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ORIGINAL ARTICLE

# C4d immunostaining is an independent predictor of cardiac allograft vasculopathy and death in heart transplant recipients

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## Summary

Antibody-mediated rejection (AMR) occurs in 10–20% of patients after heart transplantation. C4d immunostaining is one parameter used in its diagnosis. This study aimed to determine whether C4d staining has prognostic significance for mortality, coronary allograft vasculopathy (CAV), cell-mediated rejection (CMR), and graft dysfunction in patients post-transplantation. Consecutive patients receiving an endomyocardial biopsy between 2007 and 2008 were selected. Left ventricular function, angiography, episodes of AMR/CMR, and death were noted. C4d was graded from 0 to 3 (immunostaining). Cox proportional models (recurrent events analysis) were used to evaluate C4d staining with mortality, graft dysfunction, CAV (≥grade 2), and episodes of ≥2R-CMR. We analyzed 2525 biopsy specimens ($n$ = 217). During a follow-up of 4.5 ± 2 years, 35 died, 49 had graft dysfunction, seven had ≥grade 2 CAV, and 95 episodes of CMR occurred. A one-grade increase in C4d staining was associated with an increase in mortality (HR 1.57; 95% CI 1.0–2.5), a higher risk of CAV (HR 2.4, 95% CI 1.04–5.4), and a trend toward graft dysfunction (HR 1.42; 95% CI 1.0–2.09). C4d was not associated with CMR. C4d immunostaining was a significant predictor of CAV and death but not subsequent episodes of CMR. There was also a trend toward increased graft failure.

## Introduction

Antibody-mediated rejection (AMR) remains a diagnostic and therapeutic challenge in heart transplantation patients. Despite advances in immunosuppressive therapies, the incidence of AMR has not changed [1,2]. Attempts to recognize those at risk include pre- and post-transplant evaluation of recipient human leukocyte antigen (HLA) class I and class II antibodies. AMR may result from donor-specific antibodies (DSA) existing pretransplant or developing *de novo* after transplant. These antibodies bind to the graft, activate the classical complement pathway, and release split products including C4d and C3d which covalently bind to endothelium [3,4]. The presence of C4d on post-transplant biopsy tissue acts as a surrogate marker of complement activation. The diagnosis and extension of AMR in heart transplantation takes multiple factors into consideration including histopathology, documentation of graft dysfunction, and the presence of DSA, although the absence of DSA does not rule out AMR [5]. Asymptomatic AMR is often defined as the presence of pathological changes consistent with AMR, in the absence of symptoms of heart failure or graft dysfunction. AMR has been associated with coronary artery vasculopathy (CAV) and death (diagnosed based on both histology and immunohistochemistry for CAV and immunohistochemistry after death) [6,7]. This retrospective cohort study aimed to determine whether C4d staining alone, in the absence of confirmed

 

AMR, has prognostic significance in predicting death, graft dysfunction, CAV, or cell-mediated rejection (CMR) in patients after heart transplantation.

## Methods

### Patient population

Consecutive heart transplant patients who underwent right ventricular endomyocardial biopsy between July 2007 and June 2008 ($n$ = 217) were selected retrospectively. In these patients, all subsequent biopsies were evaluated for the presence of C4d staining (up to July 2012). Post-transplant biopsy specimens were obtained by protocol at standard intervals post-transplant (weekly for 1 month, followed by biweekly for 1 month, followed by monthly until 6 months, and at 6, 9, 12, 18, and 24 months) or for cause (symptoms, left ventricular (LV) dysfunction). After 2 years, biopsies were performed for the following clinical indications: clinical heart failure, graft dysfunction on echocardiogram, follow-up of a positive biopsy for CMR or AMR as part of increased surveillance protocols, or following adjustment of immunosuppressive therapy. The study was approved by the research ethics board.

