Optimal Cut-off Point for Immunoperoxidase Detection of C4d in the Renal Allograft: Results from a Multicenter Study

Gretchen S. Crary[1],
Department of Pathology and Laboratory Medicine, Hennepin County Medical Center

Yassaman Raissian[2],
Department of Laboratory Medicine & Pathology, Mayo Clinic

Robert C. Gaston[3],
Division of Nephrology, University of Alabama

Sita M. Gourishankar[4],

Division of Nephrology and Immunology, University of Alberta

Robert E. Leduc[5],
Division of Biostatistics, University of Minnesota

Roslyn B. Mannon[6],
Department of Nephrology, University of Alabama at Birmingham

Arthur J. Matas[7], and
Department of Surgery, University of Minnesota

Joseph P. Grande[8]
Department of Laboratory Medicine & Pathology, Mayo Clinic

Abstract

Background—Although C4d deposition in peritubular capillaries has been identified as a strong risk factor for subsequent renal allograft loss, the optimal cut-off for the fraction of peritubular capillaries needed to establish a positive stain in formalin-fixed paraffin-embedded material has not been systematically defined. The objective of this study was to establish the threshold for positive staining that best predicts renal outcome in renal biopsies in a multicenter study in which local and central pathology were compared.

Methods—Unstained renal biopsy slides were obtained from 296 patients. The percentage of peritubular capillaries staining positively for C4d was detected by immunoperoxidase staining.

Results—The percentage C4d deposition ranged from 0% to 90% with 44% (129/296) having a positive percentage of C4d staining. The median for positive cases was 25%. Local C4d+ results were reported qualitatively, with 28% recorded as positive for C4d. Using a centrally-determined cut-off of 10%, tests for agreement of local and central C4d staining were fair (Kappa 0.40, 95% CI 0.29-0.51). Raising the centrally-determined cut-off to 25% or 50% did not change the Kappa values (0.44 and 0.41, respectively). By Cox proportional hazards model, C4d positivity (centrally-determined assessment) using a cut-off of 10% was the strongest predictor of time to graft loss (HR 2.66, 95% CI [1.68, 4.21]). Centrally-determined C4d positivity correlated with Banff scores indicative of acute inflammation, but not with scores indicative of fibrosis/atrophy or transplant glomerulopathy.

Conclusions—Our findings indicate that C4d positivity, defined as ≥10% by immunoperoxidase, is a strong predictor of graft loss.

Keywords

C4d; graft survival; immunoperoxidase

INTRODUCTION

Peritubular capillary deposition of C4d has been identified as a risk factor for the development of progressive graft dysfunction and loss (1-3). Peritubular capillary staining for C4d and presence of donor specific antibodies have been incorporated into the revised Banff '97 classification scheme as criteria for the diagnosis of acute humoral rejection (4). Moreover, in patients with chronic graft dysfunction, C4d predicts adverse outcome and is associated with a variety of histopathologic alterations, including transplant glomerulopathy (1, 5). Based on these considerations, it has been recommended that staining for C4d be performed on all renal allograft biopsies (6).

Methods for detection of C4d deposition in peritubular capillaries include immunofluorescence performed on frozen tissue and immunoperoxidase performed on

formalin-fixed paraffin-embedded tissue. There are advantages to both methods for detection of C4d – for example, it is recognized that detection of C4d by immunofluorescence provides the most sensitive and specific marker for C4d positivity (7-9). However, immunofluorescence studies require the acquisition of additional tissue, which may not be practical for protocol biopsies or for retrospective studies. Unfortunately, there is considerable variation between centers on thresholds for "focal" or "diffuse" positive staining, both by immunofluorescence and by immunoperoxidase, rendering it difficult to compare results for C4d positivity between centers. Furthermore, there are no data in the literature that systematically correlate various cut-off points for the detection of C4d with outcome. At the Banff 2007 Meeting on Allograft Pathology, the need to correlate C4d cut-offs with long-term outcome was emphasized (6).

The purpose of this study was to establish an optimal cut-off for C4d deposition, as assessed by central analysis of formalin-fixed paraffin-embedded tissues stained by the immunoperoxidase method. Study patients were enrolled in the Deterioration of Kidney Allograft Function (DeKAF) Study, a multicenter study to define clinical and histopathologic features that predict adverse outcome in renal biopsies performed for new onset late graft dysfunction (10). In this cohort of patients, recruited from seven centers in the US and Canada, we demonstrate that a cut-off of 10% of peritubular capillaries to establish a positive C4d stain provided the best fit for the association of C4d positivity with graft loss.

