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ORIGINAL ARTICLE

Novel insights into pretransplant allosensitization in heart transplant recipients in the contemporary era of immunosuppression and rejection surveillance

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Summary

Solid-phase assays (SPA) have facilitated detection and definition of antibodies to human leukocyte antigens (HLA) and major histocompatibility complex class I chain-related antigen A (MICA). However, clinical consequences of pretransplant SPA results in heart transplantation have been studied insufficiently in the current era of immunosuppression and rejection surveillance. Pretransplant sera, panel-reactive antibodies (PRA), pretransplant crossmatch, and clinical data were retrospectively analyzed in 264 adult heart transplant recipients. The specificity of HLA and MICA antibodies and C1q-binding activity of donor-specific antibodies (DSA) were defined using SPA. Pretransplant HLA antibodies were detected in 57 (22%) individuals, in 28 individuals (11%); these antibodies were DSA after transplant. Preformed DSA and elevated peak PRA were independent predictors of pathologic AMR, which occurred in 19 individuals (7%). The increasing number of DSA and the cumulative mean fluorescence intensity of DSA were associated with AMR. C1q-binding assay was a suboptimal predictor of AMR in our cohort. Pretransplant allosensitization and MICA antibodies were related neither to impaired graft survival nor to other adverse clinical events during a median follow-up of 39 months. Identification of preformed DSA by SPA, in addition to PRA monitoring, may predict AMR in the contemporary era of heart transplantation.

Introduction

The presence of antibodies directed against human leukocyte antigens (HLA) and especially the presence of donor-specific antibodies (DSA) has been associated with allograft rejection, dysfunction, and loss [1–3]. To identify heart transplant recipients at increased risk of antibody-mediated rejection (AMR), most centers perform panel-reactive antibody (PRA) testing as a measure of presensitization and also carry out either prospective or retrospective complement-dependent cytotoxicity (CDC) crossmatch testing [4]. The introduction of solid-phase techniques has facilitated determination of alloantibody specificities and thus enabled heart transplant allocation in sensitized individuals

based on virtual crossmatching [5]. Assessment of complement-fixing ability of alloantibodies using recently developed solid-phase assays (SPA) has been proposed as approach to determine the cytotoxic potential of alloantibodies. In several studies, C1q binding antibodies correlated with higher incidence of AMR [6–8]. However, there is conflicting evidence regarding association between mortality and morbidity of heart transplant recipients and the presence of pretransplant HLA antibodies as assessed by SPA [9–15]. Similarly, the clinical significance of antibodies directed against major histocompatibility complex class I chain-related antigen A (MICA) is not well understood [16,17].

In this study, we aimed to evaluate the relationship between HLA and MICA antibodies detected by SPA before transplant and the clinical events in heart transplant recipients. We also assessed the pathogenicity of DSA and evaluated their complement binding capacity using the C1q assay.

Methods

Study design and study population

This was a single-center retrospective study which included heart transplant recipients transplanted at our center between April 2005 and December 2012. In this time period, a total of 321 heart transplants were performed and pretransplant sera were available for analysis in 264 adult heart transplant recipients. All individuals received induction therapy with polyclonal anti-human thymocyte immunoglobulin (Thymoglobulin, Genzyme Polyclonals) 1.25 mg/kg administered at the time of surgery and daily in the following 3–10 days until target through levels of calcineurin inhibitor were reached. In 2005–2007, standard immunosuppression consisted of cyclosporine A with initial target through level of 250–300 ng/ml, mycophenolate mofetil 3000 mg daily and prednisone. After 2007, we used tacrolimus with initial target trough level of 10–15 ng/ml, mycophenolate mofetil 2000 mg daily and prednisone. The initial dose of prednisone was 1 mg/kg.day with subsequent tapering to less than 0.3 mg/kg.day at 1 month and 0.1 mg/kg.day at 12 months after transplantation. Withdrawal of corticosteroids was considered after the 12th month post-transplant in individuals with a low risk of acute rejection. This was followed by scheduled endomyocardial biopsy within 3 months after weaning. Acute cellular rejection (ACR) episodes ≥ grade Banff 3A/ISHLT 2R (or ≥ grade Banff 2/ISHLT 1R early after transplantation) were treated with intravenous methylprednisolone 1000 mg for 3 consecutive days. Treatment of AMR is described in the Results section.

