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The Impact of C4d Pattern and Donor-Specific Antibody on Graft Survival in Recipients Requiring Indication Renal Allograft Biopsy

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We examined the pattern of PTC C4d by immunohistochemistry and DSA in 297 kidney recipients with indication biopsies, and evaluated their predictive value for graft survival. Median biopsy time was 5.1 months posttransplant. Patients were followed for 17.9 ± 9.4 months postbiopsy. An 18.5% had focal and 15.2% had diffuse C4d, with comparable graft survival (adjusted graft failure HR: 2.3, $p = 0.001$; HR:1.9, $p < 0.02$, respectively). 31.3% were DSA+, 19.5% class I and 22.9% class II DSA. Only those with class II DSA had worse outcome (adjusted HR:2.5, $p = 0.001$ for class II only; HR:2.7, $p < 0.001$ for class I/II DSA). Among patients with <10%C4d, 23.9% had DSA, compared to 68.9% with diffuse staining. For patients biopsied in first-year posttransplant presence of DSA, regardless of C4d positivity in biopsy, was a poor prognostic factor (adjusted graft failure HR: 4.2, $p < 0.02$ for C4d−/DSA+; HR:4.9, $p = 0.001$ for C4d+/DSA+), unlike those biopsied later. We have shown that focal C4d had similar impact on graft survival as diffuse pattern. During the first-year posttransplant either class I or II DSA, and afterward only class II DSA were associated with worse graft survival. DSA was predictive of worse outcome regardless of C4d for patients biopsied in first year and only with C4d positivity afterward, supporting the importance of assessment of both DSA and C4d pattern in biopsy.

Key words: Anitbody mediated rejection, complement C4d, donor-specific antibodies, graft biopsy, graft survival, kidney transplantation

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Introduction

With the technical advances in methods used for detection of antibodies directed at the allograft and widespread use of the histological marker of complement activation, C4d, the contribution of human leukocyte antigen (HLA) antibodies to graft injury has been more frequently recognized in recent years. Diagnostic criteria for acute and chronic antibody-mediated rejection (AMR) include the presence of circulating donor-specific antibodies (DSA) and histological evidence of their action, i.e. diffuse peritubular capillary (PTC) C4d staining (1,2). Applying these criteria, a significant number of patients with only one of these two criteria would only be suspected to have graft dysfunction due to antibody-mediated injury and not treated. Despite the increased sensitivity of the current methods for detection of DSA, there are HLA antigens and alleles not represented in the panels used. Moreover, not all non-HLA antigens are represented in the available panels. As a result, the circulating DSA cannot be detected in some patients. Moreover, noncomplement fixing antibodies may not be traced by C4d staining; conversely, in some cases C4d deposition may be the result of complement activation by lectin pathway (3). It has been shown that using immunohistochemistry (IH), focal PTC C4d staining turns out to be diffuse with immunofluoroscence (IF) in significant number of biopsies (3–6). As a result of these caveats, histological evidence of antibody-mediated graft injury cannot always be demonstrated. These shortcomings mean that in some patients with ongoing AMR, current diagnostic criteria will not be fulfilled and as a result, appropriate treatment may not be offered (7). The extent of this important problem and its impact on long-term graft outcomes are not well studied.

We sought to examine the pattern of PTC C4d staining and prevalence of DSA in nonselected renal transplant recipients who required indication graft biopsies and to evaluate the concordance between the two. Moreover, we attempted to examine the predictive value of DSA and C4d pattern alone and in combination for graft survival. We hypothesize that the current criteria for diagnosis of AMR requiring demonstration of DSA and diffuse PTC C4d staining in addition to graft injury are too strict and leave a significant number of patients without proper diagnosis and treatment.

Methods

This is a retrospective cohort study of renal allograft recipients biopsied at our center for cause between January 2005 and December 2007. Study protocol was approved by the University of Maryland Institutional Review Board. Only patients tested for DSA were eligible for this analysis. The objectives of the study included (1) to examine the pattern of PTC C4d staining in indication biopsies and its predictive value for graft survival; (2) to examine the prevalence and type of DSA in patients who required diagnostic biopsies and its association with graft outcome; (3) to evaluate the association of C4d staining with DSA in such patients; and finally (4) to examine the conjoint predictive value of C4d pattern and DSA for graft survival.

The indication for biopsies generally included unexplained increase in serum creatinine (SCr), or its slow decline early posttransplant, proteinuria and positive urine cytology for BK viruria. Biopsies were all evaluated by a single pathologist using Banff criteria(1,2,8,9). PTC staining for C4d was performed by IH, using polyclonal rabbit anti-human C4d antibody (ARP, Belmont, MA), 1:200 dilution on BenchMark automated system and ultraview DAB enhancement. Sera were screened for DSA using HLA class I and II mixed beads (LABScreen beads, One Lambda Inc., Canoga Park, CA), with further testing by single antigen beads (LABScreen beads, One Lambda Inc.). The assays were performed according to the manufacturer's protocol. Mean fluorescence intensity (MFI) $\geq$1000 was defined as positive.

The majority of recipients had received induction therapy in the form of basiliximab (Simulect, Novartis Pharmaceuticals, NJ), a 5–7-day course of thymoglobulin (Genzyme, Cambridge, MA) (rATG) (1.5 mg/kg) or alemtuzumab (Campath-1H, Genzyme, Cambridge, MA) 30 mg. Patients were maintained on mycophenolate mofetil (CellCept, Roche, Nutley, NJ) 1000 mg twice daily, tacrolimus with target 12-h trough level of 10–15 ng/mL (by immunoassay) during the first year and 6–10 ng/mL thereafter, and tapering dose steroid. Dose adjustments were made as indicated.

