original article

Donor–specific HLA-DQ antibodies may contribute to poor graft outcome after heart transplantation

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BACKGROUND: HLA-DQ donor-specific antibodies (DSA) are implicated in allograft dysfunction after renal and lung transplantation. Limited data exists on the impact of HLA-DQ antibodies on heart transplant patients.

OBJECTIVE: To investigate the impact of DSA formation on allograft function and outcomes in heart transplant patients.

DESIGN: Retrospective cohort study.

SETTING: Collating post-transplantation patient data from computerized database in a tertiary hospital in Riyadh, Saudi Arabia from January 2006 to October 2014.

PATIENTS AND METHODS: We excluded recipients with positive preoperative complement-dependent-cytotoxicity crossmatch grafts and those with preformed DSA. Anti-HLA antibodies were identified using Luminex-based assay in sera collected before transplantation with a routine endomyocardial biopsy the first year and then annually.

MAIN OUTCOME MEASURES: Primary outcome measures were all-cause mortality, development of antibody mediated rejection, treated acute cellular rejection (ACR) and cardiac allograft vasculopathy (CAV).

SAMPLE SIZE: 127 patients.

RESULTS: DSA formation occurred in 43/127 (34%), with 33/43 (77%) targeting HLA-DQ antigens alone (n=7) or in combination with -DR, -A or B antibodies (n=26). Most (76%) were male and the mean (SD) age was 36 (14) years. Ten patients developed -A, -B or -DR antibodies without -DQ antibodies also present. Treated ACR (P=.011), reduced left ventricular ejection fraction (P<.001), CAV development (P=.003), and all-cause mortality (P=.01) were all significantly more prevalent in the DSA-positive cohort.

CONCLUSION: HLA-DQ donor-specific antibodies were the most common type detected and may play a significant role in poor outcomes post-cardiac transplantation. This emphasizes the importance of HLA-DQ matching and monitoring for DSA formation in order to minimize post-transplantation immunological risk.

LIMITATIONS: Retrospective design comes with inherent biases, results from single institute, with a particularly young cohort.

CONFLICT OF INTEREST: None.

Heart transplantation remains the treatment of choice, if possible, for patients with end-stage heart disease. Availability of heart donors remains a serious concern and hence, their rarity and value warrants particular importance for clinicians to achieve the greatest possible result with all transplantations.[1] The median survival of paediatric and adult heart transplant recipients is 11 years for all patients, and up to 13 years for those surviving the first year, based upon data collated between 1982 and 2013.[1] The leading causes of death post-transplant are graft failure, infection, multiple organ failure and acute rejection, with cardiac allograft vasculopathy as the 6th commonest cause of death.[1]

There is increasing evidence that allosensitization represents an important factor in heart transplantation. These antibodies can target major histocompatibility (MHC) class I, MHC class II or non-MHC antigens, with sensitizing events that can lead to their production including pregnancy, blood transfusion, infection, prior transplant, prior cardiac surgery with homograft material[2] and insertion of a ventricular assist device as a bridge to transplant.[3] Alloantibodies to human leukocyte antigens (HLAs) in patients awaiting heart transplant are associated with prolonged waiting times for transplantation, increased risk of post-transplant cellular rejection, antibody-mediated rejection and cardiac allograft vasculopathy (CAV), producing a significant effect on mortality.[4,5]

Post-transplantation monitoring of HLA antibodies is an effective tool for predicting long-term graft outcome.[6,7] Although no standardized antibody threshold defining a sensitized patient currently exists, some centers define a sensitized patient to have a panel-reactive antibody (PRA) screen greater than 10%,[6] whereas other centers define it as greater than 25%.[8]

The ability to detect individual anti-HLA antibody types in transplant patients and their relative abundance, in the form of mean fluorescent intensity (MFI), has facilitated the exploration of donor-specific antibodies (DSA) and their impact on graft outcome. The presence of DSA has been found to be a good diagnostic indicator and predictor of antibody-mediated rejection (AMR), CAV and acute allograft dysfunction.[9-11] The aim of this study was to investigate the associations between DSA formation, graft rejection, allograft function and mortality in heart transplant recipients.