### Pathology

All specimens were fixed in 10% buffered formalin and examined in detail by two of the authors (JB and AL), who were blinded to patient outcomes. The sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Dako diagnostics, Hamilton, CA, USA). CMR was graded according to ISHLT standardized cardiac biopsy grading, where 1R=interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage, 2R=two or more foci of infiltrate with associated myocyte damage, and 3R= diffuse infiltrate with multifocal myocyte damage ± edema ± hemorrhage ± vasculitis [8]. Only the presence of significant cellular rejection defined as ≥ grade 2R was used in the statistical analysis, as grades ≥2R would trigger a change in therapeutic management. Immunostaining for C4d capillary deposition was performed on all specimens using an anti-human antibody (rabbit polyclonal, [ALPCO, Salem, NH]) and was graded from 0 to 3, where 0 = none; 1 = weak staining in a few areas; 2 = moderate staining in several areas, but not all capillaries; and 3 = strong staining in all capillaries (Fig. 1) [9].

### Outcomes

Outcomes, including graft dysfunction, CAV, CMR, and death, were recorded during the follow-up period. Graft dysfunction was characterized using serial transthoracic echocardiogram and defined as left ventricular ejection fraction (LVEF) ≤40%. Characterization of CAV was based on ISHLT nomenclature, where CAV 0 = no detectable angiographic lesions; CAV 1 = angiographic left main <50% or primary vessel with maximum lesion of <70%, or any branch stenosis <70% (including diffuse narrowing) without allograft dysfunction; CAV 2 = angiographic LM ≥50%, a single primary vessel ≥70% or isolated branch stenosis ≥70% in braches of 2 systems, without allograft dysfunction; and CAV 3 = angiographic LM ≥50%, or two or more primary vessels ≥70% stenosis or isolated branch stenosis ≥70% in all three systems; or ISHLT CAV1 or CAV2 with allograft dysfunction (defined as LVEF ≤45%, usually in the presence of regional wall motion abnormalities) or evidence of significant restrictive physiology [10]. Grades of CAV ≥ 2 were used in our statistical analysis as percutaneous intervention would be considered for this group. Review of angiogram reports and all-cause mortality was recorded during the follow-up period.

### Statistical analysis

Multivariable Cox proportional hazards models were used to evaluate the association between C4d staining and mortality and graft dysfunction. We adjusted these analyses by clinically important variables including donor age, ischemic time, and pretransplant panel reactive antibodies (PRA). Results are presented graphically using adjusted Kaplan–Meier curves. Due to the small number of events, we used univariable Cox proportional hazard model to evaluate the association between C4d staining and CAV. Cox proportional hazard with recurrent events analysis was used to analyze the association of C4d staining with episodes of ≥2R-CMR adjusted for recipients age, sex, PRA, and transplant year. C4d staining was entered in these models as continuous time-varying covariate. The assumption of proportional hazards was satisfied in all the models. A value of $P < 0.05$ was considered statistically significant.

## Results

The study sample was comprised of 2525 biopsy specimens from 217 patients (1–24 biopsies/patient), followed for 4.5 ± 2 years after enrollment, and a mean of 4.9 ± 3.7 years post-transplant. Seventy percent of patients were men, with an average age of 45 ± 13 years at transplant. Forty-three patients (20%) were transplanted between the years of 1988 and 1999, while 83 patients (38%) were transplanted between the years of 2001 and 2005 and 91 patients (42%) were transplanted between years of 2006 and 2008. Of 2525 biopsy samples, 83.5% were C4d negative. C4d 1, 2, and 3 occurred in 10.5, 5.4, and 0.6% of biopsies, respectively. Patient characteristics are shown in Table 1.