Outcome and statistical analysis

In a cross sectional analysis, the prevalence of different patterns of C4d staining in the index biopsies, i.e. minimal (<10% PTC), focal (10–50% PTC) and diffuse (>50% PTC) and the presence of class I/class II DSA were determined.

We then examined the association of PTC C4d positivity in the biopsy and/or DSA with graft outcome after the index biopsy. The primary outcome of this analysis was death-censored graft survival. Graft loss included graft failure before censoring or death.

Data were retrieved on age, race, gender, retransplantation, diabetes, chronic hepatitis C, peak PRA, donor source, donor/recipient HLA mismatch, induction agent and biopsy findings. The groups were compared using Student's *t*-test, one way ANOVA, chi-square, Fisher's exact test, or Kruskal–Wallis rank test as needed. Kaplan–Meier survival estimates and Cox proportional hazard regression models to adjust for the potential confounding effects of variables with statistitcal difference between the groups were used to evaluate the association between the predictor variables and graft survival. Logistic and multinomial regression models were used to examine the association between C4d pattern and DSA. Model assumptions were tested using appropriate statistical diagnostics. Stata/SE 10.1 (StataCorp, College Station, TX) was used for statistical analysis.

Results

We identified 297 patients who underwent indication biopsy and DSA screening during the study period. For those who had multiple biopsies during this period, the first biopsy was used for analysis. Patient and transplant-related characteristics are summarized in Table 1. The median time from transplantation to biopsy was 5.1 months (range 1 week–16 years), with 60.6% performed during the first year (2.4 $\pm$ 3.1 months) and 39.4% later (59.9 $\pm$ 40.8 months, median: 49.3). The mean interval between the biopsy and DSA screening was 19.7 days; 243 (81.8%) within a week and 262 (88.2%) within a month from biopsy. Patients were followed for up to 48.5 months (17.9 $\pm$ 9.4) after the biopsy, until last follow-up visit, graft failure or death.

Table 1: Patient and transplant-related characteristics of the entire cohort and subgroups defined by PTC C4d staining

	Entire cohort N = 297	Negative C4d N = 197	Focal C4d N = 55	Diffuse C4d N = 45	p-Value
Age (year)	51.4 $\pm$ 13.0	51.4 $\pm$ 13.0	51.1 $\pm$ 14.8	51.4 $\pm$ 11.0	0.98
Male (%)	66.7	70.1	58.2	62.2	0.20
African-American (%)	54.9	53.3	52.7	64.4	0.38
Diabetes (%)	31.4	30.2	37.7	28.9	0.53
HCV (%)	18.1	16.1	21.6	22.7	0.303
Retransplant (%)	15.5	14.7	14.5	20	0.66
Deceased-donor (%)	76.4	75.6	78.2	77.8	0.94
HLA-MM	4.2 $\pm$ 1.6	4.0 $\pm$ 1.7	4.3 $\pm$ 1.3	4.4 $\pm$ 1.4	0.29
Peak PRA (%) (N = 244) <10%/10–40%/>40%	70.1/9.6/20.4	68.9/19.1/13.0	65.2/2.2/28.9	50.0/16.7/33.3	0.04
Induction (%) none/BSX/ATG/Campath	5.2/44.8/30.7/19.3	6.3/48.2/26/718.9	5.6/35.2/37.0/22.2	0/42.2/40/17.8	0.19
ACR[2] (%)	16.2	12.2	21.8	26.7	<0.03
Grade I/II/III	9.8/4.3/2.0	7.6/3.1/1.5	14.6/3.6/3.6	13.3/11.1/2.2	
IF/TA[1] (%) mild/moderate/severe	47.8/25.9/5.4	48.7/25.4/5.1	49.1/29.1/3.6	24.4/42.2/8.9	0.87
Arteriolar hyalinosis (%)	33.7	35.5	38.2	35.6	0.93
Transplant glomerulopathy(%)	9.1	4.1	16.4	22.2	<0.001

[1]Interstitial fibrosis/tubular atrophy.
[2]Acute cellular rejection.

