ORIGINAL ARTICLE

# Clinical utility of C3d binding donor-specific anti-human leukocyte antigen antibody detection by single antigen beads after kidney transplantation—a retrospective study

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## SUMMARY

Development of donor-specific antibodies (DSA) after renal transplantation is known to be associated with worse graft survival, yet determining which specificities in which recipients are the most deleterious remains under investigation. This study evaluated the relationship of the complement binding capacity of post-transplant *de novo* anti-human leukocyte antigen (HLA) antibodies with subsequent clinical outcome. Stored sera from 265 recipients previously identified as having *de novo* DSA were retested for DSA and their C3d binding capacity using Luminex-based solid-phase assays. Most recipients had anti-HLA class II-reactive DSA (class I = 12.5%, class II = 68.7%, class I and class II = 18.9%). The recipients that had C3d binding DSA (67.5%) had a significantly higher incidence of antibody-mediated rejection and any rejection. They also had significantly lower kidney survival, with the lowest survival in those that had both anti-HLA class I and class II C3d binding DSA. Concurrent biopsy comparison revealed a 96.2% positive predictive value and 47.4% negative predictive value for C4d peritubular capillary (Ptc) deposition. Anti-HLA class I and class II C3d binding DSA carried a twofold and 1.5-fold increased risk of kidney loss, respectively, in multivariate analysis.

*Transplant International* 2018; 31: 424–435

**Key words**
alloantibody, antibody biology, biopsy, graft survival, risk assessment/risk stratification

Received: 18 April 2017; Revision requested: 20 May 2017; Accepted: 13 December 2017;
Published Online 04 January 2018

## Introduction

Post-transplant development of donor-specific antibodies (DSA) has been shown to be associated with significantly worse graft survival after renal transplantation [1–5]. However, there appears to be some heterogeneity in DSA pathologic potential (reviewed in Ref [6.]). Studies indicate that complement activation is important for graft destruction, especially in the setting of acute antibody-mediated rejection (AMR) [7]. The ability to distinguish DSA complement activation by detecting vascular endothelial C4d deposition within biopsy specimens has enhanced the ability to diagnose acute and chronic AMR [8,9] as well as aided in graft survival prognostication in some [10,11], but not all [12,13] studies. Thus, there is renewed interest in utilizing *ex vivo* assays that determine the presence of circulating DSA and their




doi:10.1111/tri.13106

complement activating capacity for post-transplant immune monitoring to elucidate AMR severity and prognosis [11,14–29].

Early flow cytometry-based studies using human leukocyte antigen (HLA) molecule-coated beads demonstrated the ability to detect IgG-dependent bead deposition of various immunoglobulin-driven complement fragments [26]. With the relatively recent availability of more sensitive solid-phase technologies, Luminex-based assays to detect the presence of DSA and DSA complement activating capacity via C4d [18,30], C1q [15,16,18–24] or C3d [15,17] binding have been developed and reported.

Detection of C4d and C1q binding anti-HLA antibodies in pre- and post-transplant sera has been correlated with a higher AMR risk and worse graft survival [19,22,31]. Antidonor HLA antibody C3d binding at the time of AMR diagnosis has been shown to identify recipients with a significantly poorer graft survival [15,17]. Comparison with a C1q binding assay showed the C3d binding assay to be more powerful at identifying recipients developing graft loss at 1 and 3 years post-AMR [15,17], outperforming biopsy C4d detection in one study [17].

In this study, we examined the relationship between *in vitro* DSA C3d binding capacity, biopsy C4d detection, and kidney graft outcome following post-transplant *de novo* DSA development.

## Methods

### Patient Sera

Since 2006 we have been testing kidney and kidney/pancreas transplant recipients post-transplant for the presence of circulating DSA either as part of routine screening or as part of the workup for allograft dysfunction. Review of our DSA database identified 265 patients of 2760 transplanted between 1/2002 and 11/2013 that had both a previously identified post-transplant DSA and stored sera available to reanalyze for this study. All patients received an ABO-compatible transplant with a negative T- and B-cell flow cytometric cross-match. All recipients in this study had their immediately pretransplant sera reanalyzed by Luminex single antigen beads, and none were not found to have anti-HLA IgG DSA. Patient demographics and transplant characteristics are summarized in Table 1. All recipients received thymoglobulin induction, and steroid-sparing maintenance immunosuppression consisting of rapamune and microemulsion cyclosporine. In 112 of 265 (42.3%) of cases, the available reanalyzed sera were procured within 30 days of initial DSA detection, in 82 of 265 of cases (30.9%) between 30 and 365 days, and in 71 of 265 of cases (26.8%) >365 days.

The use of the clinical data in this study was approved by our local institutional review board, protocol #2017H0111.

### AMR treatment

Acute AMR episodes were treated with steroids, plasmapheresis (PP) (three consecutive days followed by three times a week for a minimum of 1 week (one volume exchange), and IVIG (1 g/kg) after each PP. If the biopsy revealed a mixed cellular and antibody-mediated rejection, the patient was initially treated with steroids and antithymocyte globulin × 7 days followed by PP and IVIG. If the biopsy revealed chronic AMR without acute AMR, the patients were treated with steroids alone.

### Qualitative Anti-HLA antibody screening

Solid-phase multiplex testing (FlowPRA) was performed using a flow cytometer to determine the presence of alloantibody according to the manufacturer's recommendations (One Lambda, Canoga Park, CA, USA). Briefly, fluorescent microbeads coated with either HLA class I molecules or HLA class II molecules were mixed with patient sera. Bound antibody was detected with a PE-conjugated anti-human IgG using a Coulter Epics2 flow cytometer. A negative serum control was used to establish background cutoffs, and a serum was considered positive if it was 6% above the cutoff and exhibited peak architecture consistent with alloantibody binding.