Post-transplant follow-up

Clinical and echocardiographic follow-up was guided mainly by screening endomyocardial biopsies in the initial postoperative period, as reported previously [18,19]. A total of 3270 biopsy procedures were performed. In the later post-transplant period, all subjects continued in clinical and echocardiographic follow-up at regular 3- to 6-month intervals. We did not perform scheduled coronary angiography to diagnose cardiac allograft vasculopathy (CAV) due to limited therapeutic consequences of angiographic findings. Coronary angiography was performed in individuals with symptoms, with abnormal electrocardiogram suggesting CAV, or with unexplained left ventricular systolic dysfunction. CAV was defined in this study as angiographic or postmortem finding of at least one coronary artery stenosis with reduction in lumen > 25%. The occurrence of pathological AMR (grade ≥ 1), ACR (grade ≥ Banff 2) [20,21], left ventricular systolic dysfunction in the absence of acute rejection (ejection fraction < 40%) and CAV of were analyzed until December 2013 (or death/retransplantation).

Analysis of endomyocardial biopsies

Endomyocardial biopsy specimens were routinely stained with hematoxylin–eosin. Immunohistochemistry was performed on 3-μm-thick paraffin sections using immunoperoxidase-based indirect method to detect C4d deposition and CD 68 expression on macrophages, as reported previously [19]. Acute cellular rejection was graded both according to the 1990 [20] and the 2005 ISHLT classification [21], and AMR was evaluated using the 2005 ISHLT classification [21]. All abnormal biopsies in terms of AMR were reviewed by two expert pathologists (L.V. and M.K.) and reclassified according to the latest 2011 ISHLT classification of AMR [22].

Panel-reactive antibody testing and complement-dependent cytotoxicity crossmatch test

Before transplantation, the presence of HLA-specific antibodies was assessed in all recipients using the CDC assay [1]. Panel-reactive antibodies (PRA) were expressed as a percentage of positive tests within a panel of lymphocytes from 30 healthy donors. Patients' serum samples were retested every 12 months or after a sensitizing event (pregnancy, blood transfusion, every month after VAD implantation, etc.). The maximum PRA (peak PRA) and the last pretransplant PRA were recorded. Pretransplant CDC crossmatch test was performed in all recipients. Individuals with pretransplant PRA ≥ 10% were transplanted only after a negative CDC crossmatch test was prospectively confirmed.

Antibody detection by SPA and HLA typing

Whereas cytotoxic tests were routinely used for clinical purposes, SPA for HLA and MICA antibodies were analyzed retrospectively for this research project. Serum samples were obtained from blood samples drawn for pretransplant crossmatch and kept frozen at – 20 °C until further testing. The specificity of HLA and MICA antibodies was defined by LABScreen Mixed and Single Antigen class I and class II beads (One Lambda Inc., Canoga Park, CA, USA), as reported previously [15]. Mean fluorescence intensity of 1000 and 2000 units was adopted as a cut-off point for positivity of class I and class II antibodies, respectively. Sera from patients on VAD were treated with AdsourbOut (One Lambda Inc.) before analysis due to nonspecific binding on polystyrene beads. Patients and donors were typed for HLA- A, B, DRB1 with commercially available molecular methods (HLA class I and II) (SSP, SSOP). In instances where antibody specificities in pretransplant sera were directed against antigens of the donor allograft (donor-specific antibody, DSA), we also used SA and C1q screen kits (One Lambda Inc.) to test whether these antibodies had capacity to fix complement. Briefly, sera were inactivated by heating for 30 min at 56 °C, spiked with the complement component C1q (5 µ), and incubated with 5 µl beads for 20 min. After incubation, phycoerythrin-labeled anti -C1q antibody was added. Samples were then washed and measured on the Luminex 200 analyzer (Luminex Inc., Austin, TX, USA). Positive and negative control sera were included in each assay.

Statistical analysis

Categorical data were expressed as percentages and compared using chi-squared analysis and Fisher's exact test. Continuous variables were expressed as mean and standard deviation, or median and interquartile range in case of abnormal distribution. They were compared using the Student's *t*-test for unpaired data or by the nonparametric Mann–Whitney test or Wilcoxon test where appropriate. Univariate Cox regression models were used to identify predictors of time-dependent events. The variables that were significant on univariate analysis ($P < 0.1$) were entered into multivariable Cox regression models using forwards and backwards stepwise elimination. Kaplan–Meier curves were constructed to demonstrate the relationship between selected variables and time to events. Receiver operator characteristics (ROC) analysis was performed to assess predictive performance of selected variables. For all tests, a probability value of $P < 0.05$ was considered significant. Analysis was performed using statistical software SPSS (Chicago, IL, USA) for Windows, version 17.0.

Ethics

The study was approved by the local ethics committee.