PATIENTS AND METHODS

Using our institution's electronic database, we performed a retrospective cohort study and identified patients who underwent cardiac transplantation surgery between January 2006 and October 2014. We included heart transplant recipients operated on during this study period with available pre-HLA typing and panel reactive antibody screening data. Patients were excluded if they had undergone cardiac re-transplantation, had a positive preoperative complement-dependant cytotoxicity (CDC) crossmatch result, had evidence of pre-formed DSA, or died within 1 week of transplantation. Heart transplant donors were expatriates after brain death, and had no relation to their transplant recipients. Ethical approval was provided by our institution's ethical board.

Immunosuppression of patients

Induction therapy consisted of anti-thymocyte globulin (ATG), with an initial dose of 3 mg/kg. Thereafter, the dose was adjusted depending on CD3 level and/or absolute total lymphocyte counts. In most cases, patients received ATG for the first 3 days post-transplantation. Until 2014, almost 98% received corticosteroids as part of their induction therapy for 1 year post-transplantation. Since then, a wean-off process has been introduced that takes place after 6-9 months of steroid therapy, depending on patients' rejection free period. Maintenance immunosuppression consisted of tacrolimus, mycophenolate and corticosteroids.

Patients that were found to have formed DSA had treatment adjusted by closer monitoring and more aggressive immunosuppression (higher trough level of tacrolimus and increased dose of mycophenolate mofetil).

HLA typing

HLA-A, -B, -C, -DR, -DQ typing of all recipients and donors were determined by the DNA molecular typing method using reverse sequence specific oligonucleotide probes (SSOP) according to the manufacturer's instructions (One Lambda, Canoga Park, CA, USA and/or Immucor, Stamford, CT, USA). When the patients showed anti-DP antibodies, the corresponding donor was typed for HLA-DP by the same platforms.

Detection of HLA-antibodies

Patient sera were primarily screened for the presence or absence of HLA antibodies. Sensitized patients consequently tested for class I (HLA-A, -B, and -C) and class II (HLA-DR, -DQ, and -DP) HLA antibodies using single antigen beads (SAB) on a Luminex platform according to the manufacturer's instructions (One Lambda, Canoga Park, CA, USA). DSA were considered positive if the MFI was 2000 or more.[12]

Complement-dependent cytotoxicity crossmatch

CDC crossmatches, performed for all patients, were

performed on donor peripheral T lymphocytes or lymph nodes, using the basic NIH (CDC) and enhanced anti-human globulin (CDC-AHG) methods. Patient historic and current serum (collected at the time of transplantation) were incubated in a serial doubling dilution (neat,1:2–1:8) with donor's T for 30 min. The cells were then incubated with rabbit complement for 60 minutes and then stained with acridine orange/ethidium bromide. To differentiate between IgG and IgM antibodies, sera were tested with and without treatment with dithiothreitol (DTT). The level of cytotoxicity was scored according to ASHI scoring system.

Flow crossmatch

Three-color flow cytometry crossmatches were performed. Patient historic and current serum were incubated with donor's pronase treated T and B cells for 30 min. T and B lymphocytes were stained for 10 min with phycoerythrin (PE) and peridinin-chlorophyll-protein (PerCp) conjugated monoclonal antibodies specific for CD3 and CD19, respectively. The presence of bound antibodies was determined using a fluorescein isothiocyanate-conjugated (FITC) anti-human IgG. Flow cytometric analysis was performed using a FACSCalibur instrument and Cell Quest software (BD, PharMingen, San Jose, CA). Flow crossmatch results were analyzed based on median channel shift (MCS) over background. A positive crossmatch was reported, if the MCS value was more than 2.5 standard deviations of control serum.