RESULTS

The study cohort consists of 296 patients who underwent a clinically-indicated renal biopsy for new-onset deterioration of function (defined as 25% increase in serum creatinine or new onset of proteinuria) for which both local and central C4d data are available. The percentage of peritubular capillaries staining positively for C4d was assessed in a blinded fashion. A representative photomicrograph showing diffuse positive and negative immunoperoxidase staining for C4d is shown in Figure 1. The percentage of C4d positivity ranged from 0% to 90% with 44% (129/296) having a positive percentage of C4d staining; among those with positive percentage C4d, the median percentage was 25%. Local C4d+ results were reported qualitatively, with 28% (83/296) recorded as positive for C4d. Using a centrally-determined cut-off of 10%, the Kappa score for agreement of local and centrally-determined C4d positivity was 0.40 [95% CI 0.29-0.51]. Raising the cut-off for centrally-determined C4d values did not appreciably change the Kappa values – using a centrally-determined cut-off of 25%, the Kappa value was 0.46 [95% CI 0.35-0.57], and using cut-off of 50% for centrally-determined positivity, the Kappa value was 0.42 [95% CI 0.30-0.54]. The overall proportion of observed agreement ranged between 69%-79% for all putative cut-offs examined. The proportion of specific agreement for C4d+ negativity (range 76%-87%) was higher than the proportion of specific agreement for positivity (54%-60%), indicating that observed agreement was superior on cases where either the local or central result was negative rather than on cases where at least one result was positive, for each putative cut-off value.

Cox proportional hazards models were used to estimate the unadjusted risk of graft loss by local and central C4d results. Selection of an optimal cut-off for central C4d positivity was performed by a maximum Chi square search (Table 1), or, since all models have the same number of degrees of freedom, equivalently by AIC (Akaike Information Criterion). The model with a C4d cut-off of 10% provided the best-fitting model by these measures. A Kaplan-Meier graph of centrally-determined C4d positivity using a cut-off threshold of 10% is given in Figure 2A.

To correct for over-fitting ("optimism") in the fitting of the model as a result of multiple comparisons employed in the maximum Chi-square search, 2-fold cross-validation was employed. Cross-validation analysis estimated a statistically significant association of C4d+ positivity (likelihood ratio χ^2=19.9, p<0.0001) with a hazard ratio of 2.66 (95% CI [1.68, 4.21]), compared with a 2.88 hazard ratio for the direct univariate analysis.

Harrell's c-index corrected for over-fitting via bootstrapping shows the discrimination of C4d positivity is also greatest at a 10% cut-off (c=0.63), whereas the discrimination of local C4d positivity (c=0.53) is comparable to the null model (c=0.50). Calibration of the model with positivity cut-off of 10% was assessed through bootstrapping the difference between predicted model-based and observed Kaplan-Meier estimates of year 1 and year 2 survival to estimate the degree of over-fitting in the model (11). The direct estimate of survival and its standard error at year 1 post-biopsy was 81.0%±3.0%, with an estimated survival rate corrected for optimism of 81.2%. Likewise, at year 2, the direct estimate of survival was 71.0%±4.4% while the estimate corrected for optimism is 71.1%. Thus, the model appears to be well-calibrated in prediction of time to graft loss.

Increasing the centrally-determined C4d-positive threshold beyond 10% weakened the association between C4d positivity and outcome – using a centrally-determined cut-off of 40%, there was no significant association between C4d positivity and outcome. Of note, there was no significant association between locally-determined C4d positivity (cut-off not specified) and outcome (HR 1.47, 95% CI 0.9-2.38, p=0.121) (Table 1).

To further demonstrate the risk associated with relatively low levels of C4d positivity, the time to graft failure analyses was compared between the three subgroups defined by central C4d+ results of <10%, 10-24%, and ≥25%. A Kaplan-Meier graph showing these three risk groups is given in Figure 2B. In proportional hazards regression comparing to the group with <10% central C4d, the hazard ratio for graft failure for the group with central C4d from 10-24% was 3.3 (95% CI [1.8,6.0], p<0.001), while the estimated hazard ratio for those with ≥25% central C4d was 2.7 (95% CI [1.6,4.5], p<0.001). The two higher risk groups did not differ significantly in risk of graft failure (p=0.534).