### DSA identification

Sera which screened positive for alloantibody by qualitative anti-HLA antibody screening were subsequently tested for donor-specific antibody (DSA) with multiantigen bead and single antigen bead testing using test kits for either class I or class II antigens, according to the manufacturer's recommendations (Gen-Probe, currently Immucor, Stamford, CT, USA). The mean fluorescent intensity (MFI) and identification of discrete DSA specificities were determined on a Luminex 100 reader. Beads with MFI values above 2000 were considered indicative of antibody presence.

**Table 1.** Demographics and transplant characteristics for 265 recipients

| | |
|---|---|
| Age in years (mean ± SD) | 45.1 ± 12.1 |
| % AA recipient (*n*) | 35% (94) |
| % Recipient male gender (*n*) | 60.8% (161) |
| % Recipient-sensitized pretransplant (*n*)* | 32.8% (87) |
| % Retransplant (*n*) | 12.1% (32) |
| Transplant type (kidney/kidney and pancreas) | 233/29 |
| % Recipient diabetes (*n*) | 31.3% (83) |
| Recipient BMI (mean ± SD) | 29.5 ± 6.7 |
| % Delayed graft function (*n*) | 3.8% (10) |
| % Donor AA race (*n*) | 14.7% (39) |
| % Donor male gender (*n*) | 47.5% (126) |
| Donor age in years (mean ± SD) | 38.5 ± 13.9 |
| Donor BMI (mean ± SD) | 27.8 ± 7.3 |
| CIT in hours (deceased donors only, mean ± SD) | 10.8 ± 4.6 |
| % Biopsy-proven acute rejection (*n*) | 55.1% (146) |
| Months to 1st acute rejection (median, mean ± SD, range) | 14.0, 21.8 ± 23.2, 0.03–99.9 |
| % Death-censored kidney loss (*n*) | 46.4% (123) |
| % Death-censored pancreas loss (*n*/total) | 24.1% (7/29) |
| % Death | 15.5% (41) |
| Years follow-up time (median, CI†) | 9.3 (8.3–9.8) |
| Months to first anti-HLA IgG DSA (median, mean ± SD, range) | 17.5, 26.5 ± 26.9, 0.1–122 |
| Highest DSA MFI (mean ± SD) | 11 627 ± 6646 |

SD, standard deviation; AA, African American; CIT, cold ischemia time; DSA, donor-specific antibody; MFI, mean fluorescence intensity.

*sensitized—defined as cPRA > 0.

†CI—95% confidence interval, median and CI by reverse Kaplan–Meier method.

### DSA C3d binding assay

A single stored serum sample previously verified to possess DSA was selected for each patient. The presence of DSA was reverified by single antigen bead testing as above and also assayed using a commercially available kit which detects C3d binding DSA to single antigen beads on a Luminex platform according to the manufacturer's protocol. (Lifecodes C3d assay; Immucor, Stamford, CT, USA).

### Biopsies

C4d staining was performed using indirect immunofluorescence (on frozen sections). Briefly, frozen sections were cut and air-dried for 30 min. All slides were fixed in ice-cold acetone for 10 min and again air-dried for 10 min. Following PBS rinse, Quidel anti-C4d monoclonal antibody (Quidel Corporation, cat no. A213, San Diego, CA, USA) was applied for 1 h at room temperature (1:40 dilution). Next FITC-labeled goat anti-mouse immunoglobulin G secondary antibody (Beckman Coulter, Marseille, France, cat no. PN IM0819) was applied at room temperature (1:50 dilution). Immunofluorescence was examined using ultraviolet light microscopy. Most recipients (229/265, 86.4%) had a least one biopsy either prior to ($n$ = 87), or at the time of ($n$ = 142), the study sera analyzed. Peritubular capillaritis and C4d staining were scored using the Banff 2015 criteria [32].

### Statistical analyses

Student's t test was used to compare means between groups for continuous variables (donor and recipient age and BMI, cold ischemia time, time to acute rejection and DSA detection, follow-up time, and DSA MFI). Pearson's chi-squared test was used for statistical comparison of proportions between groups (all other dichotomous variables). Kaplan–Meier survival curves were compared by log-rank test**.** Survival time for Cox regression analysis was calculated as the time from the first serum collection date with detectable anti-HLA IgG DSA to the date of graft removal or return to dialysis. In 40% of cases (105/265), the collection date of serum used for repeat anti-HLA IgG DSA plus C3d testing and first anti-HLA IgG DSA detection were the same. For 26% of cases (69/265), the serum used for repeat anti-HLA IgG DSA plus C3d testing was collected >1 year after the first anti-HLA IgG DSA detection date, and for the remaining 34% of cases (91/265), the serum collection dates used for repeat anti-HLA IgG DSA plus C3d testing and first anti-HLA IgG DSA detection were <1 year apart. Recipient age at the time of transplant was used for all analyses and used as a fixed value. All post-transplant events (acute rejection, antibody-mediated rejection, biopsy results) were determined prior to, or at the time of, serum testing for C3d binding and treated as non-time-dependent variables. All statistical analyses were performed using IBM SPSS version 21.0.0 statistical software (Armonk, NY, USA) except reverse Kaplan–Meier method for follow-up time performed using R statistical programming. A Cox regression model for renal allograft loss was used where indicated.



