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Sex Related Differences in the Risk of Antibody-Mediated Rejection and Subsequent Allograft Vasculopathy Post-Heart Transplantation: A Single-Center Experience

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Background. Pregnancies may result in antibodies against HLA, a risk factor for antibody-mediated rejection (AMR) and subsequent cardiac allograft vasculopathy (CAV) after heart transplantation (HTx). The aim of this study was to evaluate sex differences in the incidence of AMR events and subsequent risk of CAV among HTx recipients. **Methods.** The study comprised 160 patients (51 [32%] women) who underwent HTx in 2008 to 2014. The cumulative effect of AMR events was calculated by AMR score (sum of myocardial biopsy grading divided by number of biopsies taken during 3 years post-HTx). **Results.** Females had higher levels of anti-HLA I antibodies pre-HTx compared to males which was associated with a history of pregnancies, total number of children and with a higher AMR score at 6 months post-HTx ($P < 0.05$). Women demonstrated a significant increase in the total incidence of AMR events (27 vs. 7%, $P = 0.001$) and in AMR scores at 6, 12, 24 and 36 months post-HTx compared to men ($P < 0.05$). There were no differences in cellular rejection between the groups. A history of AMR events was associated with a significantly increased risk of severe CAV onset (hazard ratio, 7.0; 95% confidence interval, 1.5-31.5; $P = 0.012$). **Conclusions.** Women are at higher risk for AMR post-HTx which subsequently increases their risk for CAV. Females recipients may benefit from closer surveillance to identify AMR at an earlier stage post-HTx, and targeted immunosuppressive therapy to attenuate the development of CAV.

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Several studies have established that multiparous women are more prone to develop antibodies against HLAs.¹⁻³ These antibodies can increase the risk for post heart transplantation (HTx) complications, because they are directed to donor major histocompatibility complex class I and II HLA antigens that are expressed on allograft endothelial cells. To detect allosensitization, transplant candidates undergo testing that expose HLA antigens from random individuals to the recipient's serum. The results are calculated and presented as a percentage panel reactive antibody (PRA), reflecting the frequency of donors considered incompatible for the patient

based on the identified antibodies. Higher PRA was found to be associated with reduced allograft survival mostly due to immune-related causes, antibody-mediated rejection (AMR) and development of cardiac allograft vasculopathy (CAV), a leading cause of long-term mortality after HTx.³⁻¹¹

Female sex has been identified as a risk factor for rejection post HTx,¹² but there is limited data on the incidence of immune mediated complications posttransplant among women compared with men. Thus, the aim of this study was to evaluate the sex differences in the incidence of AMR events and subsequent risk of CAV and long-term survival among a cohort of HTx recipients.

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METHODS

Patient Population

The study cohort consisted of all 160 consecutive patients who underwent HTx at our institution between January 1, 2008, and May 31, 2014. All patients received perioperative induction therapy with rabbit antithymocyte globulin. Maintenance immunosuppression therapy posttransplant included calcineurin inhibitors (CNIs) (tacrolimus or cyclosporine,) mycophenolate mofetil or azathioprine, and prednisone. In our institution, we have used a strategy of tapering steroid dose within the first month after transplantation until complete withdrawal, and replacing CNI based immunosuppression with sirolimus at 1 year post-HTx according to the patient clinical status and rejection history. The study protocol was reviewed and approved by institutional review board at Mayo Clinic, Rochester, MN.

Patient Follow-Up

Routine endomyocardial biopsies (EMB) were performed to screen for both cellular rejection (CR) and AMR. All EMBs were routinely stained for C4d and were reviewed and reclassified according to histopathology and immunopathology findings assessed by the 2013 International Society for Heart and Lung Transplantation (ISHLT) grading scale. Endomyocardial biopsies were performed weekly for 4 weeks after transplantation, beginning 2 weeks after the last rabbit antithymocyte globulin dose, every 2 weeks until 2 months posttransplant, monthly from 3 to 6 months, every 3 months until the end of the first year, and yearly afterward. The frequency of biopsies subsequently varied based on clinical symptoms suggesting allograft rejection. EMB were also performed 10 to 15 days after any biopsy specimens that showed CR of grade 2R or higher and AMR of grade 1 or higher, and 2 weeks after any significant change in the immunosuppression regimen. No change in immunosuppression therapy has been made for nonclinical AMR event.