Diagnosis of Rejection

In all transplanted patients, surveillance endomyocardial biopsies (EMBs) were performed at 2, 4 and 8 weeks post-transplantation, then again at 3, 4, 5, 6, 8, 10 and 12 months, as per our institutions protocol. Acute cellular rejection (ACR) was diagnosed based on current International Society for Heart and Lung Transplantation (ISHLT) guidelines,[13,14] with ISHLT grade of 2R or more considered "treated ACR". The diagnosis of AMR was made on clinical grounds based on serial, post-transplant DSA profiles, allograft functional assessments by echocardiogram and catheterization, and EMB findings, including histological and immune-pathological findings.[14]

Diagnosis of cardiac allograft vasculopathy

At our institution, routine coronary angiograms are performed 1 year after heart transplantation and then every 2 years after that. They are also performed in the case of unexplained left ventricular dysfunction. Staging of CAV is performed using the recommended ISHLT nomenclature.[15]

Investigated Outcomes and Analysis

Primary outcomes were all-cause mortality, development of AMR at a clinical level, treated ACR and CAV. Secondary outcomes included graft function and hemodynamic findings.

Patients were divided into two groups: DSA-positive and DSA-negative. A decrease in left ventricular ejection fraction (LVEF) was defined as an LVEF <45%. We compared baseline characteristics of these groups for significant differences. For categorical data, Pearson's chi-square tests were used, whilst continuous data were analyzed by t tests. Survival analysis was performed by the Kaplan-Meier method, and groups were compared using the log-rank test. This analysis was also performed with regards to freedom from CAV and drop in LVEF. Univariate Cox regression was then performed to find relevant hazard ratios for mortality, using the covariates age, gender, BMI, presence of DSA, peak MFI of DSA, development of CAV, AMR, treated ACR and a drop in LVEF. Any factor found to have a univariate significance level of $P \leq .2$ was included in the multivariate Cox regression model. We used SPSS software (version 20.0.1 for Windows, SPSS Inc., Chicago, Il, USA) for statistical analysis and the R package survminer 0.4.2 for the Kaplan Meier survival curves.

RESULTS

Of 148 consecutive patients fulfilling the inclusion criteria, 21 patients were excluded from the final analysis (1 for retransplantation, 2 for positive CDC crossmatch, 9 with preformed DSA and 9 who died within 1 week after transplantation). Consequently, 127 cases were included in the final analysis, with 43 (34%) developing de novo DSA . No statistically significant difference was observed between the DSA-positive group and DSA-negative group for mean age of donor (P=.839), BMI of donor (P=.941), mean BMI of patient (P=.06) and gender proportions (P=.609) (**Table 1**). Patients in the DSA-positive group were significantly younger than the DSA-negative group (P=.017).

Of the DSA-negative cases, 16% (12/77) developed CAV compared to 40% (16/40) in the DSA-positive group, with a relative risk (RR) of 2.57 (95% confidence interval (CI) 1.35 to 4.89) (**Table 2**). Similarly, a decrease in LVEF was experienced by 4.8% in the DSA-negative group compared with 28% in the DSA-positive group with a relative risk of 5.79 (95% CI 1.99 to 16.88). An increased risk of rejection occurred in the DSA-positive group, with the most prominent effect on antibody-mediated rejection (RR 7.81, 95% CI 2.33 to 26.22). The increased risk of treated ACR was more modest, but also statistically significant (RR 2.28, 95% CI 1.20 to 4.33).

Kaplan-Meier analysis demonstrated a significantly longer time free from CAV (*P*=.005) and LVEF (*P*<.001) in the DSA-negative group when compared to the DSA-positive group.

For all 127 patients, the 3-year, 5-year and 7-year survivals were 87.7%, 85.5% and 78.8%, respectively. Chi square analysis demonstrated a significant increase in the risk of death across the study period in the DSA-positive group (11/43, 26%) compared with the DSA-negative group (8/84, 9.5%); RR 2.69 (95% CI 1.17 to 6.18) and in the HLA-DQ positive only patients (**Table 2**). The log-rank test demonstrated a significant difference in survival in favor of DSA-negative group (*P*=.018). At 3 years, 92.1% of the DSA-negative group patients were alive compared to 80.0% of the DSA positive group. By 9.5 years, 86.3% of the DSA-negative group was alive compared to 64.7% in the DSA-positive group (**Figure 1**) .