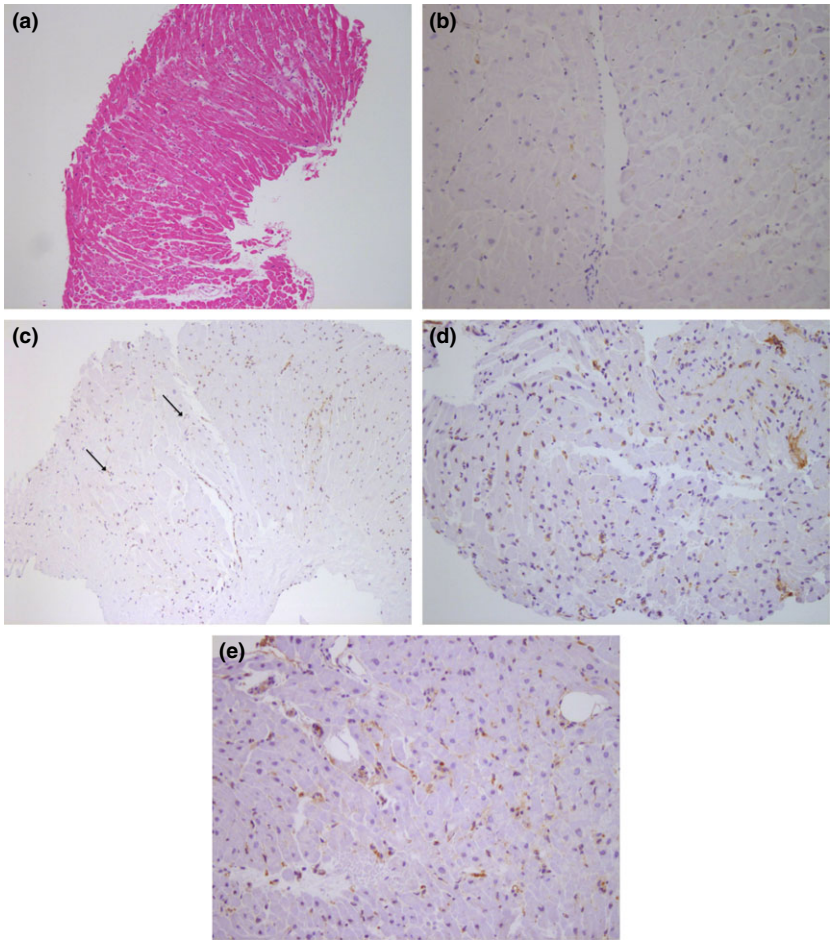


**Figure 1** Pathologic grading scheme. Selected biopsy specimens demonstrating the spectrum of C4d staining. A 45-year-old women with AMR 0 (a–b). Note absence of capillary staining of C4d. (a: hematoxylin and eosin, b: C4d; original magnification × 10). A 63-year-old women with C4d grade 1 (c). There are rare capillaries which show faint staining. There is also nonspecific staining of endothelial cells and larger vessels. (C4d; original magnification × 10). A 31-year-old women with C4d grade 2 (d). There is C4d deposition seen in the some capillaries of moderate intensity, but are not seen in all vessels. (C4d; original magnification × 20). A 36-year-old women with C4d grade 3 (e). All capillaries stain in this biopsy and the color is much darker. (C4d; original magnification × 10 (E)).

### C4d and mortality

Of the 217 patients, 35 (16%) died during the follow-up period. Of these, 7 (20%) died from multi-organ failure, 8 (23%) from sepsis, and 6 (17%) from complications of malignancy. Six deaths (17%) were due to cerebral vascular accidents (2 from intracerebral hemorrhage, one from a thrombotic event, and the remaining three unknown). There were 6 deaths (17%) secondary to congestive heart failure, in which 2 (6%) were due to CMR. There was one (3%) death from ruptured abdominal aortic aneurysm and one death from complications of systemic amyloidosis. On multivariable analysis, C4d staining was a significant predictor of death, after adjusting for donor age and ischemic time (HR 1.57; 95% CI 1.0–2.46; $P = 0.048$) (Fig. 2).

### C4d and graft dysfunction

Ninety-one percent of patients ($n = 200$) had echocardiograms performed at our institution. Forty-nine patients (23%) had graft dysfunction, defined as an LVEF ≤40%. On multivariable analysis, there was a trend toward increased risk of graft dysfunction with more pronounced C4d staining (HR 1.42 per 1 grade increase; 95% CI 1.0–2.09; $P = 0.074$) (Fig. 3).

### C4d and CAV

One hundred and nine patients (50%) underwent angiography between 2008 and 2012. Seven patients (3%) developed CAV grades 2–3 during the follow-up period. On univariable analysis, C4d staining was associated with higher risk of CAV (HR 2.4, 95% CI 1.04–5.4, $P = 0.04$).