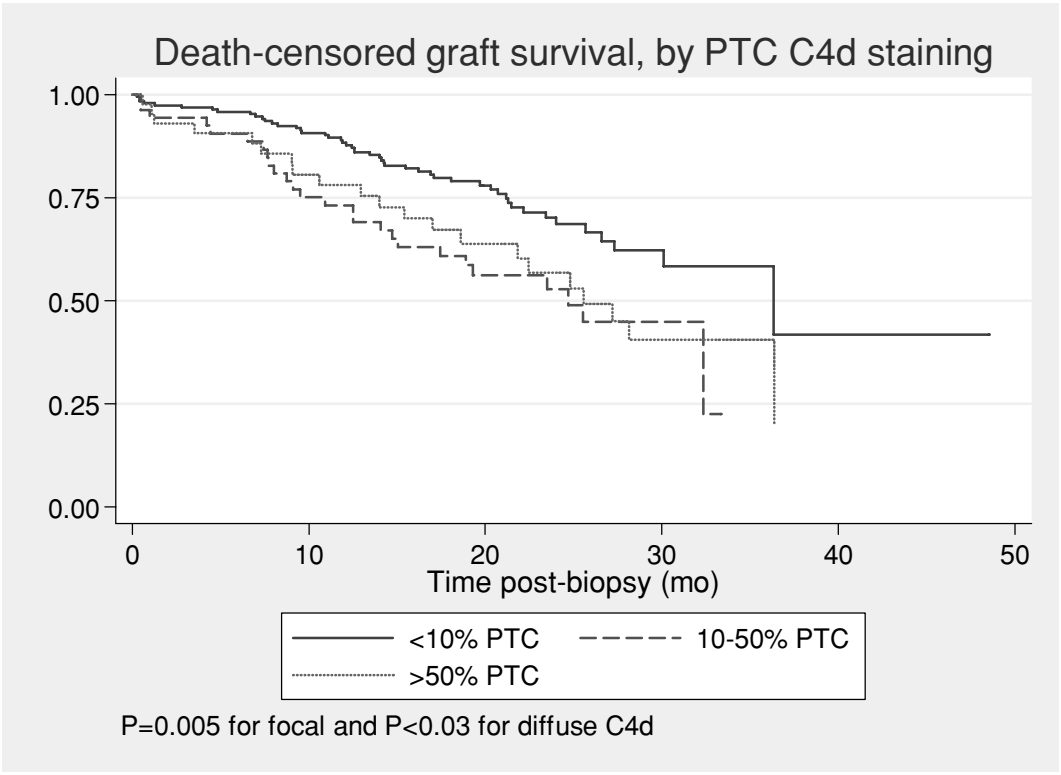

Figure 1: Graft survival estimates after biopsy by C4d grade, overall and by time of biopsy.

C4d staining, pattern and clinical outcomes

Examining the C4d staining pattern, 197 (66.3%) had <10%, 55 (18.5%) had focal and 45 (15.2%) diffuse PTC staining. The risk of acute cellular rejection (ACR) in biopsy increased with increase in the extent of C4d staining, 12.2%, 21.8% and 26.7% of the cases, respectively ($p < 0.03$). When we analyzed the C4d pattern in early as compared with late biopsies, the corresponding figures for focal and diffuse staining were 20.0% and 17.8%, respectively, for first year and 16.2% and 11.1%, respectively, for later biopsies. As depicted in Figure 1, graft survival from the time of biopsy with focal pattern was comparable to diffuse staining, both different from minimal or negative staining (HR:1.99, $p = 0.005$, 95%CI:1.2–3.2 and HR:1.78, $p < 0.03$, 95%CI:1.1–3.0, respectively) (Table 3). The three subgroups were comparable regarding the majority of variables listed in Table 1. Even after adjusting for the time of biopsy (first-year posttransplant vs. later), ACR, arteriolar hyalinosis and severity of interstitial fibrosis and tubular atrophy (IF/TA), focal and diffuse staining were both associated with worse graft survival (HR for graft loss:2.3, $p = 0.001$, 95%CI:1.4–3.7; HR:1.9, $p < 0.02$, 95%CI:1.2–3.3, respectively) (Table 3). Interestingly, despite adding DSA as a covariate to the model, focal C4d remained a significant independent predictor of graft failure (adjusted HR:2.2, $p = 0.002$; 95%CI:1.4–3.7), with a stronger

association compared with diffuse pattern (adjusted HR:1.7, $p < 0.06$; 95%CI:1.0–3.0). Since data on peak PRA were missing in 53 cases and more proximal variables on the causal pathway, i.e. cellular and antibody-mediated graft injury and DSA, were adjusted for, it was not included in the model. The other reported histological features for the groups with focal and diffuse C4d included tubular injury in 58.2% and 55.6% ($p = 0.79$), peritubular capillaritis in 9.1% and 20% ($p = 0.15$), glomerulitis in 8.9% and 14.5% ($p = 0.53$) and transplant glomerulopathy in 16.4% and 22.2% ($p = 0.46$), respectively.

Examining the association of C4d with this outcome only for those biopsied during the first year ($N = 180$), similar results were observed (HR:2.9, $p = 0.008$, 95%CI:1.3–6.4 for focal and HR:2.7, $p < 0.0.02$, 95%CI:1.2–6.1 for diffuse group) after adjusting for ACR, areteriolar hyalinosis and IF/TA. The same association was present for those biopsied later ($N = 117$) (adjusted HR:2.1, $p < 0.03$, 95%CI:1.1–4.0 and HR:1.7, $p = 0.18$, 95%CI:0.8–3.7, respectively), although it was not statistically significant for the diffuse pattern.

DSA, pattern and clinical outcomes

The median time from transplantation to DSA screening was 7.5 months (range:1 week–16 years), with 56.9% performed during the first-year posttransplant (2.5 ± 3.1 months) and 43.1% later (59.8 ± 41.4 months, median:48.7). Ninety-three patients (31.3%) were DSA positive, 58 (19.5%) with class I and 68 (22.9%) with class II DSA; 33 (11.1%) with both class I and class II DSA. For 169 patients screened during the first year, corresponding numbers included 46 (27.2%), 34 (20.1%), 29 (17.2%) and 17 (10.1%), respectively. Examining the association of DSA with graft survival, those with class I only DSA did not have higher risk of graft failure from the time of sampling compared with DSA-negative patients (HR:1.2, $p = 0.63$, 95%CI:0.5–2.7), while those with class II DSA, alone or in combination with class I DSA had worse graft survival (HR for graft failure: 3.0, $p < 0.001$, 95%CI:1.8–5.0 and HR:3.2, $p < 0.001$, 95%CI:1.9–5.4, respectively) (Figure 2). The four subgroups were not comparable regarding gender, race, retransplantation and ACR. Adjusting for these variables, in addition to the sampling time (first-year vs. later) did not change this association (adjusted HR:2.5, $p = 0.001$, 95%CI:1.4–4.3 for class II only; and HR:2.7, $p < 0.001$, 95%CI:1.6–4.7 for class I/II DSA) (Table 3). Analysis of those tested during the first year showed that any form of DSA was predictive of higher risk of graft failure (HR:2.9, $p < 0.03$, 95%CI:1.1–7.4 for class I only; HR:4.1, $p = 0.003$, 95%CI:1.6–10.5 for class II only; and HR:3.4, $p = 0.007$, 95%CI:1.4–8.3 for both class I/II). Adjustment for the same variables did not affect these associations (adjusted HR:3.2, $p < 0.02$, 95%CI:1.2–8.3; HR:5.8, $p = 0.002$, 95%CI:1.9–18.0; and HR:4.5, $p = 0.005$, 95%CI:1.6–12.6, respectively). In contrast, for patients screened after the first year, only positivity for class II DSA, either alone or with Class I, was predictive of worse graft survival (HR for graft failure:1.9, $p = 0.04$, 95%CI:1.0–3.6 for class II only and HR:2.9, $p = 0.001$, 95%CI:1.5–5.6 for class I/II DSA). Adjustment for the potential confounders only modified the strength of this association (adjusted HR:1.9, $p < 0.05$, 95%CI:1.0–3.7 and HR:2.2, $p < 0.04$, 95%CI:1.0–4.4, respectively).

