d was compared to ABMR, no difference in graft survival was found (78 and 64%, respectively; p = 0.10, log-rank = 2.76).




### Inter and intra-observer reproducibility of C4d interpretation

Intra-observer concordance rate for the presence or absence of C4d was excellent, with a Kappa value (*k*-value) of 0.77 (p < 0.001). Agreement rate were 91.7% for the presence and 89.1% for the absence of C4d. A high *k*-value was also found for grading C4d as negative, focal or diffuse staining (*k*-value = 0.73; p < 0.001). The agreement rates were 89.1, 72 and 91.3% for scoring C4d as negative, focal and diffuse, respectively. The lowest concordance rate was observed for interpreting C4d staining as focal, when ten biopsies were scored as C4d negative and two as C4d diffuse in the first interpretation changing to C4d focal in the second analysis.

Inter-observer reproducibility for the presence or absence of C4d was worse, with a *k*-value was 0.60 (p < 0.001). Agreement rates were 93% for presence and 75% for absence of C4d. For grading, *k*-value was even worse (0.57; p < 0.001), with an agreement rate of 75% for absence of C4d, 64% for grading C4d as focal and 95% as a diffuse staining.

## Discussion

Several studies have been showing that C4d accumulation in PTCs occurs not only in ABMR but also in cellular rejection and even in biopsies without rejection (i.e., acute tubular necrosis, drug toxicity).[2,3,8-11,18] So far, the pathogenic and clinical significance of those findings is incompletely understood. Humoral and cellular alloresponses might affect the kidney graft either independently or concurrently, with endothelium antigens of capillaries and arteries acting as targets for both humoral and cellular immune responses, respectively.[9,19,20]

This study showed a 31% prevalence of C4d deposition in biopsies of kidney grafts similar to other studies, in which C4d has varied from 30 to 45% in average.[2,3,9,10,13,14] Lower prevalence of C4d positivity has been reported in indication biopsies, as in the

**Table 4** Clinical and pathologic determinants of kidney graft outcome in Multivariate Cox Regression

| Variable | Crude Hazard Ratio (95%IC) | p | Adjusted Hazard Ratio (95%IC) | p |
|---|---|---|---|---|
| eGFR (MDRD) | 0.94 (0.91-0.97) | < 0.001 | 0.95 (0.91-0.98) | 0.005 |
| PRA > 20% | 1.03 (1.01-1.04) | < 0.001 | 1.02 (1.00-1.04) | 0.038 |
| C4d deposition in PTC | 0.89 (0.44-1.78) | 0.74 | Non included | |
| Banff category | 2.81 (0.86-9.20)* | 0.08 | 0.47 (0.11-2.17)* | 0.33 |

IC: Confidence interval; eGFR: estimated glomerular filtration rate at the time of graft biopsy; PRA: panel reactive antibodies; PTC: peritubular capillaries. *for humoral rejection.

study of Mengel *et al.*[11] (20.7%) and Cheunsuchon *et al.*[12] (16.4%). This variability can be explained by several factors, such as different staining techniques and percentage of PTC positivity, different biopsy settings, time since transplant, and case selection.

Acute pathologic changes other than rejection and normal biopsies were grouped as NR category. Twenty one percent of these biopsies stained positively for C4d, a prevalence that did not differ from other series,[9,10,13,18] independently of the technique employed. However, it is not possible to exclude that in some of these first biopsies with acute graft injury C4d deposits preceded the development of cellular or ABMR, that would be later diagnosed in subsequent biopsies,[21] which were not done in our study.

Mauiyyedi *et al.*[3] reported IF C4d deposition in 15% of the biopsies with ACR type I and II. Using the same pathological criteria, Nickeleit *et al.*[9] described the prevalence of C4d based on morphologic features present in biopsy: 43% in tubulo-interstitial rejection and 45% in transplant endarteritis. Using IHC, prevalence rates of C4d positive staining were similar: 20% of indication biopsies with ACR,[11,13] 21.4% in ACR Banff I, II or III[10], 24% in a series of protocol and diagnostic biopsies,[22] and 29% in the present study.

The correlation of Banff morphologic features and C4d in acute rejection has been emphasized in some studies.[3,9-11,13] Our results are in agreement with previous reports, since both tubulitis and arteritis were significantly more frequent in C4d negative biopsies (71% of ACR biopsies), whereas glomerulitis and neutrophils in PTC predominated in biopsies with C4d deposits, the majority of them with ABMR.