Central histopathologic analysis of hematoxylin & eosin (H&E), periodic acid-Schiff (PAS), trichrome, and silver-stained sections was performed according to the Banff '07 Classification Scheme modified to include assessment of inflammation and tubulitis in areas of atrophy as well as in non-atrophic regions (6). Distribution of Banff scores according to C4d status is summarized in Table 2. By the Wilcoxon two-sample test, positive C4d staining correlated with the presence of peritubular capillary infiltrates (p<0.001), acute inflammation (Banff i score, p=0.004), acute tubulitis (t, p=0.026) and a marginal but not significant association with transplant glomerulopathy (Banff cg-score, p=0.051). Positive C4d staining was significantly associated with inflammation in areas of atrophy (p=0.001) and tubulitis in areas of atrophy (p=0.030). Positive C4d staining did not significantly correlate with chronic Banff scores, including interstitial fibrosis (ci), tubular atrophy (ct), vascular sclerosis (cv), arteriolar hyalinosis (ah), or mesangial matrix expansion (mm).

The number of centrally-defined C4d positive biopsies, using a cut-off threshold of 10%, as a function of locally-determined primary or secondary clinicopathologic diagnosis is shown in Table 3. By Fisher's Exact Test, C4d positivity was associated with a local diagnosis of acute cellular rejection (p<0.001), antibody-mediated rejection (p=0.024), and chronic allograft nephropathy (CAN) (p=0.039). Acute tubular necrosis was significantly associated with a negative C4d status (p=0.016).

Twenty-three percent of the 296 biopsies studied were C4d-positive (centrally-determined), 12% had acute rejection (Banff i2 t2 or greater), and 12.5% had both acute rejection and

positive C4d staining. The presence of positive C4d staining was at least as strong a predictor of adverse outcome as the presence of acute rejection (Figure 3). Compared to patients with biopsies that were C4d-negative and had no evidence of rejection (neither), hazard ratios for patients with biopsies that were C4d-positive, acute rejection-positive, or both ranged from 2.4 to 4.0. However, these differences in hazard ratios for the latter three groups were not significantly different.

DISCUSSION

Although C4d has been touted as a marker of humoral immunity and adverse outcome (12, 13), interpretation of results is highly method-dependent (immunofluorescence versus immunoperoxidase, and cut-offs for positivity ranging from "focal" to "diffuse"). Studies have suggested that immunofluorescence detection of C4d is more sensitive than peroxidase-based methods, but it may be impractical to obtain an additional biopsy core for immunofluorescence studies at some centers. Furthermore, the clinical utility of various cut-off points for positive C4d staining, particularly by immunoperoxidase, has not been previously established.

In this study, Kappa values for agreement between central and local determination of C4d were fair (0.40 for a cut-off of 10%) and did not appreciably change when higher cut-off values for centrally-determined C4d positivity were employed. These Kappa values are similar to those obtained in a study comparing immunofluorescence and immunoperoxidase detection of C4d which were performed on the same biopsy cases (Kappa 0.3) (9). Based upon a maximum Chi-square search, a cut-off of 10% for central C4d positivity best predicted graft failure/death. The cross-validated estimate of the hazard ratio of central C4d positivity using a 10% cut-off was 2.66 (95% CI [1.68, 4.21], Likelihood ratio χ^2=19.9, 1 df, p<0.001). The association of C4d positivity with graft failure/death became less significant as higher cut-off thresholds for centrally-determined C4d were employed; there was no significant association between C4d positivity and graft loss when a threshold of 50% was employed. Those with central C4d between 10% and 24% (inclusive) had a significantly higher risk of failure than those with <10% C4d, and furthermore had similar risk of graft-failure compared to those with ≥25% C4d, strengthening the assertion that relatively low levels of C4d are associated with a higher risk of graft failure. There was no significant association between local C4d determinations and outcome, as defined by time to graft failure/death (p=0.121). This may be due to the center-to-center variation in method to detect C4d and the lack of a validated threshold for determination of C4d positivity.

One limitation of our study is an inability to investigate whether a lower cut-off than 10% would have significantly more or less predictive power than the currently selected cut-off of 10% due to insufficient numbers of events among individuals with central C4d >0% and <10%. This question may become open to analysis as additional events accrue with continued follow-up.