**Table 1.** Demographic baseline and patient characteristics in 217 heart transplant patients.

| Population characteristics | All patients mean ± SD/*n* (%) |
|---|---|
| Recipient | |
| Age (years) | 45 ± 13 |
| Male gender | 152 (70%) |
| Donor | |
| Age (years) | 34 ± 14 |
| Male gender | 130 (60%) |
| CMV positive | 100 (46%) |
| Ischemia time (min) | 143 (63–192)* |
| Cause of cardiomyopathy | |
| Ischemic | 75 (35) |
| Idiopathic | 35 (16) |
| Congenital | 15 (7) |
| Hypertrophic | 15 (7) |
| Valvular | 8 (4) |
| Other | 69 (31) |
| Year of transplant | |
| 1998–2000 | 43 (20) |
| 2001–2005 | 83 (38) |
| 2006–2008 | 91 (42) |

*Reported as median with interquartile range.
CMV: cytomegalovirus

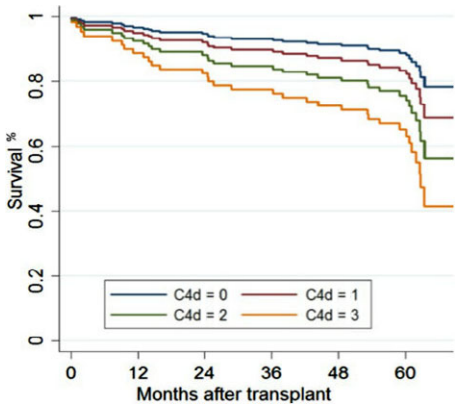


**Figure 2** Adjusted survival based on C4d grading. Kaplan–Meier analysis of patient survival. For a grade increase in C4d staining, the mortality risk was 67% higher (HR 1.57; 95% CI 1.0–2.46; $P = 0.048$) after adjusting for donor age, ischemic time, and pretransplant percentage PRA. C4d staining was graded from 0 to 3, where 0 = none; 1 = weak staining in a few areas; 2 = moderate staining in several areas, but not all capillaries; and 3 = strong staining in all capillaries.

### C4d and CMR

There were 95 episodes of CMR (≥ 2R) in 56 patients. C4d staining was not significantly associated with episodes of CMR (hazard ratio 0.66; 95% CI, 0.4–1.2; $P = 0.18$) after adjusting for recipient's age, sex, PRA, and transplant year.

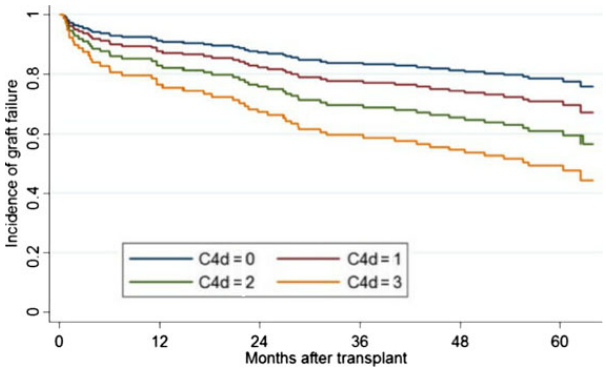


**Figure 3** Adjusted incidence of graft dysfunction based on C4d grading. Kaplan–Meier analysis of graft dysfunction. On multivariable analysis, there was a trend toward significance with one-grade increase in C4d staining and graft dysfunction (HR 1.42; 95% CI 1.0–2.09; $P = 0.074$) after adjusting for donor age, ischemic time, and pretransplant percentage PRA. C4d staining was graded from 0 to 3, where 0 = none; 1 = weak staining in a few areas; 2 = moderate staining in several areas, but not all capillaries; and 3 = strong staining in all capillaries.