Results

A total of 264 heart transplant recipients were followed for a median time of 39 months (19–66 months). No patient was lost from follow-up. Detailed baseline demographics of these patients are listed in Table 1.

Pretransplant markers of allosensitization

Complement-dependent cytotoxicity assays revealed any positive PRA in 100 (38%) patients, with a peak PRA ≥ 10% in 54 (21%) patients and last pretransplant PRA ≥ 10% in 20 (8%) patients. Eight (3%) patients were highly sensitized

Table 1. Study group characteristics.

	Study group characteristics (n = 264 pts)
Age of recipient (years)	50 ± 12
Gender of recipients(%)	211 males (80%) 53 females (20%)
Diabetes mellitus	73 (28%)
Etiology of heart failure	Ischemic 100 pts (38%) Dilated cardiomyopathy 124pts (47%) Other 40pts (15%)
Retransplantation	2 pts (0.7%) with cardiac allograft vasculopathy
Previous non-VAD cardiac surgery	64 pts (24%)
Ventricular assist device before transplantation	68 pts (26%)
Type of VAD	Pulsatile VAD (Thoratec) 18 pts (7%) Continous flow VAD (Levitronix) 3 pts (1%) Continous flow VAD (Heart Mate II) 47 pts (18%)
Time on VAD (days)	116 (61–291)
Any previous cardiac surgery	123 pts (47%)
Age of donor (years)	38 ± 12
Gender of donors (%)	196 males (74%) 68 females (26%)
Cold ischemia (min)	140 (115–170)
Time from reperfusion to termination of extracorporeal circulation (min)	40 (30–45)
Duration of hospitalisation in ICU (days)	2 (4–7)
Calcineurin inhibitor at the initial hospitalization (%)	Cyclosporine A 75 pts (28%) Tacrolimus 189 pts (72%)

Table 2. Comparison of allosensitization status in individuals with and without antibody-mediated rejection with corresponding *P*-values. (*p <0.05, **p <0.01, ***p<0.001).

	Individuals with AMR (n = 19)	Individuals without AMR (n = 245)	*P*-value
Cytotoxic tests			
Any positive PRA (%)	7 (37%)	93 (38%)	1.0
Peak PRA ≥ 10%	7 (37%)	47 (19%)	0.078
Positive last PRA	4 (21%)	46 (19%)	0.765
Last PRA ≥ 10%	3 (16%)	17 (7%)	0.164
Positive CDC crossmatch	2 (10%)	7 (3%)	0.131
Solid-phase assays			
Class I or II HLA antibodies	8 (42%)	49 (20%)	0.039*
Isolated class I HLA antibodies	4 (21%)	34 (14%)	0.493
Isolated class II HLA antibodies	1 (5%)	7 (3%)	0.454
Simultaneous Class I and II HLA antibodies	3 (16%)	8 (3%)	0.036*
MICA	2 (10%)	32 (13%)	1.0
Donor-specific antibodies (class I or II)	7 (37%)	21 (9%)	0.001**
Isolated class I DSA	3 (16%)	15 (6%)	0.129
Isolated class II DSA	1 (5%)	2 (0.8%)	0.201
Simultaneous class I and II DSA	3 (16%)	4 (2%)	0.009**
Number of DSA ≥ 2	5 (26%)	11 (4%)	0.003**
Number of DSA ≥ 3	5 (26%)	4 (1.6%)	0.000***
Number of DSA ≥ 4	3 (16%)	1 (0.4%)	0.001**
C1q-binding DSA	2 (10%)	2 (1%)	0.027*
Combination of cytotoxic and solid-phase assays			
Class I or II anti-HLA antibodies and/or peak PRA ≥ 10%	11 (58%)	89 (36%)	0.084
Preformed DSA and/or peak PRA ≥ 10%	10 (53%)	62 (25%)	0.015*
Donor–recipient HLA missmatch			
Number of missmatches in HLA antigens A, B, DR (available n = 240 pts)	5.2 ± 0.9	4.8 ± 1.0	0.110
Number of mismatches in HLA antigens A and B (available n = 240 pts)	3.4 ± 0.7	3.3 ± 0.8	0.163
Number of mismatches in HLA antigens DR (available n = 240 pts)	1.7 ± 0.5	1.5 ± 0.6	0.430

CDC, complement-dependent cytotoxicity; DSA, donor-specific antibodies; HLA, human lekocyte antigen; PRA, panel-reactive antibodies.

with a peak PRA ≥ 80%. The pretransplant CDC crossmatch test was positive in 8 (3%) individuals with last PRA < 10%. The crossmatch test was weakly positive in five patients and strongly positive in one patient. Details on the strength of positivity are unavailable in the remaining two patients. The crossmatch test results were found out retrospectively in these individuals and did not result in routine enhancement of immunosuppression. The remaining patient with a positive crossmatch test was highly sensitized with peak PRA of 92%. He underwent pretransplant desensitization by immunoadsorption and intravenous immunoglobulin G (IVIG) with reduction in PRA to 36%. Pre-operative immunoadsorption resulted in a negative crossmatch test in this case. This was followed by additional immunoadsorption and IVIG on the second post-transplant day.