By central histopathologic analysis, positive C4d scores correlated with the presence of peritubular capillary infiltrates, acute inflammation, acute tubulitis, and inflammation in areas of atrophy. Other investigators have found C4d positivity in approximately 30-40% of biopsies with acute cellular rejection, and an association with steroid-resistant acute rejection (12-15). Our data confirm this finding, as the presence of C4d in biopsies demonstrating acute cellular rejection is associated with adverse outcome (14-18). Widespread peritubular capillary staining for C4d in association with peritubular capillary inflammatory infiltrates and/or glomerulitis is thought representative of acute humoral rejection (13, 19). Positive C4d staining has also been identified in 25-40% of patients with chronic antibody-mediated rejection, as defined by presence of donor-specific antibody,

double-contouring of glomerular capillary loop basement membranes (transplant glomerulopathy), mononuclear inflammatory cell infiltrates within peritubular capillaries, and arterial intimal thickening with mononuclear cell infiltrates (5, 18, 20-22). In patients with transplant glomerulopathy, the presence of peritubular capillary staining for C4d has been associated with adverse outcome (23). However, the prevalence of C4d staining in the presence of transplant glomerulopathy is only 25-40% (23, 24).

Histopathologic correlates of positive C4d staining did not correlate with chronic Banff scores, including interstitial fibrosis, tubular atrophy, vascular sclerosis, arteriolar hyalinosis, or mesangial matrix expansion. One possible explanation for the lack of correlation of C4d staining with chronic lesions is that there is considerable variability of C4d staining in sequential biopsies, with both late appearance and disappearance of C4d staining observed in serial biopsies obtained from the same patient (20). Nevertheless, a positive C4d stain in patients with chronic allograft dysfunction, when present, is a strong marker of adverse outcome. In a retrospective analysis of 80 patients with CAN but no evidence of transplant glomerulopathy, peritubular capillary staining for C4d was associated with a worse outcome than C4d-negative biopsies (25).

Based on these considerations, we conclude that immunoperoxidase detection of C4d, with a cut-off threshold of 10%, predicts adverse graft outcome in this cohort of patients with graft dysfunction. In patients with acute cellular rejection, positive C4d staining may predict adverse outcome and may provide the basis for alternative therapeutic strategies to target a humoral immune response. Although a positive C4d predicted adverse outcome in patients with chronic histopathologic alterations, C4d was not a sensitive marker for the presence of transplant glomerulopathy. Future studies are needed to develop an optimal model that predicts graft failure, using a cut-off value of 10% for C4d positivity in association with other biomarkers of chronic graft dysfunction. Nevertheless, validation of C4d as a definitive marker using paraffin-embedded material is an important first step in the development of therapeutic protocols to improve long-term graft function.

MATERIALS AND METHODS

Patients and Enrollment

Patients were enrolled in the Cross-Sectional Cohort of the DeKAF Study, a multicenter study to define clinical and histopathologic features that predict adverse outcome in renal biopsies performed for new onset late graft dysfunction (10). Recipients were eligible for enrollment if transplanted prior to October 1, 2005, having a baseline serum creatinine <2.0 mg/dL as of January 1, 2006, and subsequently developing new-onset dysfunction, defined as a ≥25% increase in serum creatinine, or new-onset proteinuria (albumin/Cr ratio >0.2 or protein/Cr ratio >0.5) leading to an allograft biopsy. Enrollment occurred at the time of the biopsy. The study is registered at www.clinicaltrials.gov (NCT00270712). Institutional Review Board approval was obtained at all participating sites.

The current study included patients with late-onset graft dysfunction, as defined above, for which both local and central C4d data were available. Local C4d determinations were performed in conjunction with standard histopathologic analysis of the clinically-indicated renal biopsy, and were reported as negative or positive using locally-established cut-off points. Unstained slides were submitted for central C4d staining. Serum creatinine, urine protein and creatinine, demographic information, current immunosuppression therapy, intervening illnesses, date and cause of graft loss were collected from all enrolled patients every 6 months.

Immunoperoxidase stain for C4d

Central C4d staining of formalin-fixed paraffin-embedded tissues from the clinically-indicated renal biopsies was performed by immunoperoxidase using a polyclonal anti-C4d antibody (Alpco Diagnostics, Salem, NH) following antigen retrieval (26). To facilitate consistency, slides were batched and stained on a Dako autostainer (Dako, Carpinteria, CA). C4d stains were read blindly, without clinical or pathologic information. The percentage of peritubular capillaries staining positively for C4d was recorded.