Recipient age (*P*=.038), presence of DSA (*P*=.024) and decreased LVEF (*P*=.001) were significantly associated with mortality in the univariate Cox regression analysis (**Table 3**). Recipient BMI (P=.095) and peak MFI (*P*=.079), CAV (*P*=.059) and AMR (*P*=.069) approached significance, and were included in the multivariate model. No factor was shown to be independently associated with mortality in the multivariate Cox regression analysis **(Table 3**). Of the patients who developed de novo DSA, 7 (16.3%) targeted class I antigens only, 20 (46.5%) targeted class II antigens only and 16 (37.2%) targeted both class I and class II antigens (**Figure 2**). There was a statistically significant difference between the frequency of patients who developed class I antibodies (23) compared to those targeting class II (36) (*P*=.005). The majority of DSA-positive cases developed antibodies against HLA-DQ antigens (n=33, 76.7%), either alone (n=7) or in combination with HLA-A, -B or -DR (n=26) and survival was reduced in these patients (**Figure 3**). Only 10 patients developed a DSA HLA-A, -B or -DR antibody without HLA-DQ antibody present.

Table 1. Demographic data by presence of donor-specific antibodies.

	All patients	DSA-negative patients	DSA-positive patients	*P* value
Total no. of patients	127	84 (66%)	43 (34%)	n/a
Mean Age of recipient, years	36 (14)	38 (13)	32 (13)	.017
Mean Age of donor, years	33 (9)	33 (9)	33 (8)	.839
Male sex	97 (76%)	63 (75%)	34 (79%)	.609
Mean Recipient BMI	23.9 (6.12)	24.7 (6.74)	22.5 (4.45)	.060
Mean Donor BMI	25.2 (3.86)	25.2 (3.90)	25.2 (3.82)	.941

Data are mean (standard deviation) or number (percentage).

Table 2. Incidence of negative outcomes in DSA-negative versus DSA-positive groups.

	All patients (%)	DSA-negative patients (%)	DSA-positive patients (%)	*P* value	DQ-positive patients (%)	*P* value
Cardiac allograft vasculopathy	28 (24%)	12 (16%)	16 (40%)	.003	33 (28%)	n/a
Decrease in left ventricular ejection fraction	16 (13%)	4 (4.8%)	12 (28%)	<.001	12 (16%)	.001
Acute cellular rejection	28 (23%)	13 (16%)	15 (37%)	.011	11 (33%)	<.001
Antibody-mediated rejection	15 (12%)	3 (3.6%)	12 (28%)	<.001	13 (41%)	.005
Death	19 (15%)	8 (9.5%)	11 (26%)	.016	10 (30%)	<.001
					9 (27%)	.014

Data are number (percentage). DSA: donor specific antibodies. Decrease in left ventricular ejection fraction was defined as left ventricular ejection fraction less than 45%. Analysis by chi-square test.

HLA-DR antibodies were also present in nearly half of the cases (21, 48.8%).

DISCUSSION

Our results are consistent with the current literature describing the impact of de novo DSA formation on the outcome of heart transplantation.[16-18] Our univariate Cox regression results demonstrated that presence of DSA, recipient age, CAV, AMR or a decreased LVEF had an impact on mortality rate at the *P*≤.2 level. Although multiple factors approach this level, only the presence of de novo DSA (*P*=.024), decrease in LVEF (*P*=.001) and recipient age at operation (*P*=.038) had a statistically significant impact on mortality rate in this analysis. However, as shown by the results of the multivariate Cox regression model, no individual factor was an independent predictor of mortality. As chi square analysis indicated that the presence of DSA had a significant impact on the risk of mortality, development of CAV, treated ACR, AMR and experiencing a drop in LVEF, we suggest that the presence of DSA alone is unlikely to directly result in a greater risk of death. Instead, mortality is impacted via the resulting complications of de novo DSA formation, particularly reduced ventricular function indicated by a drop in LVEF.