Solid-phase assays demonstrated HLA antibodies (class I or II) in 57 (22%) individuals, which were in 28 (11%) patients donor-specific. MICA antibodies were detected in 34 patients (13%). Details are shown in Table 2. In patients with DSA, DSA to class I only were present in 18 (64%) patients, DSA to class II only were found in 3 (11%) patients and DSA to both classes were detected in 7 (25%) patients. More than half of the patients with DSA had donor-specific antibodies directed against multiple donor antigens: 25% against 2 antigens, 18% against 3, 7% against 4, and 7% against 5 antigens. C1q-binding assay was positive in 4 patients (14%) with DSA.

Three individuals (33%) with a positive pretransplant crossmatch test had detectable DSA by Luminex. They had the following C1q-reactive DSA: B7 (MFI 9494); A24 (23161MFI) and B13 (16389MFI); B27 (1791MFI); and DR12 (1000MFI). Two individuals (22%) with a positive pretransplant CDC crossmatch test developed AMR. Among them, AMR was diagnosed in one patient with C1q-reactive DSA (A24, B13) and in another patient without detectable pretransplant DSA on the 13th and 43rd post-transplant day, respectively. There was an additional patient with C1q-reactive DSA (B51; 2868MFI), but a negative pretransplant crossmatch test, who developed AMR on the 9th post-transplant day.

Identification of individuals with pretransplant HLA antibodies as detected by PRA and SPA from demographic data

Positive peak PRA were associated with any previous cardiac surgery and also VAD implantation [positive in 61% of recipients with previous cardiac surgery versus 38% of the remaining individuals, ($P < 0.001$); positive in 63% of ventricular assist device recipients versus 29% of the remaining individuals, ($P < 0.001$)]. In contrast, pretransplant HLA antibodies as defined by SPA (class I or II) were most closely related to female gender of recipients. They were present in 47% of females versus 15% males ($P < 0.001$). Female gender of recipients and/or a history of previous cardiac surgery would predict the presence of pretransplant HLA antibodies (class I or II) detected by SPA with a sensitivity of 74% and specificity of 45% ($P = 0.01$). The corresponding positive and negative predictive value and accuracy were 27%, 86%, and 51%, respectively. Interestingly, among the 57 recipients with pretransplant HLA antibodies as defined by SPA methods (Class I or Class II), 63% and 75% had undetectable peak and last HLA antibodies by the CDC method, respectively.

Predictors of AMR

Analysis of endomyocardial biopsy specimens demonstrated pathological AMR in 19 (7%) individuals. The following types of AMR (ISHLT classification) were described: grade 1 (i+) in five patients, grade 1 (h+) in one patient, grade 2 in seven patients, a combination of AMR grade 1 (i+) or 2 with ACR grade Banff 2 to 3B in six patients. Eight (42%) individuals experienced early AMR within 100 days post-transplant. Nine (47%) individuals developed LV systolic dysfunction and/or acute heart failure during AMR. Eight patients received comprehensive treatment of AMR including steroids, plasmapheresis/immunoadsorption, and IVIG with addition of thymoglobulin in one case, rituximab in four cases, and rituximab with bortezomib in one case. Five individuals with a combination of ACR and AMR received steroids followed by thymoglobulin in three cases. Basal immunosuppression was increased in the remaining cases.

In Table 2, we compared allosensitization status in patients who developed AMR and those who did not, and we also carried out an univariate Cox regression analysis to examine the strength of the relationships (Table 3). The strongest predictor of AMR was the presence of preformed DSA, followed by peak PRA examined as a continuous variable. The peak PRA value appeared to provide additional prognostic information to the preformed DSA (Fig. 1) and both associations retained significance even after adjustment in a multivariable model (Table 4). Preformed DSA and/or a history of peak PRA $\geq 10\%$ predicted AMR with a sensitivity of 53% and specificity of 75% ($P = 0.015$). The corresponding positive and negative predictive value and accuracy were 14%, 95%, and 73%, respectively. The above findings remained true for prediction of early AMR

Table 3. Results of univariate Cox regression analysis. Predictors of antibody-mediated rejection, acute cellular rejection, clinical cardiac allograft vasculopathy, and survival or retransplantation.