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## Results

### DSA single antigen bead (SAb) IgG and C3d Binding

The results of the DSA C3d binding assay are summarized in Table 2. The post-transplant time of sera testing ranged from 8 days to 10 years, with a median of nearly 3 years. The majority of the recipients had only HLA class II-reactive IgG DSA (68.7%, $n = 182$), 12.5% ($n = 33$) had only HLA class I-reactive IgG, and 18.9% ($n = 50$) had both HLA class I- and class II-reactive IgG. Thus, there were a total of 83 recipients with HLA class I-reactive IgG DSA and 232 with HLA class II-reactive IgG DSA. Overall 179 of 265 of recipients had DSAs that bound C3d. The HLA class I-reactive DSA bound C3d in the C3d binding assay in 49.4% (41/83) of cases, whereas the HLA class II-reactive DSA bound C3d in the C3d binding assay in 70.3% (163/232) of cases.

### Clinical presentation and outcome versus DSA class specificity

Recipients in this study who had anti-HLA class I with or without class II DSA were more likely to have a previous or concurrent biopsy-proven acute cellular rejection (AR) ($P < 0.001$) and AMR accompanied or not with AR ($P = 0.001$). This likely explains their higher rate of renal allograft loss (66.3% vs. 37.4%, $P < 0.001$) and the lower estimated glomerular filtration rate (eGRF, calculated using the Modification of Diet in Renal Disease Study equation) at the time of initial DSA identification ($23.6 \pm 17.5$ vs. $31.5 \pm 19.1$, $P = 0.002$) (Table 3). Consistent with the above findings, comparison of death-censored renal allograft 5-year survival stratified by HLA class specificity of DSA (HLA class I versus HLA class II versus HLA class I and class II) demonstrates a significantly lower survival when HLA class I DSA is present (Fig. 1).

### Analysis of DSA IgG MFI stratified by C3d binding capacity

The DSA IgG specificity with the highest MFI value was compared between C3d binding and nonbinding groups stratified by HLA class specificity. The mean of the highest IgG specificity MFI was significantly higher for cases where the IgG binds C3d for all HLA class specificity groups ($P < 0.001$, Table 4).

### Patient characteristics stratified by C3d binding

Recipients were stratified by the presence of HLA class I and/or class II C3d binding, and their demographics and clinical characteristics were compared to the C3d-negative cohort (Table 5). In general, the recipients with C3d binding DSA were younger, and had a significantly higher incidence of AR, AMR, and kidney loss. The recipients with HLA class II binding DSA, with or without HLA class I binding DSA, had a longer mean time post-transplant to initial DSA detection and median post-transplant follow-up time.

### Clinical outcome stratified by DSA C3d binding

For the entire recipient cohort, the renal allograft 5-year survival was significantly lower when DSA binds C3d ($P = 0.003$) (Fig. 2a). Even when analyzing recipients with lower DSA IgG MFIs (<7000), renal allograft 5-year survival was significantly lower when DSA bound C3d (Fig. 2b). When stratifying cases by HLA specificity of C3d DSA binding (class I, class II, or both class I and II), the lowest 5-year graft survival is when both anti-HLA class I and class II DSAs bind C3d (Fig. 3).

**Table 2.** Recipient IgG and C3d donor HLA binding results of 265 DSA-positive recipients

| DSA HLA class specificity | IgG positive (MFI mean ± SD) | Class I C3d binding | Class II C3d binding | Any 2C3d binding |
|---|---|---|---|---|
| Anti-HLA class I | 12.5% 33/265 (6253 ± 4626) | 39.4% 13/33 | NA | 39.4% 13/33 |
| Anti-HLA class II | 68.7% 182/265 (11 662 ± 6581) | NA | 65.9% 120/182 | 65.9% 120/182 |
| Anti-HLA class I & Anti-HLA class II | 18.9% 50/265 (Class I: 7635 ± 5427) (Class II: 14 513 ± 6172) | 56.0% 28/50 | 86.0% 43/50 | 92.0% 46/50 |
| Total | 100% 265/265 | 49.4% 41/83 | 70.3% 163/232 | 67.5% 179/265 |

DSA, donor-specific antibody; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; SD, standard deviation; NA, not applicable.

**Table 3.** Demographics and transplant characteristics for 265 recipients stratified by HLA class specificity

| | Anti-HLA Class I ± HLA Class II (*n* = 83) | Anti-HLA Class II only (*n* = 182) | Signif |
|---|---|---|---|
| Age in years (mean ± SD) | 44.6 ± 11.6 | 45.3 ± 12.3 | ns |
| % AA race (*n*) | 36.1% (30) | 35.2% (64) | ns |
| % Male gender (*n*) | 54.2% (45) | 63.7% (116) | ns |
| % sensitized pretransplant (*n*) | 36.8% (32) | 30.2% (55) | ns |
| % Retransplant (*n*) | 7.2% (6) | 14.3% (26) | ns |
| Tx type (kidney/kidney and pancreas) | 74/9 | 162/20 | ns |
| % Recipient diabetes (*n*) | 30.1% (25) | 31.9% (58) | ns |
| Recipient BMI (mean ± SD) | 29.3 ± 7.7 | 29.6 ± 6.2 | ns |
| % Delayed graft function (*n*) | 4.8% (4) | 3.3% (6) | ns |
| % Donor AA race (*n*) | 18.1% (15) | 13.2% 24) | ns |
| % Donor male gender (*n*) | 47.0% (39) | 47.8% (87) | ns |
| Donor age in years (mean ± SD) | 37.1 ± 13.5 | 39.1 ± 14.0 | ns |
| Donor BMI (mean ± SD) | 27.2 ± 6.6 | 28.1 ± 7.6 | ns |
| CIT in hours (deceased donors only, mean ± SD) | 9.7 ± 3.7 | 11.3 ± 5.0 | 0.039 |
| % Biopsy-proven AR (*n*) | 73.5% (61) | 49.5% (90) | <0.001 |
| % Biopsy-proven AMR ± AR (*n*) | 36.1% (30) | 17.0% (31) | 0.001 |
| Months to 1st AR (mean ± SD) | 21.2 ± 25.0 | 22.2 ± 22.1 | ns |
| 5-year death-censored kidney survival | 34.3% | 63.6% | <0.001 |
| 5-year death-censored pancreas survival | 88.9% | 83.3% | ns |
| 5-year patient survival | 85.1% | 84.9% | ns |
| Years follow-up time (median, CI) | 11.8, 9.5-NC | 8.4, 7.8–11.2 | ns |
| Months to DSA (mean ± SD) | 25.0 ± 26.7 | 27.2 ± 27.0 | ns |
| eGFR at DSA start (mean ± SD) | 23.6 ± 17.5 | 31.5 ± 19.1 | 0.002 |