Interestingly, increased recipient age at operation was associated with reduced mortality during univariate Cox regression analysis (HR=.962). This contradicts the results of other studies, detailing the increased mortality risk associated with older operative patients.[19-21] However, these studies focus on operative patients greater than 50 or 55 years old, far older than the

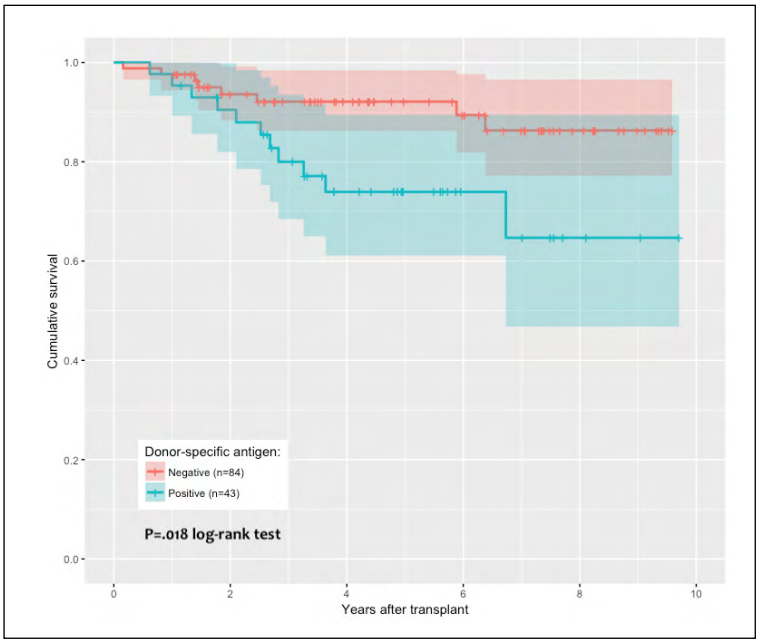

Figure 1. Cumulative survival of DSA-positive and DSA-negative cohorts over time post-transplantation. DSA: donor-specific antibodies *P*=.018, log-rank test for difference in survival. (*P*=.018, chi-square=5.555, log-rank test, Mantel-Cox).

Table 3. Univariate and multivariate Cox regression results with the hazard defined as mortality.

Factor	Univariate hazard ratio (CI)	*P* value	Multivariate hazard ratio (CI)	*P* value
Recipient age	0.962 (0.928–0.998)	.038	1.008 (0.948–1.072)	.802
Recipient male sex	0.986	.980	-	-
Recipient BMI	0.926 (0.846–1.013)	.095	0.847 (0.651–1.101)	.215
Donor age	1.031 (0.979–1.086)	.248	-	-
Donor male sex	1.128 (0.412–3.0.90)	.815	-	-
Donor BMI	1.015 (0.894–1.154)	.816	-	-
Presence of DSA	2.871 (1.148–7.181)	.024	>9000 (<0.000 – >9000)	.970
Peak MFI (Divided by factor of 100)	1.007 (0.999–1.016)	.079	1.006 (0.995–1.018)	.280
Treated ACR	1.532 (0.574–4.088)	0.394	-	-
AMR	2.578 (0.928–7.164)	0.069	2.048 (0.473–8.859)	.337
CAV	2.746 (0.926–7.836)	0.059	1.070 (0.208–5.496)	.935
Decreased LVEF	4.655 (1.803–12.021)	0.001	3.686 (0.472–28.804)	.214

DSA: donor specific antibodies. Only factors with univariate *P* values greater than .2 were included in the multivariate model. Multivariate chi-square 20.6118.190, df 7, *P*=.316, -2 log likelihood: 54.419. MFI: Median fluorescence Intensity; ACR: Acute cellular rejection; AMR: Antibody-mediated rejection; CAV: Cardiac allograft vasculopathy LVEF: Left ventricular ejection fraction.