Name of variable	Chi-square	Relative risk (95% CI)	*P*-value
1. Predictors of antibody-mediated rejection (19 pts)			
Female recipient	6.84	3.165 (1.271–7.880)	0.019
Peak PRA	13.60	1.023 (1.010–1.037)	0.005
Class I HLA antibodies	3.73	2.450 (0.960–6.240)	0.053
Class II HLA antibodies	5.98	3.640 (1.202–11.027)	0.014
Preformed DSA	14.57	5.112 (2.010–13.0)	0.000
Days in ICU	8.29	1.009 (1.002–1.015)	0.004
2. Predictors of acute cellular rejection (grade $\geq$ Banff 2, ISHLT classification) (83 events in 74 pts)			
Days in ICU	3.50	0.994 (0.989–0.999)	0.061
Tacrolimus versus cyclosporine based immunosuppression	20.29	0.356 (0.223–0.568)	0.000
3. Predictors of clinical cardiac allograft vasculopathy (31 pts)			
Number of episodes of acute cellular rejection	4.23	1.684 (1.032–2.747)	0.040
Antibody-mediated rejection	9.27	3.427 (1.474–7.968)	0.002
4. Predictors of survival or retransplantation (49 pts)			
Nonischemic versus ischemic etiology	3.41	0.590 (0.339–1.039)	0.068
Time from reperfusion to termination of extracorporeal circulation	8.77	1.014 (1.005–1.024)	0.003
Number of ACR episodes during FU	6.04	1.584 (1.100–2.280)	0.014
Presence of LV systolic dysfunction during FU	17.37	3.394 (1.842–6.253)	0.000
Clinical CAV	4.10	1.967 (1.022–3.787)	0.043

ACR, acute cellular rejection; CAV, cardiac allograft vasculopathy; DSA, donor-specific antibodies; FU, follow-up; HLA, human leukocyte antigen; LV, left ventricular; PRA, panel-reactive antibodies.

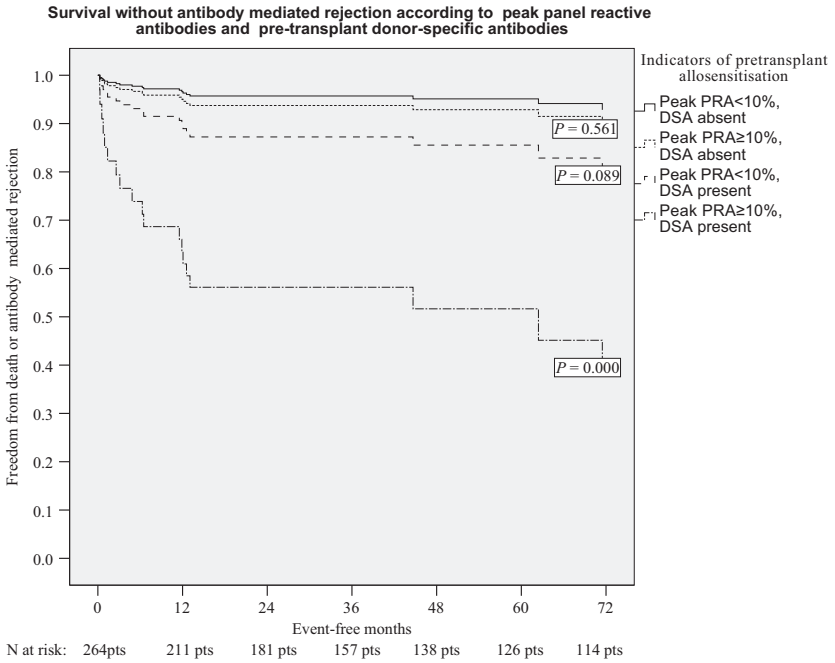

Figure 1 Kaplan–Meier curves illustrate survival without antibody-mediated rejection according to peak panel-reactive antibodies and the presence of pretransplant donor-specific antibodies. The stratum of individuals with peak panel-reactive antibodies <10% and absent donor-specific antibodies was compared with remaining groups using the log-rank test. The corresponding *P*-values are shown.

(≤100 days post-transplant), but were not valid in late AMR (Table 5).