SD, standard deviation; AA, African American; Tx, transplant; BMI, body mass index; CIT, cold ischemia time; AR, acute rejection; AMR, antibody-mediated rejection; CI, 95% confidence interval, median, and CI by reverse Kaplan–Meier method; NC, not computed; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate by Modification of Diet in Renal Disease calculation; anti-HLA C1, anti-human leukocyte antigen class I; anti-HLA C2, anti-human leukocyte antigen class II; Signif, significance.

### Correlation with biopsy microvascular inflammation and peritubular capillary (Ptc) C4d detection

In 142 of 265 (54%) of cases, a renal biopsy was obtained at the time of analyzed sera acquisition (designated synchronous biopsies). In another 87 cases, a biopsy had been obtained remote from the time of sera acquisition (designated nonsynchronous biopsies) for a total of 229 biopsies for comparison (86% of the entire cohort). DSA C3d binding results were compared to biopsy ptc C4d detection for synchronous and nonsynchronous biopsies (Table 6). In general, there was good correlation between the two methods for DSA complement binding, with the synchronous biopsies demonstrating superior correlation compared to the nonsynchronous biopsies (83.1% vs. 65.5% concordance). The sensitivity, specificity, PPV, and NPV were 83.3%, 81.8%, 96.2%, and 47.4%, respectively, when comparing the synchronous biopsies, and 69.8%, 54.2%, 80.0%, and 40.6%, respectively, when comparing nonsynchronous biopsies. From the comparison of the biopsy C4d severity score between C3d binding and nonbinding DSAs (0 = no ptc staining, 1 = <10% ptc staining, 2 = 10–50% ptc staining, 3 = >50% ptc staining), we found a statistically higher percentage of grade 3 scores in the DSA binding recipients for synchronous (82.7% vs 31.6%, $P < 0.001$) and nonsynchronous (63.2% vs 40.6%, $P = 0.037$) biopsies (Fig. 4a). Comparison of the microvascular inflammation score (0 vs. >0) between C3d binding and nonbinding DSAs demonstrated a correlation for both synchronous and nonsynchronous biopsies (Fig. 4b).

### DSA C3d Binding and Risk of Renal Allograft Loss

The relative risk of renal allograft loss was assessed by Cox regression analyses. We first investigated a model that included all patients in the cohort (which required excluding biopsy findings from the model). A history of treated rejection (cellular and/or antibody mediated) and HLA class I and anti-HLA class II C3d binding DSA were significant, independent risk factors for graft

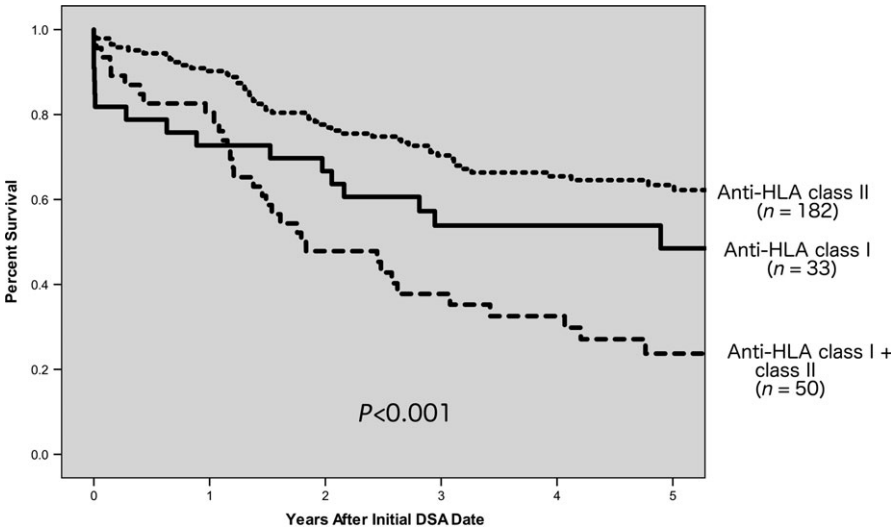



**Figure 1** Comparison of Kaplan–Meier death-censored renal allograft survival curves stratified by DSA HLA class specificity (anti-HLA class I, anti-HLA class II, or both anti-HLA class I and class II) ($P < 0.001$ by log-rank test).