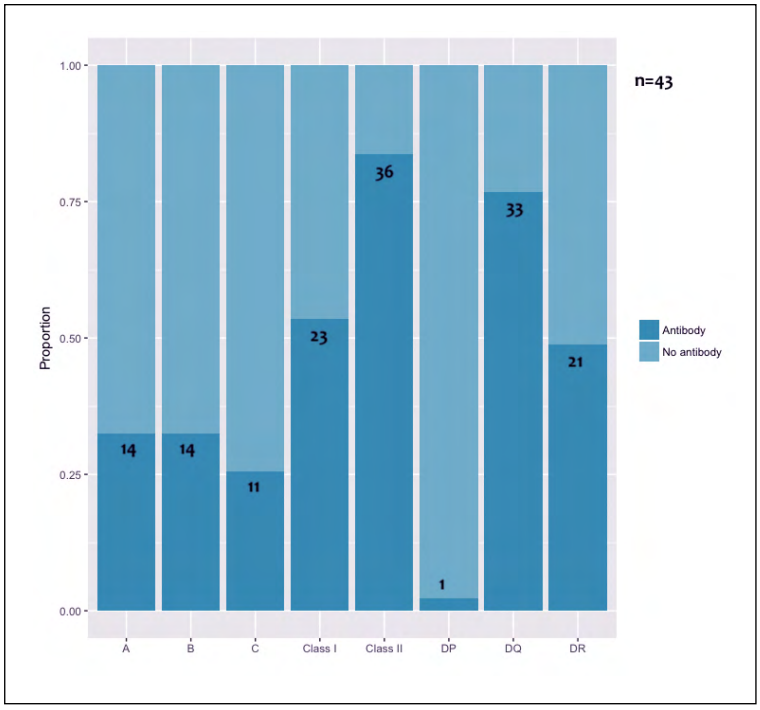

Figure 2. Number of patients and frequency of HLA antibodies in DSA-positive group (n=43), by class and group .

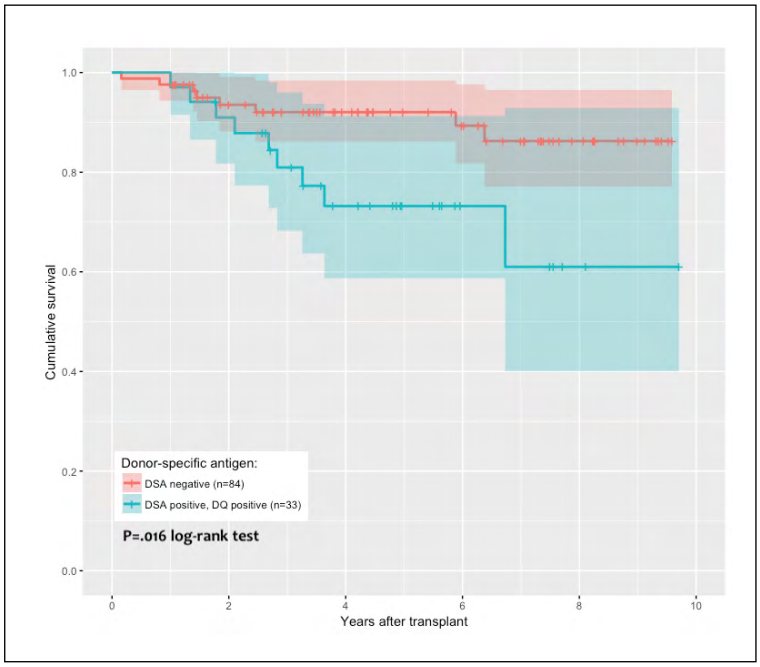

Figure 3. Cumulative survival of DSA-positive group and the DSA-negative group over time post-transplantation (*P*=.016, chi-square 5.808, log-rank test, Mantel-Cox).

mean age of 36 years in our study population. As the DSA-positive group was significantly younger than the DSA-negative group (32 vs. 38 years old, *P*=.017), which may have worked against the detrimental effect of the presence of DSA on mortality. Younger age may have also played a role in limiting the effect of the presence of DSA on mortality, therefore preventing DSA from appearing as an independent predictor of mortality during survivability analysis, as was demonstrated by Smith et al in 2011.[22]

The reason for this significant difference in age is unclear, with similar findings in other studies relating to both heart and kidney transplantation.[23,24] The frequency of antibodies targeting HLA class II antigens compared to HLA class I was statistically significant (*P*=.005), a phenomenon which was previously reported by other investigators.[16,22] The literature also shows a higher proportion of antibodies targeting the HLA-DQ and DR antigens,[16,22] which was also demonstrated during our analysis, particularly with regards to HLA-DQ. The available evidence suggests that neither HLA class I nor class II antigens are expressed in the myocardium of a non-diseased heart, but only in the interstitial structures, namely, the endothelium and dendritic cells.[25,26] It is believed that expression of class I antigens is induced in the myocardium and interstitially post-transplantation, whereas class II up-regulation is isolated to interstitial structures. Rejection was found to be associated with this increased class I expression and, specifically, increased expression of class II DQ antigens within the interstitial tissue.[26]