Among the 28 individuals with DSA, the increasing total number of DSA was associated with an increased likelihood of AMR development in the Cox regression analysis [(χ^2 12.62, P =< 0.001; RR 2.809, 95% CI (1.440–5.481)] (Fig. 2). Patients with ≥ 3 DSA also had an earlier development of AMR as compared with the remaining individuals (mean 34 ± 13 vs. 89 ± 6 months, P = 0.001). From the quantitative indexes, the cumulative mean fluorescence intensity of all DSA was also associated with subsequent AMR risk in the Cox regression analysis [(χ^2 8.77, P = 0.003; RR 1.070, 95% CI (1.015–1.128) per 1000 units]. However, it was of borderline significance in the Mann–Whitney test [median 15 141 (3298–33 963) in AMR patients versus 7548 (2346–10 517), P = 0.055] and ROC analysis [AUC 0.748, 95% CI (0.530–0.967), P = 0.053). The cumulative MFI ≥ 3234 identified among DSA-positive patients predicted development of AMR with a sensitivity of 86% a specificity of 38%. Neither the class of the targeted HLA antigen nor the C1q assay (Fig. 2) could differentiate the pathogenic DSA.

Predictors of acute cellular rejection, cardiac allograft vasculopathy, and graft survival

We also evaluated the effect of allosensitization status and other clinical characteristics on the risk of acute cellular rejection, cardiac allograft vasculopathy, and graft survival. There were 74 (28%) individuals who experienced 83 episodes of ACR grade ≥ Banff 2 (ISHLT classification), 31 (12%) patients were diagnosed with CAV, and 48 (18%) patients died and 1 (0.4%) recipient underwent re-transplantation during follow-up. The overall graft survival was 90% at 1 year and 79% at 5 years. Tables 3 and 4 show predictors of these events as identified in univariate and multivariate analyses. Neither pretransplant serology (by CDC methods or SPA) nor mismatches in HLA antigens were predictors of ACR or graft survival. The most powerful predictor of freedom from ACR was tacrolimus-based immunosuppression as compared with cyclosporine A regimen. While the association between pretransplant sensitization and CAV did not reach statistical significance, patients who developed AMR were at an increased risk of CAV. A similar relationship was found between the number of ACR episodes and the risk of CAV. Time from reperfusion to termination of extracorporeal circulation, a correlate of perioperative hemodynamic instability, and LV systolic dysfunction after transplant, in the absence of acute rejection, were independent predictors of graft survival.

Discussion

We examined the performance of conventional CDC assays and the SPA, including the C1q-binding assay, in their ability to predict clinical consequences of pretransplant

Table 4. Results of multivariate Cox regression analysis. Independent predictors of antibody-mediated rejection, acute cellular rejection, cardiac allograft vasculopathy, survival or retransplantation.

Name of variable	Chi-square	Relative risk (95% CI)	*P*-value
1. Predictors of antibody-mediated rejection			
Peak PRA	5.68	1.018 (1.003–1.033)	0.017
Preformed donor-specific anti-HLA antibodies	7.25	3.882 (1.447–10.419)	0.007
2. Predictors of acute cellular rejection (grade ≥ Banff 2, ISHLT classification)			
Tacrolimus versus cyclosporine based immunosuppression	20.29	0.356 (0.223–0.568)	0.000
Days in ICU	3.50	0.994 (0.989–0.999)	0.061
3. Predictors of clinical cardiac allograft vasculopathy			
Antibody-mediated rejection	8.19	3.427 (1.474–7.968)	0.004
4A. Predictors of survival or retransplantation- baseline			
Time from reperfusion to termination of extracorporeal circulation (min)	8.77	1.014 (1.005–1.024)	0.003
4B. Predictors of survival or retransplantation- baseline and follow-up			
Time from reperfusion to termination of extracorporeal circulation (min)	8.34	1.015 (1.005–1.025)	0.004
LV systolic dysfunction (LVEF < 40%)	15.74	3.60 (1.91–6.78)	0.000

HLA, human leukocyte antigen; ICU, intensive care unit; LV, left ventricular; PRA, panel-reactive antibodies.

Table 5. Relationship between timing of antibody-mediated rejection and pretransplant immnological findings.