Conjoint C4d/DSA, associations and clinical outcomes

In this cohort 262 patients were screened for DSA within a month of index biopsy. Among 163 patients with negative or minimal C4d, 124 (76.1%) did not have detectable DSA, while 39 (23.9%) were associated with positive screening; 12 with class I only, 16 with class II only and 11 with both class I and II specificities. Among 54 cases with focal C4d staining, 19 (35.2%) did have DSA, 4 with class I only, 10 with class II only and 5 with both class I/II DSA. In contrast, in 45 with diffuse C4d, only 31 (68.9%) were associated with DSA; 8 with class I, 7 with class II and 16 with both class I/II specificities. Compared with those with negative or minimal C4d staining, patients with focal staining had 90% higher chance of having circulating DSA ($p = 0.055$, 95%CI:0.99–3.6), whereas those with diffuse pattern were 7.9 times more likely to have positive screening ($p < 0.001$, 95%CI:3.9–16.2).

We then evaluated the predictive value of DSA class for C4d pattern. Taking those with negative/minimal C4d as the comparison group, in the subgroup with diffuse C4d, the relative risk ratio (RRR) comparing class I only DSA to negative DSA for diffuse C4d relative to minimal/negative C4d was 5.9 ($p = 0.001$, 95%CI:2.1–16.9); for class II only and Class I/II DSA the RRR were 3.9 ($p = 0.01$, 95%CI:1.4–11.0) and 12.9 ($p < 0.001$, 95%CI:5.0–33.2), respectively. However, in those with focal C4d, relative to the comparison group risk ratios for the positive DSA subgroups were not significantly different compared to negative DSA subgroup. In other words, presence of DSA did not predict the presence of focal C4d in the biopsy when compared with negative/minimal C4d. However, any kind of DSA was significant predictor of diffuse C4d compared to negative/minimal pattern. Examining the predictive value of all forms of DSA for C4d positivity, the probability of C4d positivity, either focal or diffuse, was significantly higher in the presence of DSA (OR:2.5, $p < 0.04$, 95%CI:1.1–6.0 for class I only; OR:2.7, $p = 0.01$, 95%CI:1.3–5.7 for class II only; and OR:4.8, $p < 0.001$, 95%CI:2.2–10.8 for class I/II DSA).

To further evaluate the conjoint predictive value of C4d in biopsy and circulating DSA for graft survival, we estimated the graft survival rates from the time of biopsy among these 262 patients. Since focal and diffuse C4d were similarly associated with worse graft survival, we combined the two into one group as C4d positive. Accordingly four subgroups were defined: C4d−/DSA− ($n = 124$, 47.3%),