**Table 4.** Comparison of DSA HLA IgG MFI stratified by C3d binding capacity

| DSA HLA class specificity | All recipients† | No C3d binding | C3d binding |
|---|---|---|---|
| Anti-HLA class I | 7086 ± 5140 | 3822 ± 2573 | 10 429 ± 4973* |
| Anti-HLA class II | 12 282 ± 6588 | 5604 ± 3640 | 15 027 ± 5470* |

DSA, donor-specific antibody, HLA, human leukocyte antigen.

†Mean ± standard deviation highest specificity MFI value.

*$P < 0.001$.

loss as well as recipient male gender (Table 7). Analyses of the biopsy subgroup of recipients (229/265) allowed inclusion of biopsy C4d detection as a variable. Again, a history of treated rejection (cellular and/or antibody mediated) and HLA class I C3d binding DSA were significant risk factors for graft loss. Younger recipient age also was a significant risk factor; however, neither anti-HLA class II C3d binding DSA nor biopsy ptc C4d detection reached statistical significance as risk factors for renal allograft loss.

## Discussion

We retrospectively reanalyzed sera from 265 kidney and kidney/pancreas transplant recipients identified as having developed *de novo* DSA post-transplant. The majority (68.7%) of recipients had anti-HLA class II-reactive DSA only, with 18.9% having both anti-HLA class I and class II reactivity and 12.5% only anti-HLA class I reactivity (Table 2). This *de novo* DSA HLA class reactivity distribution is similar to what we [33] and others have previously reported [17,34]. However, not all reports have noted a preponderance of anti-HLA class II reactivity [22,28,35]. When we stratified our recipient cohort using the presence of only anti-HLA class II DSA, we noted that these patients were more likely to present with an indolent, chronic active form of AMR (described in [6]) as evidenced by less biopsy-confirmed AR and AMR, better renal function at the time of DSA detection, less graft loss, and a better death-censored renal graft survival during the follow-up observation period (Table 3 and Fig. 1).

Overall, we found that in 67.5% of cases (179/265), recipient sera contained at least one DSA specificity that bound C3d in the C3d binding assay. This is similar to the 58% previously reported in 69 DSA-positive patients with AMR [17] but significantly higher than the 23% noted in 39 pediatric kidney recipients with *de novo* DSA reported by Comoli et al. [15] using the same assay. However, most patients in that study had HLA class I-reactive DSA. Only 39% of HLA class I-reactive DSAs bound C3d in our study, much closer to the 23% reported by Comoli et al.

The deleterious nature of complement binding DSA is highlighted by the lower 5-year graft survival in this study for recipients with C3d binding versus nonbinding DSA. This was found to be true even when comparing only the lower DSA IgG MFI recipients with

**Table 5.** Demographics and transplant characteristics for 265 recipients stratified by DSA C3d binding

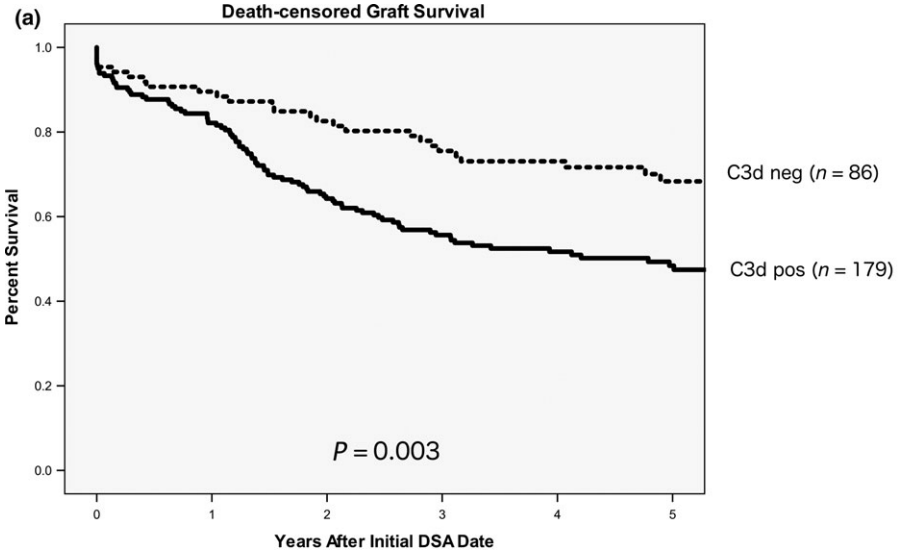
| | No C3d Binding (*n* = 86) | C3d Binding (*n* = 179) | *P*-value |
|---|---|---|---|
| Age in years (mean ± SD) | 48.8 ± 12.1 | 43.3 ± 11.7 | <0.001 |
| % AA race (*n*) | 37.2% (32) | 34.6% (62) | ns |
| % Male gender (*n*) | 55.8% (48) | 63.1% (113) | ns |
| % sensitized pre-tx (*n*) | 34.9% (30) | 31.8% (57) | ns |
| % Retransplant (*n*) | 15.1% (13) | 10.6% (19) | ns |
| Tx type (kidney/kidney and pancreas) | 78/8 | 158/21 | ns |
| % Recipient diabetes (*n*) | 34.9% (30) | 29.6% (53) | ns |
| Recipient BMI (mean ± SD) | 30.0 ± 6.3 | 29.3 ± 6.8 | ns |
| % Delayed graft function (*n*) | 4.7% (4) | 3.4% (6) | ns |
| % Donor AA race (*n*) | 16.3% (14) | 14.0% (25) | ns |
| % Donor male gender (*n*) | 47.7% (41) | 47.5% (85) | ns |
| Donor age in years (mean ± SD) | 39.3 ± 13.5 | 38.1 ± 14.0 | ns |
| Donor BMI (mean ± SD) | 27.1 ± 6.2 | 28.2 ± 7.7 | ns |
| CIT in hours (deceased donors only, mean ± SD) | 10.9 ± 5.0 | 10.7 ± 4.5 | ns |
| % Biopsy-proven AR (*n*) | 39.5% (34) | 65.4% (117) | <0.001 |
| % Biopsy-proven AMR ± AR (*n*) | 16.3% (14) | 26.3% (47) | 0.07 |
| Months to 1$^{st}$ AR (mean ± SD) | 21.4 ± 23.7 | 21.9 ± 23.2 | ns |
| 5-year death-censored kidney survival | 67.7% | 47.9% | 0.001 |
| 5-year death-censored pancreas survival (n/total) | 85.7% (2/8) | 85.0% (5/21) | ns |
| 5-year patient survival | 84.3% | 86.0% | ns |
| Years follow-up time (median, CI) | 7.2 (6.7–7.9) | 10.2 (9.5–11.3) | |
| Months to DSA (mean ± SD) | 18.9 ± 21.7 | 30.2 ± 28.4 | <0.001 |
| eGFR at DSA start (mean ± SD) | 31.5 ± 20.9 | 27.8 ± 17.8 | 0.13 |
| Highest DSA MFI (mean ± SD) | 5035 ± 3338 | 14 712 ± 5449 | <0.001 |