	Early AMR (≤100 days post-transplant) (*n* = 8 pts)		Late AMR (>100 days post-transplant) (*n* = 11 pts)	
Predictor	Positive (Sensitivity)	Other indicators of predictive capacity	Positive (Sensitivity)	Other indicators of predictive capacity
Peak PRA	–	AUC 0.64 (95% CI 0.39–0.88) *P* = 0.19	–	AUC 0.47 (95% CI 0.31–0.88) *P* = 0.77
Peak PRA ≥ 10%	4 (50%)	Specificity 80%, *P* = 0.058	3 (27%)	NS (*P* = 0.70)
Positive CDC crossmatch	2 (25%)	Specificity 97%, *P* = 0.026	0 (0%)	NS (*P* = 1.0)
Any DSA	4 (50%)	Specificity 91%, *P* = 0.005	3 (27%)	NS (*P* = 0.10)
DSA class I only	1 (12%)	Specificity 93%, *P* = 0.436	2 (18%)	NS (*P* = 0.17)
DSA class II only	0 (0%)	NS (*P* = 1.0)	1 (9%)	NS (*P* = 0.12)
DSA both classes	3 (37%)	Specificity 98%, *P* = 0.001	0 (0%)	NS (*P* = 1.0)
Any class I DSA	4 (50%)	Specificity 92%, *P* = 0.003	2 (18%)	NS (*P* = 0.28)
Any class II DSA	3 (37%)	Specificity 97%, *P* = 0.002	1 (9%)	NS (*P* = 0.35)
Number of DSA	–	AUC 0.72 (95% CI 0.51–0.95) *P* = 0.030	–	AUC 0.58 (95% CI 0.40–0.77) *P* = 0.34
Number of DSA ≥ 2	4 (50%)	Specificity 95%, *P* = 0.001	1 (9%)	NS (*P* = 0.50)
Number of DSA ≥ 3	4 (50%)	Specificity 98%, *P* = 0.000	1 (9%)	NS (*P* = 0.32)
Number of DSA ≥ 4	3 (37%)	Specificity 99%, *P* = 0.000	0 (0%)	NS (*P* = 1.0)
The highest MFI of DSA	–	AUC 0.72 (95% CI 0.51–0.95) *P* = 0.032		AUC 0.58 (95% CI 0.39–0.77) *P* = 0.34
C1q fixing DSA	2 (25%)	Specificity 99%, *P* = 0.000	0 (0%)	NS (*P* = 1.0)
DSA or peak PRA≥ 10%	5 (62%)	Specificity 74%, *P* = 0.037	5 (46%)	NS (*P* = 0.18)

allosensitization in heart transplant recipients with contemporary immunosuppression and comprehensive detection of AMR in biopsies. The main findings can be summarized as follows. First, preformed DSA and elevated peak PRA were independent predictors of AMR, especially of early AMR. Second, the increasing number of DSA and cumulative mean fluorescence intensity of DSA identified pathogenic DSA. Third, C1q-bindig assay was a suboptimal predictor of AMR in our cohort where a negative prospective CDC crossmatch was required in sensitized recipients.