Mauiyyedi *et al.*[8] found C4d in 61% of biopsies diagnosed as chronic rejection. Ranjan *et al.*[13] reported C4d in 30% of patients with chronic allograft nephropathy, rate that increased to 83% if associated to ABMR. In the study of Nickeleit *et al.*,[9] C4d was present in 23% of biopsies with striped fibrosis and in 14% of diffuse interstitial fibrosis. In our sample, C4d was present in 12% of biopsies with IF/TA, and we cannot exclude an antibody-mediated chronic rejection in these cases. It is important to note that irrespective of C4d, the presence of IF/TA correlates with late graft dysfunction and failure.

While the majority of the studies showed C4d positivity as predictive of a worse graft function and survival,[2-4,10,21,23] some did not confirm this association.[9] Moreover, in specific settings, such as protocol biopsies[11] or late acute renal allograft rejection,[24,25] there is controversy if C4d deposition is associated or not with poor function and higher rates of graft loss. Overall, we could not demonstrate this association, but graft survival of patients without ABMR but positive for C4d did not differ from those with ABMR, a finding to be further explored. We believe that our results indicate that ABMR was correctly identified in this cohort as it was associated with an adverse outcome. However, a question remains if C4d present in other histologic categories affects graft outcome. Certainly, numerous other mechanisms must be taken into account, as many different molecules have altered expression in rejection and in other types of injury potentially affecting graft outcome.

It has been discussed if the percentage of C4d distribution has distinct clinical and morphologic correlations, and how it affects graft function and survival in the short and long term. Magil *et al.*[2,26] showed a similar clinical course and biopsy findings in patients with focal or diffuse C4d. Haririan *et al.*[4] also demonstrated that graft survival was adversely influenced by C4d independent of the staining pattern, and focal C4d was a significant independent predictor of graft failure. In our study, patients with focal C4d had the lowest eGFR and graft survival at last follow-up, but this group had either an increased prevalence of DGF and a trend to more deceased donors as graft source, both potentially related to this worse outcome (Table 2). The percentage of C4d staining that accurately predicts kidney transplantation outcome is still debated, and even a cutoff of 10% for C4d positivity was reported to be a strong predictor of renal graft loss.[27]

Several studies had compared IF and IHC on frozen and paraffin sections for C4d identification and grading in kidney graft biopsies. Some of them showed higher accuracy for frozen-IF with monoclonal antibody in relation to paraffin-IHC,[14,15,28] but other authors reported an acceptable sensitivity (87.5%) and specificity (98%) of paraffin-IHC with anti-C4d polyclonal antibody.[22] Nadasdy *et al.*[29] compared frozen-IF and paraffin-IHC, suggesting a loss of sensitivity of 31% for IHC. Technical factors can contribute to a lower sensitivity of IHC, such as tissue fixation, paraffin-embeddeding process, the quality of paraffin, and a more variable intensity of staining.[15,21,28] Moreover, the staining of plasma proteins in PTCs that results from soluble C4 fixation sometimes makes interpretation of C4d less reliable.[24] Nonetheless, potential advantages of paraffin-IHC are better preservation of morphologic features, and most important, IHC can be done retrospectively in stored paraffin sections when diagnosis of ABMR is doubtful.[3,22]

We investigated the internal consistency of C4d interpretation in paraffin-IHC, by measuring its

reproducibility. Agreement rate were good for inter-observer and excellent for intra-observer interpretations, suggesting that our C4d analysis has been consistent. In only one study the agreement rates of C4d in paraffin-IHC was evaluated.[15] This study showed similar results: a good inter-observer concordance (*k* value = 0.63) and an excellent agreement for intra-observer interpretation (*k*-value = 0.83).

A limitation of our study is the lack of measurement of donor specific antibodies (DSA). There are consistent data showing DSA as predictive of worse graft function and outcome,[3,22,30] irrespective of C4d staining at least in the first year of transplant.[4] DSA were not measured in our centre when these biopsies were carried out, so we should call ABMR as "presumptive antibody-mediated rejection" based only in C4d positivity and pathologic features.

In conclusion, this study confirms that C4d staining in kidney allograft biopsies is clinically useful as a marker of antibody-mediated rejection, with defined clinical and pathological correlations in renal transplant recipients. The impact of C4d deposition in other Banff histology was not defined by the present investigation. We can speculate that even with a good reproducibility, C4d staining by immunohistochemistry in paraffin can render immunopathology less sensitive to detect antibody-mediated graft injury, which might be present in some cases of acute rejection and therefore affect graft outcome.

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