The above may explain the significance of HLA-DQ demonstrated in our analysis. When we excluded all patients who developed DSA that did not target HLA-DQ (n=10), the association between DQ presence and survival (*P*=.014), development of CAV (*P*=.001), treated ACR (*P*=.005), AMR (*P*<.001) and decrease in LVEF (*P*<.001) appeared more significant than when compared against any DSA presence (**Table 2**. Survival analysis showed a slightly more significant difference when analyzing presence of DQ DSA using the Kaplan-Meier method (log rank, *P*=.016) (**Figure 3**) and univariate Cox regression (*P*=.022), but again, no factor was shown to be an independent predictor of mortality in the multivariate model using the same methods shown above (**Table 4**).

Due to the study's relatively small sample size, especially with regards to non-DQ DSA, a causative relationship cannot be accurately inferred. Retrospective analysis, by isolating the DSA-positive group and comparing all patients with any HLA-DQ DSA (n=33) against all other DSA (n=7) did not demonstrate sig-

nificance in terms of frequency of AMR (*P*=.525), ACR (*P*=.311), drop in LVEF (*P*=.150), CAV (*P*=.216) or mortality (*P*=.644). This was also the case with Kaplan-Meier survival analysis (*P*=.711).

This relationship between HLA-DQ targeting antibodies and poor transplantation outcomes does not seem unique to this field. Similar associations were described in the context of kidney and lung transplantations.[27-29] This may represent a potential therapeutic target that stretches across several types of solid organ transplantations. The appearance of anti-DQ DSA may also be used as an indicator of potential complications, and possibly play a significant role in the monitoring of patients post-transplantation. In fact, a recent study published in 2017 demonstrated significantly increased mortality in post-heart transplant patients who developed HLA-DQ DSA, in comparison to non-DQ DSA.[18]

Although this study describes a single center's experience, our institution is one of the largest transplantation centers in the region with a highly comparable number of cases included in this study with respect to other similar studies discussed. Unmeasured bias cannot be completely ruled out in any retrospective study such as ours. However, our database is comprehensive and meticulously maintained to minimize such effects. Finally, the average age of our patients is lower than commonly reported elsewhere and therefore care should be taken before generalizing our findings to other patient populations. In conclusion, this study supports the hypothesis that the formation of de novo DSA plays a significant role in the outcome of heart transplantation, with a particularly striking effect observed when antibodies target HLA-DQ antigens, the most commonly formed DSA.

Table 4. Univariate and multivariate Cox regression results with the hazard defined as mortality, using data excluding any non-DQ DSA.

Factor	Univariate Hazard ratio (CI)	*P* value	Multivariate hazard ratio (CI)	*P* value
Recipient age	0.965 (0.929–1.003)	.065	0.992 (0.937–1.051)	.795
Recipient male sex	1.177 (0.338–4.105)	.798	-	-
Recipient BMI	0.929 (0.846–1.020)	.124	0.891 (0.754–1.053)	.175
Donor age	1.026 (0.972–1.083)	.348	-	-
Donor male sex	0.769 (0.102–5.808)	.799	-	-
Donor BMI	1.034 (0.910–1.176)	.609	-	-
Presence of DSA	3.071 (1.176–8.018)	.022	2.067 (0.469–9.111)	.337
Treated ACR	1.695 (0.626–4.593)	.299	-	-
Antibody-mediated rejection	3.396 (1.194–9.660)	.022	2.258 (0.552–9.245)	.257
Cardiac allograft vasculopathy	3.272 (1.098 – 9.749)	.033	1.866 (0.464–7.511)	.380
Decreased LVEF	6.046 (2.242–16.308)	<.001	3.430 (0.631–18.644)	.154

Only factors with univariate *P* values greater than .2 were included in the multivariate model. Multivariate chi-square 20.611, df 6, *P*=.002, -2 log likelihood: 84.959. MFI: Median fluorescence Intensity; ACR: Acute cellular rejection; LVEF: Left ventricular ejection fraction.

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