Histopathologic analysis

Allograft biopsies were read by the local pathologist, and the local pathologic diagnosis was used to guide clinical care and immunosuppressive management. Representative sections were submitted to a central laboratory for analysis. All biopsies were interpreted by the central pathologist in a blinded fashion, without knowledge of C4d status. In addition to the "standard" Banff classification scheme (6), a simplified semiquantitative estimate of inflammation in areas of atrophy was developed and scored as follows: 0 = inflammation in <10% of atrophic regions; 1 = inflammation in 10-25% of atrophic regions; 2 = inflammation in 26-50% of atrophic regions; 3 = inflammation in >50% of atrophic regions.

Statistical analysis

Agreement between local and central C4d positivity was assessed by Kappa analysis, proportion of observed agreement, and proportions of specific agreement for C4d+ positivity and for C4d+ negativity (27). Methods of survival analysis were used to analyze time-to-event outcomes. Non-parametric assessment (Kaplan-Meier) was employed to analyze time to graft failure – as defined by return to dialysis, retransplantation, or death – for biopsies staining positively or negatively for C4d. Cox proportional hazard models were used to estimate unadjusted hazard ratios for graft loss by local or central C4d results.

For both the survival methods and the agreement analyses, all observed values of percentage C4d up to 50% were assessed as putative cut-offs for determination of central C4d positivity. Values greater than 50% were not considered due to the paucity of observations with C4d above this value (41/296).

Selection of the optimal cut-off for determination of central C4d positivity was made using the maximum Chi-square search, following Mazumdar et al. (28). Briefly, the optimal cut-off for C4d positivity is determined by the largest Chi Square value (smallest p-value) for the Likelihood Ratio test in a univariate model of C4d positivity determined by the putative cut-off. Two-fold cross-validation was employed to obtain a hazard ratio estimate and p-value for the optimal cut-off to correct for the multiple testing employed in the maximum Chi Square search. The predictive performance of the final model was assessed in terms of discrimination via Harrell's c-index (11). The calibration of the final model was assessed by bootstrap estimation of the bias due to over-fitting at 1-year and 2-years post-biopsy. Both the c-index and the calibration were corrected for over-fitting ("optimism") through bootstrap estimation with 200 replications.

Associations between C4d staining and histologic features were assessed by the two-sample Wilcoxon test. Associations between C4d staining and local diagnoses were assessed by Fisher's exact test. Two-sample cross-validation, computation of the c-index and calibration estimates were performed using the R (29), using the rms package of Harrell (30). All other analyses were performed using SAS/STAT software, Version 9.2 of the SAS System for Unix.

Acknowledgments

## ABBREVIATIONS

|	
AIC	Akaike Information Criterion
CAN	Chronic allograft nephropathy
DeKAF	Deterioration of Kidney Allograft Function