## Discussion

In this large cohort of heart transplant recipients, isolated C4d immunostaining, independent of AMR, was a predictor of graft dysfunction, CAV, and death but was not associated with subsequent episodes of CMR. AMR is thought to begin with the development of circulating antibody alone (latent humoral response), followed by C4d deposition, termed the "silent phase," without any pathological or clinical manifestations [11]. This is followed by pathological changes and the presence of circulating antibodies, termed the "subclinical" phase, and lastly, by clinical manifestations, called "symptomatic AMR."[11] AMR has been associated with increased risk of graft dysfunction, CAV, and death post-transplant. Risk factors for AMR include recipient female sex and multiparity, blood transfusions, use of ventricular assist device, the presence of pretransplant anti-HLA antibodies, prior organ transplantation, and positive peri-operative cross-match [12,13]. C4d has previously been found in the renal transplant population to predict graft dysfunction as a result of antibody-mediated damage [14–16].

### C4d and mortality

We found that C4d staining, in the absence of histological findings, was associated with worse outcomes, at any stage post-transplant. In a previous study, the presence of C4d was found to be related to early post-transplant mortality, with 5 of 9 nonsurvivors demonstrating prominent immunohistochemical staining with $C4d^{+++}$ ($n = 14$) compared to only 1 patient in the C4d+ group


 

($n = 46$) [7]. Fedrigo *et al.* also found a higher mortality risk in patients who were C4d positive, in all groups (± DSA and ± graft dysfunction), compared to controls ($P < 0.0001$) [17]. However, others have not found similar results. Wu *et al.* found no difference in survival over a 5-year period among patients with AMR (defined as the presence of histological and/or immunohistochemical findings) with either reduced ($n = 22$) or preserved ($n = 21$) LV function [6]. This may be due to their small sample size. In addition, their definition of pathologic AMR was based on histology alone or in the presence of immunohistochemistry without findings of capillary endothelial swelling, interstitial hemorrhage and edema, and neutrophil infiltration. If the majority of their patients did not show visible changes on histology alone (i.e., immunohistochemistry), their population may have been in an "earlier" stage of AMR and may explain the lack of association with mortality in their study.

### C4d and CAV

In our series, C4d staining was associated with higher risk of CAV (HR 2.4, 95% CI 1.04–5.4, $P = 0.04$). The presence of C4d staining has been found to be associated with the development of both early and late CAV post-transplantation. Poelzl *et al.* showed that the development of intracoronary ultrasound (IVUS)-confirmed CAV at 1 year post-transplant was associated with at least two prior C4d-positive biopsies [18]. Wu *et al.*, also found a significant difference in development of CAV (defined as ≥30% luminal stenosis) in patients with asymptomatic AMR compared to the control group (79.1 vs. 52.4%, $P = 0.02$) at 5-year follow-up [6]. Loupy *et al.* identified the presence of C4d and C3d in their patient population and found that severe CAV occurred late post-transplantation (>7 years), with biopsies which demonstrate positive immunohistochemistry [19]. Conversely, others have not found the same relationship. A retrospective analysis by Moseley *et al.* looked at the presence of C4d and C3d staining in patients ($n = 26$) with angiographic-confirmed CAV using IVUS [9]. The authors used C4d grade ≥2 for statistical analysis, which was similar to our grading scheme. Biopsies were taken at routine intervals post-transplant up to 2 years. The authors found no significant difference in C4d staining and presence of subsequent CAV, but did find a significant difference in C3d staining. Small sample size and difference in C4d grading, as well as their short follow-up of only 2 years, may explain the differences in our results.

### C4d and graft dysfunction

In our population, graft dysfunction (LVEF ≤40%) was found to occur more frequently in patients with positive C4d staining, after adjusting for donor age, PRA, and ischemic time. Similar to our results, Gupta *et al.* found a decrease in LV function in patients with C4d staining; mean LVEF was 38 and 43%, for CMR negative and positive, respectively, in C4d-positive patients versus 60% in C4d-negative patients ($P < 0.01$) [20]. Although our results showed a trend toward significance, it is possible that our results were underpowered.

### C4d and CMR

We found no relationship between C4d staining and episodes of CMR. Gupta *et al.* looked at C4d staining and its association with reduced graft dysfunction in addition to CMR [20]. The authors found CMR occurred in 57% patients with positive C4d versus 11% of patients with negative C4d staining ($P < 0.001$). Other studies have found that the presence of C4d staining exists in the presence of CMR [7,21], and as such, it remains unclear why some cases of CMR are associated with complement activation. The authors proposed that routine assessment for AMR should occur in patients with pathologic evidence of CMR and positive C4d staining.