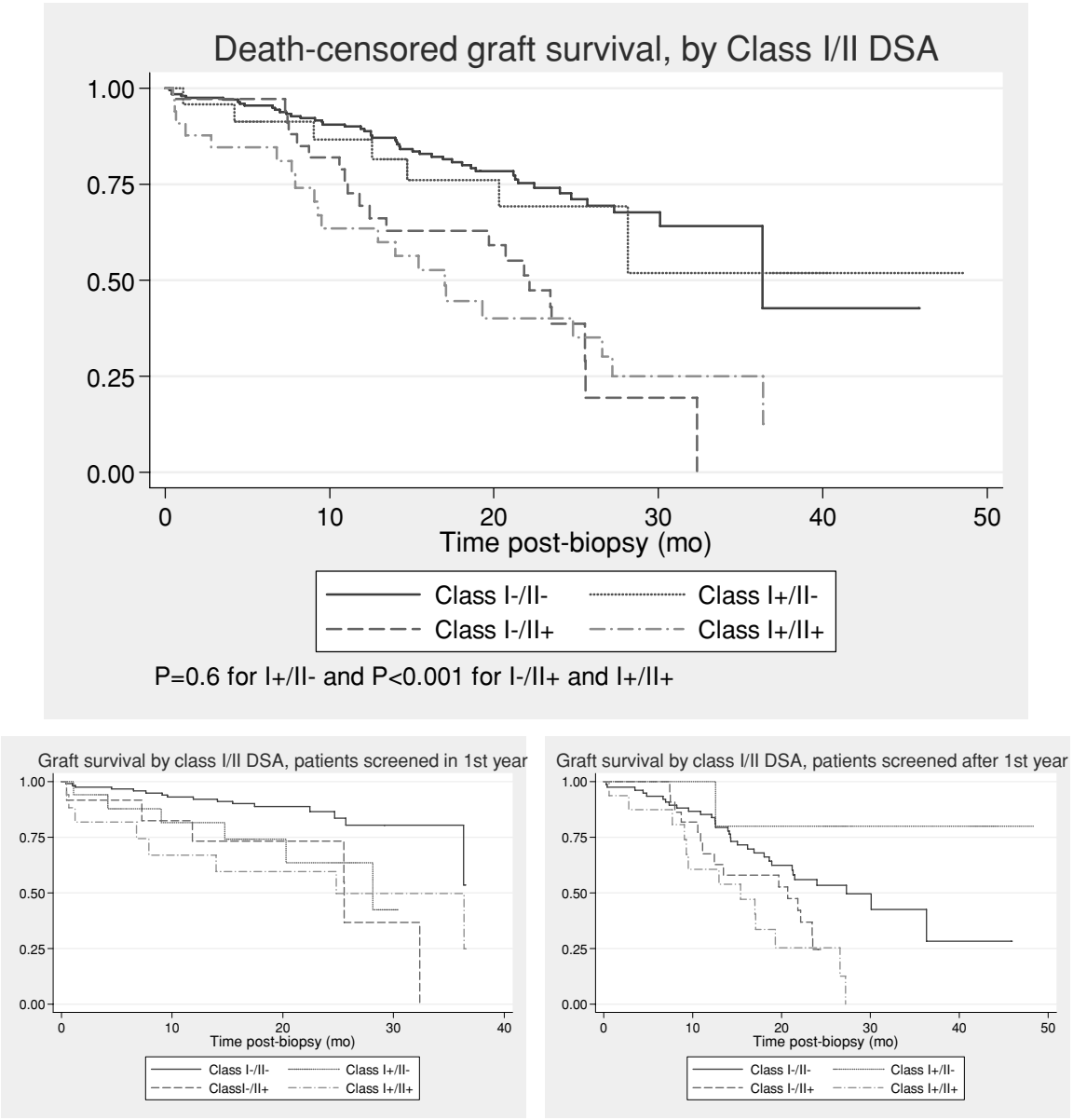

Figure 2: Graft survival estimates after biopsy by class I/II DSA status, overall and by time of DSA sampling.

C4d−/DSA+ (n = 39, 14.9%), C4d+/DSA− (n = 49, 18.7%) and C4d+/DSA+ (n = 50, 19.1%). The comparison between characteristics of the four subgroups has been summarized in Table 2.

Survival estimates for these subgroups are depicted in Figure 3. Among the C4d+/DSA+ patients 18 had received treatment for AAMR. Presence of DSA, with or without C4d positivity in biopsy, was associated with worse graft survival compared to DSA/C4d negativity (HR:2.1, $p < 0.03$, 95%CI:1.1–3.9 for C4d−/DSA+ and HR:3.1, $p < 0.001$, 95%CI:1.8–5.3 for C4d+/DSA+ patients) (Table 3). In contrast the presence of C4d in biopsy without detectable DSA was not associated with worse graft survival (HR for graft failure:1.3, $p = 0.45$, 95%CI:0.7–2.4). In this cohort, the clinical variables listed in Table 2 were largely comparable between the subgroups, except for retransplantation and ACR in the biopsy. After adjustment for the discrepant variables and the time of biopsy (first-year vs. later) presence of DSA alone was no longer independently predictive of worse post-biopsy graft survival (HR:1.6, $p < 0.13$, 95%CI:0.9–3.2), as was C4d positivity alone (HR:1.7, $p = 0.12$, 95%CI:0.9–3.2). After adjustment for the potential confounders, only C4d/DSA positivity was associated with poor graft survival (HR:3.2, $p < 0.001$, 95%CI:1.8–5.5). Among 157 patients

Table 2: Patient and transplant-related characteristics of subgroups defined by C4d and DSA status

	C4d−/DSA− N = 124	C4d−/DSA+ N = 39	C4d+/DSA− N = 49	C4d+/DSA+ N = 50	p-Value
Age (year)	51.4 ± 12.8	49.5 ± 13.4	53.3 ± 13.5	49.3 ± 12.7	0.38
Male (%)	71.8	59.0	63.3	58.0	0.22
African-American (%)	48.2	66.7	53.1	62.0	0.15
Diabetes (%)	34.5	30.6	36.2	32.0	0.95
HCV (%)	12.3	21.6	23.9	20.4	0.20
Retransplant (%)	12.9	23.1	6.1	26.0	0.02
Deceased-donor (%)	70.8	82.1	83.7	72.0	0.24
HLA-MM	3.9 ± 1.8	4.3 ± 1.4	4.4 ± 1.3	4.3 ± 1.4	0.21
Peak PRA (%) (N = 217) <10%/10–40%/>40%	73/10/17	60/10/30	77.3/6.8/15.9	41.9/11.6/46.5	0.0005
Induction (%) none/BSX/ATG/Campath	4.3/44.1/32.0/19.5	2.5/48.8/28.1/20.1	13.5/46.0/27.0/13.5	2.0/36.7/44.9/16.3	0.19
Time of biopsy (median) (mo)	7.9	16.8	1.3	2.0	0.07
ACR[2] (%)	10.5	20.5	14.3	32.0	0.008
Grade I/II/III	8.1/1.6/0.8	7.7/10.3/2.6	8.2/4.1/2.0	18.0/10.0/4.0	
IF/TA[1] (%) mild/moderate/severe	51.6/21.8/4.0	41.0/33.3/12.8	44.9/26.5/2.0	48.0/28.0/10.0	0.03
Arteriolar hyalinosis (%)	34.7	28.2	40.8	34.0	0.69