SD, standard deviation; AA, African American; tx, transplant; BMI, body mass index; CIT, cold ischemia time; AR, acute rejection; AMR, antibody-mediated rejection; CI, 95% confidence interval, median, and CI by reverse Kaplan–Meier method; DSA, donor-specific antibody; GFR, glomerular filtration rate; MFI, mean fluorescence intensity.

presumably lower alloantibody titers (Fig. 2b). The lowest 5-year survival in our study was seen in recipients that have both anti-HLA class I and II C3d binding DSA, suggesting an additive effect (Fig. 3). The role of alloantibody and complement fixation in tissue damage of renal transplants is incompletely understood. Anti-HLA antibodies may bind to the allograft causing complement-independent damage [36,37] or, via complement fixation, resulting in formation of cell-destructive membrane attack complexes and release of chemoattractants that invite additional immune cell-mediated tissue damage (reviewed in Ref. 7,36). The ability to mitigate against tissue damage in acute AMR by blocking C5 cleavage with eculizumab [38] clearly demonstrates the role of complement in that setting. The pathogenic role of complement fixation in indolent or chronic AMR remains to be defined.

Comparison of the IgG DSA MFIs between the C3d binding and nonbinding recipient sera demonstrated a significantly higher mean MFI for both HLA class I- and class II-reactive DSAs (Table 4). Previous studies have shown a similar relationship between higher IgG MFIs and either C3d [17] or C1q [16,22] binding capacity. This relationship seems logical as higher MFIs correlate with higher antibody titers, allowing for a higher density of cell surface bound anti-HLA antibodies and a much greater multivalent C1q binding avidity [39]. However, not all studies have confirmed this relationship [18,21,23]. These conflicting data may in part be due to the prozone effect, where high IgG levels lead to low MFI results that frequently are much higher after serum dilution (reviewed in [14]). This effect was demonstrated to be significant in previous C1q binding studies [40,41]. While we did not control for this effect in our study, it is worth mentioning that, contrary to the C1q assay, the serum for the IgG and C3d Luminex assays is subjected to a 1:4 dilution greatly diminishing the possibility of prozone. In addition, we noted a good DSA IgG MFI and C3d binding correlation, thus obviating a significant prozone effect influencing our results.

In 86.4% (229/265) of recipients, a kidney biopsy was available to compare evidence of DSA *in vitro* (C3d binding) with *in vivo* (C4d deposition) complement fixation. Due to the possibility that C4d deposition may



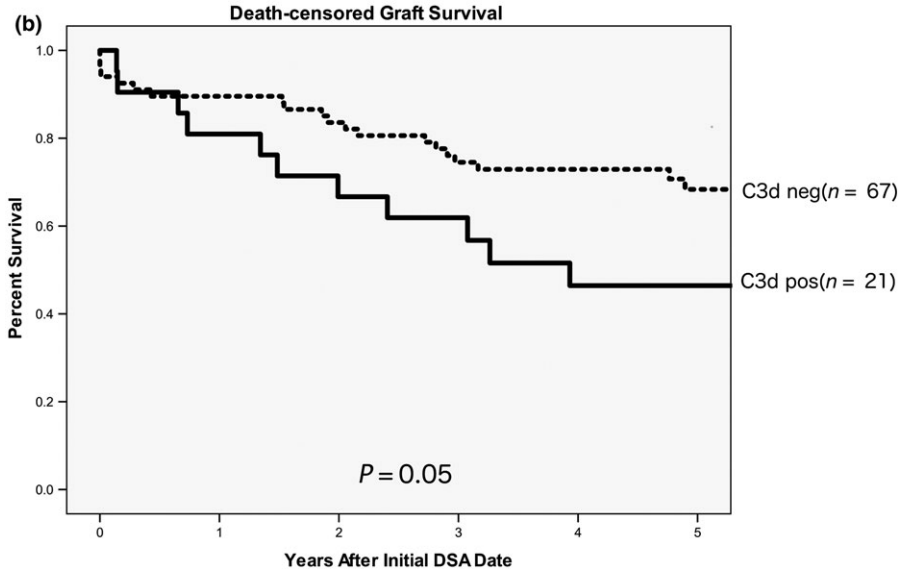




**Figure 2** Comparison of Kaplan–Meier death-censored renal allograft survival curves stratified by C3d binding status for the entire study cohort (a) and for recipients with DSA IgG MFI < 7000 (b). Statistical comparison by log-rank test.