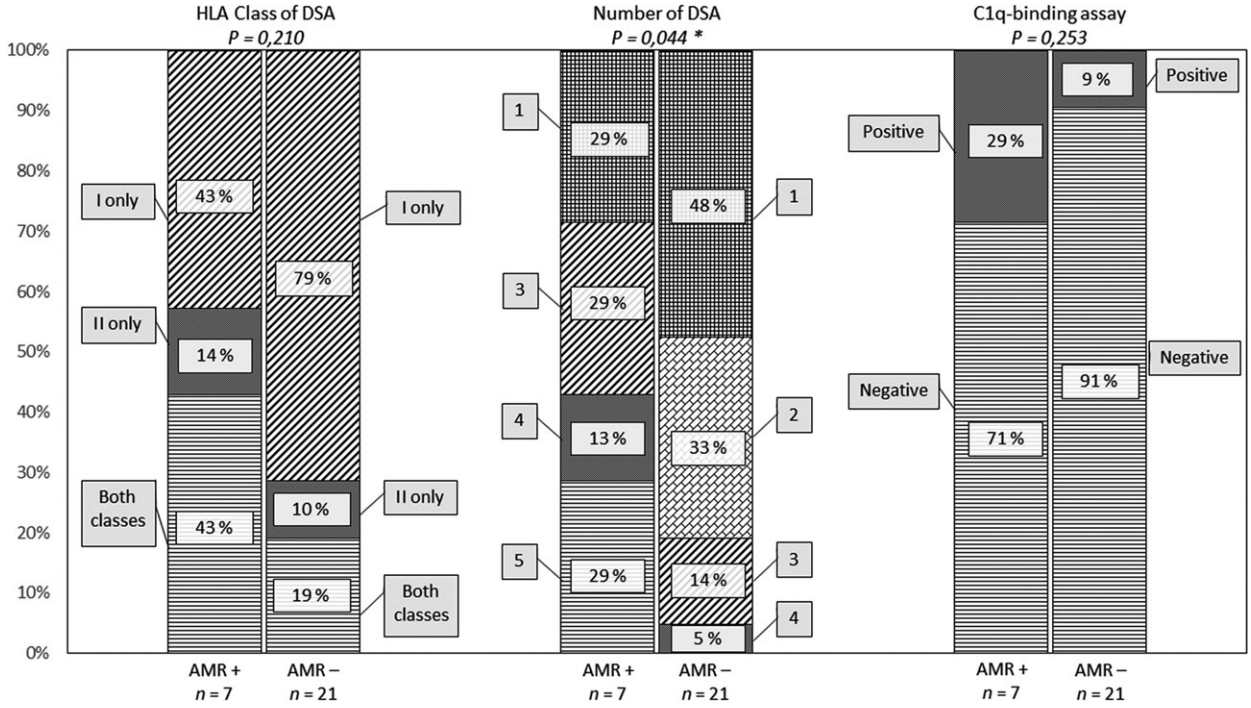

Figure 2 Relationship between class of human leukocyte antigens targeted by donor-specific antibody, number of donor-specific antibodies, positivity of C1q-binding test, and occurrence of antibody-mediated rejection. Results of the aforementioned variables are shown as relative proportion in 28 individuals with preformed donor-specific antibodies.

Finally, pretransplant MICA antibodies were not associated with adverse clinical events in the group of patients we studied.

Comparison with previous studies

Although several studies evaluated pretransplant HLA antibodies as detected by SPA in heart transplant recipients [9–15], there is still conflicting evidence regarding their clinical consequences. In most of these studies, routine C4d staining of EMB specimens as a marker of AMR was unavailable [9,10] or performed just in a part of the study group [11–14], in one study only postmortem [12]. This may have led to underestimated prevalence and inadequate treatment of AMR resulting in increased mortality observed in individuals with preformed DSA [9,10]. Other limitations of previous studies include use of CDC and SPA methods to detect HLA antibodies in different time periods in one cohort [11], missing identification of DSA [13], use of outdated schemes of immunosuppression [9–14], and limited sample size [14,15]. Our study thus provided a unique opportunity to assess the clinical effect of pretransplant allosensitization in the setting of the contemporary immunosuppression and rejection surveillance. We found convincing relationship between preformed DSA to HLA and AMR, especially with the early onset of AMR. In agreement with the largest study [10], we failed to detect association between pretransplant allosensitization and impaired graft survival, probably due to improved AMR surveillance and management. In agreement with the study of Smith *et al.* [17], pretransplant MICA antibodies were not associated with adverse clinical events in our study.

Pretransplant allosensitization and pathophysiology of antibody-mediated rejection

Another important finding of our study is the additional value of elevated peak PRA to predict AMR. Unfortunately, we could not analyze the historical sera by SPA. The explanation of these phenomena thus needs further confirmation. The elevated peak cytotoxic PRA in a patient with negative SPA at the time of transplant and subsequent AMR may represent either presence of non-HLA antibodies, or historical sensitization, with no significant levels of antibodies at the time transplant, but possible anamnestic response after transplant leading to renewed alloantibody production. The study design also allowed us to assess the pathogenicity of pretransplant DSA, which triggered pathological AMR in 25% of DSA-positive patients. In our study, the novel C1q-binding assay failed to provide additional predictive value to the standard SPA antibody testing. Previous studies with C1q-binding assay predicted AMR in

selected highly sensitized heart transplant recipients [6,7]. Such patients may have not been included in our study as we required a negative prospective CDC crossmatch in sensitized patients before transplant. Results similar to ours were recently reported in renal transplantation [8]. On the other hand, the increasing number of DSA and cumulative mean fluorescence intensity of DSA were associated with pathogenicity of DSA in our study. These results are in agreement with a report in desensitized renal transplant recipients [23].

Study limitations

There are several limitations to our study. *De novo* production of DSA has been associated both with AMR and decreased survival in heart transplant recipients; however, this was not evaluated in our study [11,24–26]. The prevalence of CAV may have been underestimated in our study group as we did not perform routine angiographic surveillance. Consequently, this could have influenced identification of CAV risk factors. Lastly, the median follow-up of 39 months allows us to assess only intermediate-term consequences of pretransplant allosensitization.

Conclusion

Identification of preformed DSA, in addition to PRA monitoring, is a helpful predictor of AMR risk in the contemporary era of immunosuppression and rejection surveillance.

Authorship

ES, TG, MK, YV, MU, IM and TSl: designed the study, collected and analyzed the data, and wrote the manuscript. ES, LK and TS: provided and analyzed the laboratory data. LV and MK: provided and analyzed the histopathological data. MK and VL: performed statistical analysis. IN, JP, VM and JK: participated on the study design and drafted the manuscript.

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