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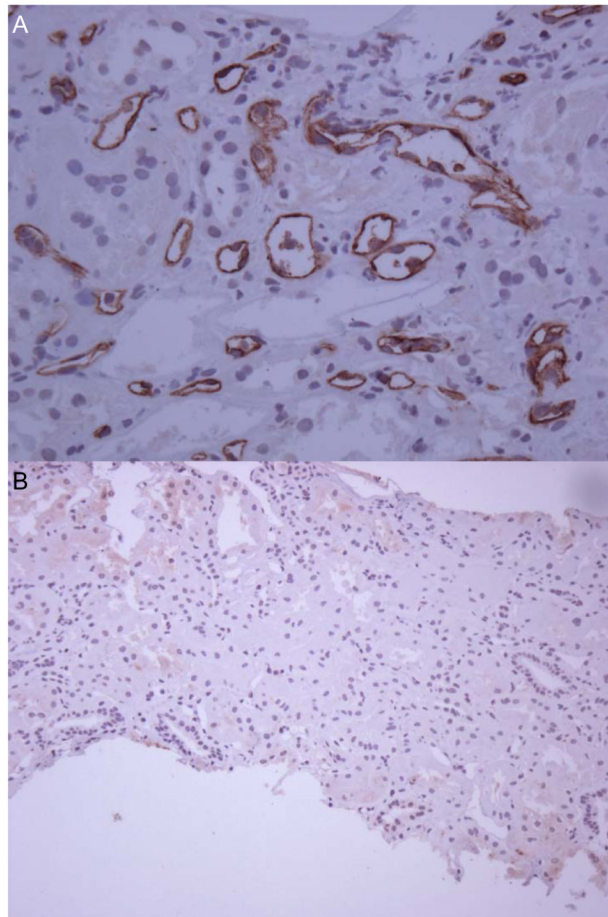
Figure 1.
Representative histopathologic section of renal biopsy stained by immunoperoxidase for C4d showing diffuse positive staining within peritubular capillaries (panel A).
Representative section of a renal biopsy showing no significant staining for C4d (panel B).
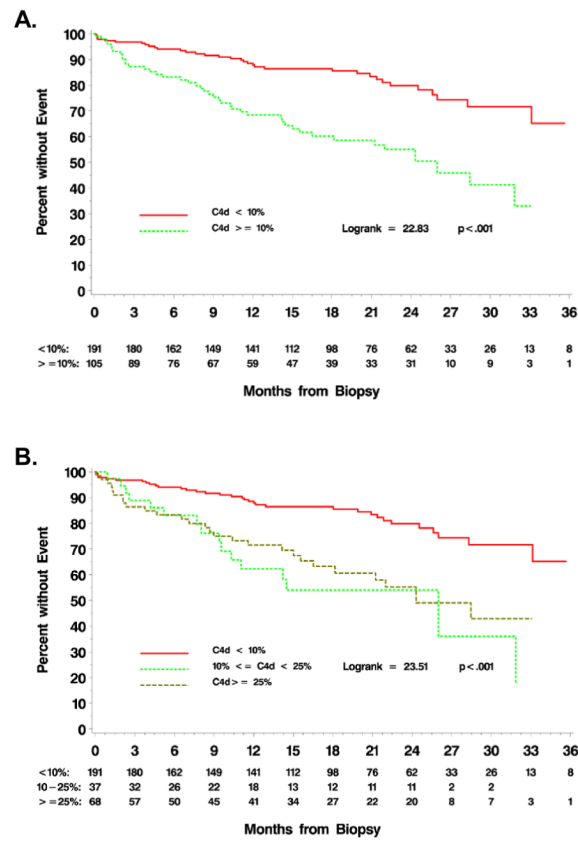
Figure 2.
Time to graft failure by C4d status. Immunoperoxidase staining for C4d was centrally
performed and read in a blinded fashion. A) Time to graft failure as a function of C4d status,
using a centrally-defined cut-off threshold of 10%. B) Time to graft failure as a function of
C4d status, using centrally-defined cut-off values of <10% (negative), 10-24%, or ≥25%.
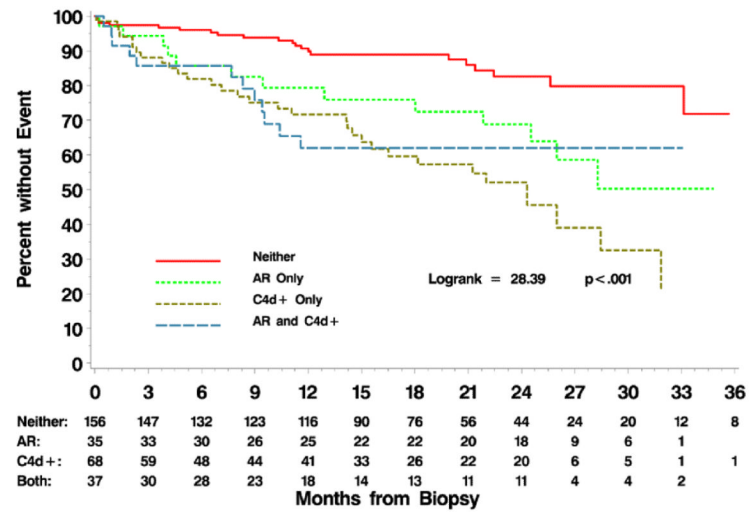
Figure 3.
Time to graft failure in patients (n=296) whose biopsies show positive C4d staining (n=68), acute rejection (n=35), both (n=37), or neither (n=156).

Table 1

Hazard ratios from univariate models for time-to-graft failure (including death) by central C4d positivity cut-off or local determination.