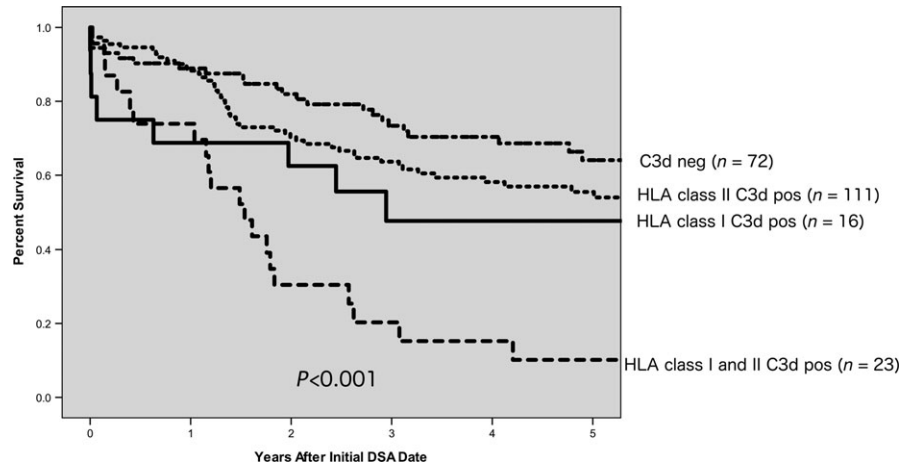



**Figure 3** Comparison of Kaplan–Meier death-censored renal allograft survival curves stratified by HLA specificity of C3d binding DSA. Statistical comparison by log-rank test.

vary over time [42,43], biopsies were designated as synchronous ($n = 142$) if obtained close to time of the serum acquisition date (arbitrarily defined as within 30 days), and nonsynchronous ($n = 87$) if >30 days from the serum acquisition date. In the majority of nonsynchronous cases, the indication biopsy predated

the serum date (73.6%, 64/87). There was better correlation of assay results for synchronous compared to nonsynchronous biopsies (83.1% vs. 65.5% concordance) with better positive and negative predictive values (Table 6). Various factors may account for differential C3d binding and biopsy C4d results. First, complement binding DSA titers may be low or decrease over time and/or with treatment such that later post-biopsy *in vitro* sera testing fails to detect DSA complement fixation, whereas the previous endothelial C4d deposition may persist in the graft. Second, HLA molecule density and/or conformation on the beads may not reflect their *in vivo* properties. Additionally, complement used for the C3d assay is derived from normal human sera which could differ in level or function from *in vivo* recipient complement. Finally, lack of biopsy C4d detection in the setting of AMR with DSA-related graft injury has been described [34,43,44], which lead to elimination of Ptc C4d detection as a necessary prerequisite for AMR diagnosis by Banff criteria [45]. However, one would generally expect the C3d assay also to be negative in this setting.

**Table 6.** Comparison of DSA C3d binding and biopsy C4d results

| (a) Synchronous | C3d Neg | C3d Pos |
|---|---|---|
| C4d Neg | 12.7% (18) | 2.8% (4) |
| C4d Pos | 14.1% (20) | 70.4% (100) |
| **(b) Nonsynchronous** | **C3d Neg** | **C3d Pos** |
| C4d Neg | 14.9% (13) | 12.6% (11) |
| C4d Pos | 21.8% (19) | 50.6% (44) |

(a) Concordant results: 118/142 = 83.1%; Discordant results: 24/142 = 16.9%; PPV = 96.2%, NPV = 47.4%; Sensitivity = 83.3%, Specificity = 81.8%.

(b) Concordant results: 57/87 = 65.5%; Discordant results: 30/87 = 34.5%; PPV = 80.0%, NPV = 40.6%; Sensitivity = 69.8%, Specificity = 54.2%.

The biopsy C4d severity scores were found to be higher for the C3d binding DSAs compared to the non-binding DSAs suggesting the higher DSA titer [indicated by the higher mean DSA IgG MFI value for the C3d binding DSAs (Table 4)] leads to greater IgG binding that enhances complement C1q binding avidity for both *in vitro* (C3d binding) and *in vivo* (C4d) complement binding.

Both anti-HLA class I and class II C3d binding nearly double the risk of graft loss by Cox regression analyses for the entire recipient cohort. When including only the biopsied cohort, anti-HLA class I, but not class II C3d binding or ptc C4d detection reached significance as a factor predicting graft loss (Table 7). The loss of significance of HLA class II C3d binding DSA for predicting