[1]Interstitial fibrosis/Tubular atrophy.
[2]Acute cellular rejection.

biopsied in the first-year DSA positivity was predictive of higher risk of graft failure (adjusted HR:2.4, p = 0.12, 95%CI:0.8–6.9 for C4d+/DSA−; HR:4.2, p < 0.02, 95%CI:1.3–13.5 for C4d−/DSA+; and HR:4.9, p = 0.001, 95%CI:1.8–13.2 for C4d+/DSA+ subgroups, respectively). However, for those biopsied later only patients with positive C4d/DSA had worse graft survival (adjusted HR:1.4, p = 0.4, 95%CI:0.6–3.6; HR:1.1, p = 0.8,, 95%CI:0.5–2.5; and HR:3.0, p = 0.003, 95%CI:1.5–6.1, respectively).

When we added severity of IF/TA and arteriolar hyalinosis to the multivariate model, only those with both C4d/DSA positivity in the whole cohort had worse graft survival (HR for graft failure compared to C4d−/DSA− patients:2.7, p = 0.001; 95%CI:1.5–4.6). However, for patients biopsied during the first year presence of DSA, regardless of C4d, was a poor prognostic finding (adjusted HR:4.0, p < 0.03, 95%CI:1.2–13.2 for C4d−/DSA+ and HR:5.5, p = 0.001, 95%CI:2.0–15.1 for C4d+/DSA+ subgroups). C4d positivity alone in these patients was associated with worse graft survival only with borderline significance (adjusted HR: 2.7, p = 0.08, 95%CI:0.9–7.9).

Transplant glomerulopathy

Among this cohort 27 had transplant glomerulopathy (TG) in the biopsy, 22 (81.5%) diagnosed after first-year post-transplant. Eight (29.6%) were associated with negative or minimal PTC C4d staining, nine (33.3%) with focal and ten (37.0%) with diffuse C4d (p < 0.001). The odds of TG was 4.6 fold higher for focal C4d compared to negative/minimal staining (p = .003, 95%CI:1.7–12.6) and 6.8 fold for diffuse C4d (p < 0.001, 95%CI:2.5–18.3). Seven patients with TG (25.9%) did not have detectable DSA, 1 (3.7%) had only class I, 8 (29.6%) had only class II and 11 (40.2%) had both class I and II DSA (p < 0.001). Transplant glomerulopathy was associated only with the presence of class II DSA, either alone or with class I (OR:1.2, p = 0.88, 95%CI:0.14–9.9 for class I only; OR:8.3, p < 0.001, 95%CI:2.8–24.8 for class II only; and OR:14.1, p < 0.001, 95%CI:4.9–40.0 for class I and II DSA).

Examining the association of TG with C4d/DSA status, C4d positivity alone was not associated with this finding. However, DSA alone or in combination with C4d positivity was predictive of TG (OR:5.8, p = 0.02, 95%CI:1.3–25.4 for C4d−/DSA+; and OR:17.9, p < 0.001, 95%CI:4.9–65.2 for C4d+/DSA+ group).

Discussion

In this study, we analyzed the prevalence of DSA and pattern of C4d staining in patients requiring indication renal allograft biopsies. In 297 biopsies, one per patient, one-third showed PTC C4d positivity by IH, 18.5% focal and 15.2% diffuse. We found that focal and diffuse PTC staining patterns were similarly associated with graft failure, independent of major clinical and concomitant histological variables and the presence of DSA. Time of biopsy did not affect the observed association. According to consensus recommendations, only diffuse PTC C4d staining fulfills the criteria for AMR (1,7). Our observation argues against this recommendation and suggests that, at least using IH, focal and diffuse pattern should be treated similarly. It was previously shown that a significant number of biopsies with focal C4d staining using IH have diffuse positivity with IF (4–6). The reported prevalence of C4d positivity and its association with clinical outcomes in the literature have been variable due to the use of different staining techniques and percentage of PTC positivity for inclusion, and different biopsy settings. While the majority found C4d positivity predictive of worse graft survival