### Challenges of AMR

Antibody-mediated rejection remains challenging to diagnose and treat. In a survey performed prior to the recent consensus conference on AMR, 53% of centers made the diagnosis based on LV dysfunction even in the absence of microscopic findings [5]. This international AMR survey found that a variety of diagnostic and treatment strategies were used, which may contribute to the various findings in the studies discussed. More so, the different grading scales of C4d among the respective centers, as well as the lack of a commercially available monoclonal antibody [22], may also explain the varying results.

Despite these potential limitations, Kobashigawa *et al.* evaluated the uniformity of C4d staining in seven centers and found high reproducibility with minor variations, when using site-specific immunoperoxidase stains, with the same biopsy tissue among the sites [5]. However, the group could not establish a uniform method to grade severity, with only 5 (16%) centers adopting the Banff scoring system, while the majority used center-derived scores [5]. Criteria for the pathological scoring of AMR have been recently published by the ISHLT working group [22]. Implementing a uniform grading system is the first step needed for future studies that correlate pathological findings with clinical outcomes.

In our population, we demonstrated that C4d staining alone was an independent predictor of mortality, with an association with graft dysfunction. Understanding the spec-

trum and continuum of C4d as a marker/contributor in the pathogenesis of graft disease continues to evolve. More data are needed to identify whether treatment of patients with C4d staining alone or those with asymptomatic AMR improves survival, reduces mortality, graft dysfunction, or progression of CAV. If so, more studies will be needed to define the timing of treatment strategies, as current recommendations for therapy require both pathological findings and graft dysfunction, by which point treatment may be less effective [5,23]. Factors clinicians use to decide on treatment include pAMR grading [22], the presence of DSA, as well as LVEF (≤45% or 25% decrease from baseline). Moreover, a recent survey of ISHLT members found that 83% of responders would treat pAMR 0 with a decrease in LV function and DSA alone, with a significant increase in treatment as pAMR grade increases ($P < 0.001$). In all groups, the presence of DSA was more often treated than DSA-negative patients ($P < 0.05$). Although the response rate was low ((14%), the survey clearly highlighted the varied clinical practices of physicians treating AMR [24]

### Limitations

This study is limited by its retrospective design in a single institution with a relatively small sample size. However, all patients were managed systematically with prespecified protocols for the diagnosis and treatment of AMR. The role of C3d and C1q was not evaluated in our population as it was not routinely available in the laboratory. From this retrospective review, we were unable to distinguish between biopsies that were part of standard protocol with those which were done for another indication. This may have possibly highlighted a higher risk group with worse outcomes. In addition, only 50% of our patients underwent coronary angiography, which may limit the analysis, as well as its ability to only identify late CAV. Although our center does use intravascular ultrasound routinely, it is possible that the severity of cases were underestimated. We did not evaluate the impact of DSA post-transplantation, as only 47% of patients had data available. Post-transplant antibody testing was only implemented partway through and therefore not available in this retrospective study in 53% of patients. Similarly, for many of these patients, HLA typing of donors and recipients was not performed in the era in which they were transplanted, so systematic HLA mismatch data are not available. Lastly, the analysis of the effect on C4d on CAV was limited to univariable analysis due to the small number of events. Univariable analysis provides the opportunity for type 1 error or false-positive results. This association needs to be further explored in larger studies using multivariable analysis.

## Conclusions

In conclusion, our findings show that the presence of C4d immunostaining alone, independent of AMR, was an independent predictor of mortality and CAV, with a trend toward reduced graft function. C4d was not found to be predictive of CMR. The association between C4d and adverse outcomes post-transplant highlights the need for management strategies (duration to next biopsy, measurement of DSA, evaluation of graft function) for the continuum of AMR.

## Authorship

AL, ACA, JB, KT, DD and HJR: designing research/study, analyzed data, and wrote the paper. AL and ACA: performed the research and contributed important reagents. AL, ACA, and HJR: analyzed the data. AL: collected the data.

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