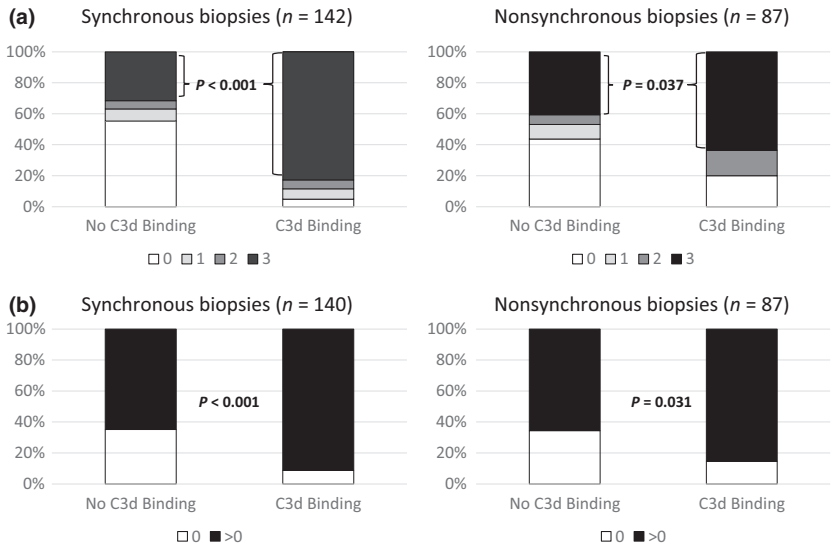



**Figure 4** (a) Comparison of biopsy peritubular capillary (Ptc) C4d staining score for synchronous and nonsynchronous biopsies stratified by DSA C3d binding status. Statistical significance for the difference in the percent of C4d severity score = 3 biopsies between C3d versus non-C3d binding DSA was <0.001 for synchronous biopsies and 0.037 for nonsynchronous biopsies. Severity score: 0 = no ptc staining, 1 = <10% ptc staining, 2 = 10–50% ptc staining, 3 = >50% ptc staining. (b) Comparison of biopsy microvascular inflammation score for synchronous and nonsynchronous biopsies stratified by DSA C3d binding status. Statistical significance for the difference in the percent of microvascular inflammation score >0 biopsies between C3d versus non-C3d binding DSA was <0.001 for synchronous biopsies and 0.031 for nonsynchronous biopsies. Severity score (glomerular + peritubular capillaritis) as per Banff 2013 criteria.

**Table 7.** Independent risk factors for renal allograft loss by Cox regression analysis

| | Cox regression (hazard ratio, confidence interval) | *P*-value |
|---|---|---|
| Entire Study Cohort ($n$ = 265) | | |
| Age (HR per year, CI) | 0.99, 0.97–1.01 | 0.115 |
| HLA class I C3d binding | 2.14, 1.39–3.31 | 0.001 |
| HLA class II C3d binding | 1.54, 1.02–2.33 | 0.039 |
| Rejection history | 3.28, 2.12–5.08 | <0.001 |
| Recipient Gender (Male) | 1.60, 1.09–2.34 | 0.017 |
| Biopsy Group Cohort ($n$ = 229) | | |
| Age (years) | 0.98, 0.97–1.00 | 0.034 |
| HLA class I C3d binding | 1.95, 1.26–3.02 | 0.003 |
| HLA class II C3d binding | 1.68, 0.85–3.31 | 0.102 |
| Rejection history | 2.15, 1.33–3.47 | 0.002 |
| C4d positive biopsy | 1.71, 0.88–3.35 | 0.103 |

Nonsignificant variables in the models include recipient gender, race (African American versus other), antibody-mediated rejection (AMR) history, maximum DSA IgG MFI, DSA HLA specificity regardless of C3d binding capacity (class I, class II, both), transplant type, retransplantation, and microvascular inflammation score (Biopsy Group Cohort only).

graft loss appears to be due to the low incidence of graft loss in the nonbiopsied, non-C3d binding anti-HLA class II cohort (only one of 19 grafts). Without inclusion of this nonbiopsied group with a strong negative prediction of graft loss in the absence of anti-HLA C3d binding in the biopsy cohort, anti-HLA C3d binding loses its statistical significance as a predictor of graft loss. However, as biopsied patients represent a selected subgroup of the entire cohort chosen to compare the influence of C4d biopsy findings versus anti-HLA DSA C3d binding, we feel analyses using the entire cohort are more important regarding the significance of anti-HLA class II C3d binding. These results are similar to those reported by Sicard et al. regarding the hazard ratio of C3d binding DSA for graft loss [17]. Importantly, anti-HLA IgG DSA maximum MFI was not a significant explanatory covariate in any of our multivariable models, indicating that the C3d binding characteristic of DSAs provides prognostic information independent of the IgG MFI value.

Previous studies correlating graft outcome with post-transplant DSA C3d binding versus C1q binding have found a stronger correlation of graft loss with C3d binding suggesting this assay may be superior for predicting graft loss [15,17]. It has been proposed that this is because the C3d binding assay bears a closer relationship to the actual process of DSA-associated complement cascade activation resulting in graft injury, whereas anti-HLA DSA binding to purified C1q molecules in the C1q binding assay may not always predict downstream C3 fragment generation. Regarding the C4d DSA Luminex binding assay, to our knowledge, no comparison of the strength of correlation between the C4d binding assay and either C1q or C3d binding assays with graft outcome has been reported.

In conclusion, identification of *de novo* post-transplant C3d binding DSA identifies those recipients at highest risk of both diffuse ptc C4d deposition on kidney biopsy and subsequent 5-year graft loss. Additionally, the C3d binding assay can identify which DSA specificities bind complement for those recipients with multiple DSA. This information may prove useful for targeting which patients and which DSA to treat following identification as well as recipient counseling regarding renal graft survival prognosis.

## Authorship

RPP: participated in research design, data collection, data analysis and writing of the manuscript. IB: participated in data collection, data analysis and writing of the manuscript. PA: participated in data collection and data analysis. AR: participated in data collection and writing of the manuscript. NRD: participated in data collection and data analysis. MLH: participated in data collection and writing of the manuscript.

## Funding

No funding was obtained for this study.

## Conflict of interest

The authors of this manuscript have conflict of interests to disclose as described by Transplant International. Ivan Balazs was an employee of Immucor, Inc. during the conduction of this study.

## Acknowledgements

We would like to acknowledge Laura Smith and Angela Butler for their help in preparation of this manuscript.

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