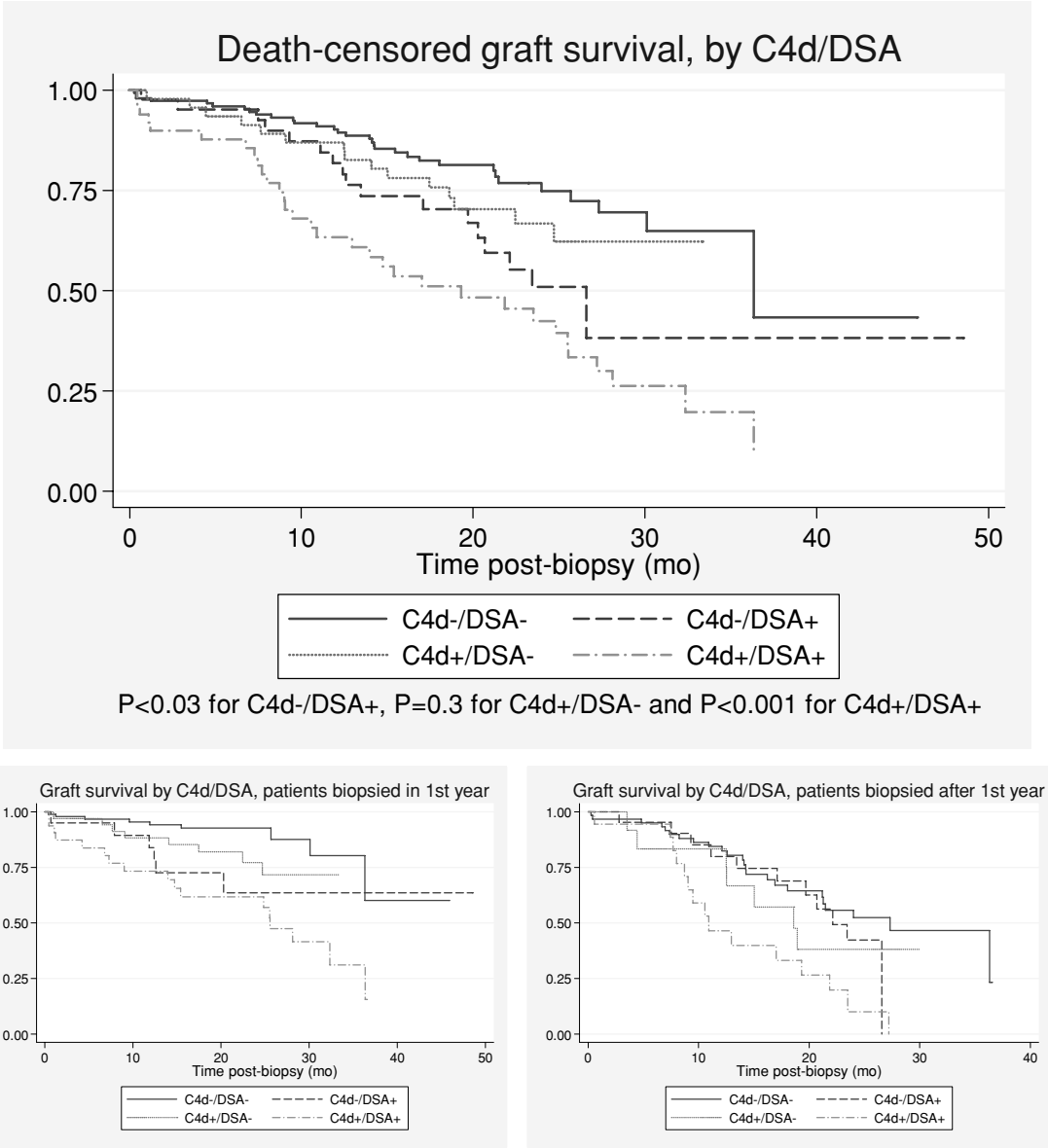

Figure 3: Graft survival estimates after biopsy for the subgroups defined by C4d/DSA together, overall and by time of biopsy.

and function, some did not confirm this association (10–19). Similar variability also exists regarding the pattern of C4d positivity and its impact on graft function or survival. Using IH, the reported prevalence for focal and diffuse staining varies between 8.5–24% and 12.2–42%, respectively (20,20–24). While David-Neto et al. (20) found comparable association with graft survival for the two, Poduval et al. (23) reported worse graft survival only with diffuse pattern. Nickeleit et al. (17) observed 12% focal and 18% diffuse C4d staining by IF, with no differences regarding histology and graft function. Kedainis et al. (25) reported focal staining in 20.9% and diffuse C4d in 9.5% of indication biopsies, with significantly worse graft survival for diffuse C4d compared to C4d-negativity. Although the focal C4d group had intermediate graft survival rates, the differences with the other two were not significant.

The other important finding in this study was the high prevalence of detectable DSA in patients who required indication graft biopsy. Close to one-third of these recipients were found to have DSA, 19.5% with class I and 22.9% with class II antibodies, 11% had both. This is in contrast with much lower prevalence among general transplant recipient population. In a longitudinal study of 1229 patients with yearly testing for up to 5 years, 5.5% had DSA (26), associated with worse graft survival and

Table 3: The unadjusted and adjusted association between graft survival and subgroups defined by C4d pattern, DSA and C4d/DSA conjointly

	Unadjusted HR	95% CI	p-Value	Adjusted HR	95% CI	p-Value
C4d < 10% PTC (N = 197)	1	–	–	1	–	–
C4d 10–50% PTC (N = 55)	1.99	1.2–3.2	0.005	2.3[1]	1.4–3.7	0.001
C4d > 10% PTC (N = 45)	1.77	1.1–3.0	<0.03	1.9[1]	1.2–3.3	<0.02
No DSA (N = 204)	1	–	–	1	–	–
DSA Class I (N = 25)	1.2	0.5–2.6	0.63	1.2[2]	0.5–2.7	0.67
DSA Class II (N = 35)	3.0	1.8–5.0	<0.001	2.5[2]	1.4–4.3	0.001
DSA Class I & II (N = 33)	3.2	1.9–5.4	<0.001	2.7[2]	1.6–4.7	<0.001
C4d−/DSA− (N = 124)	1	–	–	1	–	–
C4d−/DSA+ (N = 39)	2.1	1.1–3.9	<0.03	1.6[3]	0.9–3.2	0.13
C4d+/DSA− (N = 49)	1.3	0.7–2.4	0.34	1.7[3]	0.9–3.2	0.12
C4d+/DSA+ (N = 50)	3.1	1.8–5.3	<0.001	3.2[3]	1.8–5.6	<0.001

[1]Adjusted for time of biopsy and ACR, arteriolar hyalinosis and interstitial fibrosis/tubular atrophy in the biopsy.
[2]Adjusted for time of sampling, gender, race, retransplantation and ACR.
[3]Adjusted for time of biopsy, retransplantation and ACR in the biopsy. With further adjustment for arteriolar hyalinosis and interstitial fibrosis/tubular atrophy, only C4d+/DSA+ status was associated with graft failure.
Groups were comparable regarding the other variables listed in Tables 1 and 2 and in Figure 1.

function. In another study in 54 patients DSA was detected in 15, 13 with graft failure during study period compared to four failures among 22 HLA-antibody-negative patients (27). In a cross-sectional study of 249 patients, Cardarelli et al. (28) found DSA in 4.4%. In contrast, Everly et al. (29) reported that 31% of 52 patients with AR tested positive for DSA, which was an independent risk factor for graft failure. Furthermore, Bohmig et al. (10) did not find statistically significance difference in 12-month graft function between those with positive vs. negative FCXM at the time of biopsy.