C4d Positivity	HR (95% CI)[a]	Chi-Square	P-value	AIC[b]	C-index[c]
>0%	2.75 ([1.73,4.38])	19.719	<0.001	775.214	0.63
≥5%	2.85 ([1.79,4.54])	21.239	<0.001	773.848	0.63
≥10%	*2.88 ([1.83-4.52])[d]*	*22.849*	*<0.001*	*773.479*	**0.63**
≥15%	2.40 ([1.53,3.78])	15.235	<0.001	780.939	0.60
≥20%	2.24 ([1.42,3.55])	12.537	<0.001	783.368	0.59
≥25%	2.08 ([1.30-3.32])	9.763	0.002	785.781	0.58
≥30%	1.81 ([1.11,2.94])	5.789	0.016	789.179	0.56
≥40%	1.42 ([0.84,2.42])	1.743	0.187	792.713	0.52
≥50%	1.33 ([0.76-2.35])	1.007	0.317	793.385	0.51
Local C4d positive (cut-off unknown)	1.47 ([0.90-2.38])	2.430	0.119	792.108	0.53

[a] Hazard Ratio (HR) and 95% Confidence Interval (CI)

[b] Akaike Information Criterion, a penalized likelihood, and measure of general. Smaller values are better.

[c] Harrell's C-index, an analogue of area under the Receiver Operator Characteristic Curve for censored data, bootstrap-corrected for optimism using 200 bootstrap samples. Values range from 0 to 1, with the uninformative null model having a value of 0.5. Larger values indicate better discrimination between high and low risk cases. The values are rounded to two decimal places, with the maximal value occurring at the 10% cut-off.

[d] The table contains the hazard ratio estimated directly from the data. The cross-validated estimate of the hazard ratio for central C4d positivity defined by a 10% cut-off is 2.66 (95% CI [1.68,4.21]), with likelihood ratio test χ^2=19.9, 1 df, p<0.001.

Table 2

Selected centrally-determined Banff scores as a function of centrally-determined C4d positivity (≥10%).

Central C4d Status	Central Banff i-score p=0.004				Total
Frequency/Row Percent	0	1	2	3	
Negative	117 69%	21 12%	18 10%	13 8%	169
Positive	48 49%	21 22%	22 23%	6 6%	97
Total	165	42	40	19	266

Central C4d Status	Central Banff t-score p=0.026				Total
Frequency/Row Percent	0	1	2	3	
Negative	127 75%	11 7%	18 11%	13 8%	169
Positive	59 61%	11 11%	20 21%	7 7%	97
Total	186	22	38	20	266

Central C4d Status	Central Banff ptc-score p<0.001				Total
Frequency/Row Percent	0	1	2	3	
Negative	90 53%	55 33%	11 7%	13 8%	169
Positive	28 29%	44 45%	18 19%	7 7%	97
Total	118	99	29	20	266

Central C4d Status	Central Banff cg-score p=0.051				Total
Frequency/Row Percent	0	1	2	3	
Negative	121 72%	14 8%	12 7%	22 13.02%	169
Positive	58 60%	12 12%	8 8%	19 20%	97
Total	179	26	20	41	266

Central C4d Status	Inflammation in Atrophic Regions P<0.001				Total
Frequency/Row Percent	0	1	2	3	
Negative	67 40%	51 30%	37 22%	14 8%	169
Positive	17 18%	35 36%	34 35%	11 11%	97
Total	84	86	71	25	266

*
P-values computed via the Wilcoxon Two-Sample test.

Table 3

Local primary or secondary diagnoses by centrally-determined C4d status

Local Primary or Secondary Diagnoses	Central C4d Status				
	Negative		Positive		
	N	Pct*	N	Pct*	P-value**
Rejection: Cellular	26	13.7	32	30.5	<0.001
Rejection: Antibody-Mediated	10	5.3	14	13.3	0.024
Borderline Changes	14	7.4	6	5.7	0.639
ATN	10	5.3	0	0.0	0.016
CAN	79	41.6	57	54.3	0.039
CNI Toxicity	56	29.5	29	27.6	0.789
Glomerulopathy (de novo)	12	6.3	5	4.8	0.795
Arterial Nephrosclerosis	21	11.1	5	4.8	0.086
Polyoma Virus	7	3.7	1	1.0	0.267
Recurrent Disease	20	10.5	20	19.0	0.050
Transplant Glomerulopathy	37	19.5	25	23.8	0.456
No Abnormalities	6	3.2	1	1.0	0.428
Other	52	27.4	23	21.9	0.331
No Diagnosis	1	0.5	0	0.0	>0.999
Total Cases	*169*	*100*	*97*	*100*	

*
Percents are percents of C4d negative (resp. positive) cases. Percentages in a given column may add to more than 100% due to individual participants having one or several secondary diagnoses.

**
P-values are computed via Fisher's Exact Test.