In our cohort, the prevalence of DSA during first year was lower (27.4%) than those screened later (36.7%). The prevalence of class I DSA during this period was higher than class II antibody; in contrast, class II DSA was found more often after the first year. Interestingly, in the entire cohort, only the presence of class II DSA was found to be predictive of graft failure after adjusting for a number of clinical variables, including time of screening. However, among those screened during the first year any form of DSA was associated with worse graft survival; although presence of class II DSA was associated with higher risk of graft failure compared with class I (HR: 5.8 vs. 3.2, respectively). These observations are in concert with other reports showing the adverse effects of class II DSA on graft outcomes (30). In this cohort, TG was highly associated with the presence of class II DSA, either alone or with class I antibody, similar to observation reported by another group (31). Although data presented in this report and by others demonstrate the negative impact of DSA on graft outcomes, Bartel et al. (32) have shown that in patients with good, stable graft function, the presence of DSA is not necessarily predictive of worse graft function or survival.

Although great progress has been made in recognizing and treating ACR, it is not clear how to identify all patients with antibody-mediated graft injury and how to treat them. Current guidelines require a set of criteria for diagnosis of AMR (7). However, these criteria may be too strict for diagnosis, hence for attempting in its treatment. In this study, we examined the concordance between C4d positivity and the presence of DSA, in addition to conjoint predictive value of the two for graft survival. We observed a significant discordance between these two essential components of the diagnostic criteria. Among patients with negative or minimal C4d staining, 23.9% had DSA, while among those with focal and diffuse C4d staining in biopsy 35.2% and 68.9% did have identifiable DSA. One could speculate that the titer of DSA, the stage of its development, and its properties, in addition to our current technical limitations may account for the observed discrepancies. This observation suggests that reliance on the recommended guideline will exclude significant number of patients with ongoing graft injury who do not fulfill all the criteria. The impact of these discrepancies on the outcomes became clear by showing that focal PTC C4d staining had negative predictive value for graft survival similar to diffuse staining and the fact that presence of DSA, alone or with C4d positivity similarly affected graft survival in patients biopsied during the first year. Among patients who underwent biopsy during the first-year, despite adjusting for clinical and histological variables, DSA positivity was associated with almost 4–5-fold higher risk of graft failure regardless of the C4d pattern. In contrast, C4d positivity without DSA was not a poor prognostic factor. Similarly, TG was associated with the presence of DSA irrespective of C4d positivity, while for diagnosis of chronic AMR evidenced by TG, diffuse PTC C4d staining is a requirement (1,2). Mauiyyedi et al. (14) found that diffuse C4d by IF was associated with DSA in 90% of cases, and the pathology of C4d+ biopsies was not different in the presence or absence of DSA. In the study by Kayler et al. (21), DSA were detected in 92% with diffuse, 44% with focal and 26% with negative C4d. In the study by Worthington et al. (24) only 5.4%

of patients had DSA, all with diffuse C4d staining in the biopsy. Among 132 patients presented by Kedainis et al. (25) the percentages of DSA positivity with diffuse, focal and negative C4d staining in biopsies were 79.3, 68.8 and 9.9, respectively. The potential reasons for the discrepancy between PTC C4d deposition in biopsy and detectable DSA include low titer DSA, non-antibody-mediated complement activation, noncomplement fixing antibody, HLA DSA that is not routinely tested in the available antigen panels, like HLA-DP, and non-HLA antigens (33–37). Moreover, non-DSA HLA antibodies directed at shared epitopes may explain some DSA-negative/C4d positivity in biopsy (38, 39). With increased sensitivity of the detection methods and inclusion of nontraditional target antigens in the panels, we could improve the chance of detection of DSA, if present.

We acknowledge the limitations of this study. It was a retrospective evaluation and carries the associated shortcomings. We did not account for all confounding factors, an important one being the dose or blood level of maintenance immunosuppressive agents during the study period. However, we tried to include major clinical and histological variables that could confound the results.

In summary, we have shown that focal PTC C4d deposition has similar impact on graft survival as diffuse pattern when IH is used. Therefore, we suggest that the two should be treated the same for diagnosis of AMR. Furthermore, we found a high prevalence of DSA positivity among renal transplant recipients requiring indication graft biopsy. This study showed that during the first-year posttransplant either class I or class II DSA is associated with worse graft survival; although class II DSA was a worse prognostic factor. After the first year, only class II DSA is associated with poor graft survival. Another important observation in this investigation was the significant discrepancy between C4d staining in biopsy and associated DSA status. Notably, for patients biopsied during the first-year presence of DSA, regardless of C4d status was an independent predictor of worse graft survival. In contrast, for those biopsied later, only patients with both C4d and DSA positivity experienced worse outcome. Further studies are needed to better define the diagnostic criteria for acute and chronic AMR, with consideration of the time posttransplant.

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