The following rejection scores were calculated for each patient at 6, 12, 24, and 36 months:

- (1) CR score was calculated as $0R = 0$, $1R = 1$, $2R = 2$ and $3R = 3$, based on 2004 ISHLT R grading, and represented the total number of rejections divided by the total number of biopsies performed during the study period.
- (2) AMR score was calculated as $p\ AMR\ 0 = 0$, $p\ AMR\ 1 = 1$, $p\ AMR\ 2 = 2$ and $p\ AMR\ 3 = 3$, based on 2011 ISHLT AMR grading, and represented the total number of rejections divided by the total number of biopsies performed during the study period.

Cardiac allograft vasculopathy was routinely assessed by the both coronary angiogram and intravascular ultrasound (IVUS) of the left anterior descending artery at 2 months after HTx and then annually in all patients. Cardiac allograft vasculopathy was defined angiographically based on the 2010 ISHLT CAV grading scale. Cardiac allograft vasculopathy classification using IVUS was: 0, normal IVUS; 1, mild atherosclerosis by IVUS; 2, moderate atherosclerosis by IVUS; 3, severe atherosclerosis by IVUS.

The development of CAV posttransplant was based on both coronary angiogram and IVUS of the left anterior descending artery at 2 months after HTx and then annually in all patients. The definition of CAV angiographically was based on the

2010 ISHLT CAV grading scale. The methods for conducting IVUS have been described elsewhere.^{13,14} Briefly, IVUS was performed during routine coronary angiography after intracoronary administration of 100 to 200 μ g nitroglycerin. Mechanical pullback (0.5 mm/s) was performed from the mid to distal left anterior descending coronary artery to the left main coronary artery with a 20-MHz, 2.9F, monorail, electronic Eagle Eye Gold IVUS imaging catheter (Volcano Therapeutics Inc., Rancho Cordova, CA) and a dedicated IVUS scanner (Volcano Therapeutics). The presence of CAV was based on visual assessment of an experienced intervention cardiologist. Cardiac allograft vasculopathy classification using IVUS was: 0, normal (without visible intimal thickening); 1, mild atherosclerosis (any visible intimal thickening < 20% occlusive); 2, moderate atherosclerosis (any visible intimal thickening < 50% occlusive); 3, severe atherosclerosis (any visible intimal thickening > 50% occlusive).

Single Antigen Bead Analysis of Alloantibodies

Pretransplant sera were screened periodically for HLA antibodies using a panel of up to 100 different color-coded beads each coated with purified single HLA class I and class II antigens (LABScreen Single Antigen Beads; One Lambda) using Luminex based technology (Luminex Corp., Austin, TX). Donor typing performed was at low-to-medium resolution and serologic equivalents were reported. In cases with more than 1 allele, if only 1 bead was positive, whereas the other was negative, based on the low to medium resolution typing and considering the common well defined allele, the bead that would correspond to the donor type was considered as donor-specific antibodies (DSA). Donor-specific antibodies were defined as HLA antibodies to the HLA antigens shared by the donor and were defined at serological equivalent levels. At our institution, mean fluorescence intensity greater than 300 is defined as a positive result for the presence of DSA.

Panel-Reactive Antibody Calculation

Panel-reactive antibody was calculated for all heart transplant candidates based on a panel of HLA antigens obtained in all organ transplant donors and recipients at our center. PRA I and II was calculated based on the presence of HLA type I and II antibodies respectively (positive alloantibodies were defined as mean fluorescence intensity >300). Calculated PRA closest to time of HTx was used for analysis of sensitization.

Statistical Analysis

Data are expressed as mean values with standard deviations or as medians with interquartile ranges, according to data distribution. Categorical data are presented by absolute numbers with percentages. Differences between women and men were compared by Independent samples Student *t* test or Mann-Whitney *U* test (for skewed data) and χ^2 test, for continuous and categorical data, respectively. Correlations between various patient's characteristics and CR and AMR scores were examined by Pearson correlation coefficient. Survival curves were estimated using the Kaplan-Meier method, whereas differences between curves were evaluated with the Log rank test. In all tests, *P* value less than 0.05 was considered to be statistically significant. Statistical analysis was

TABLE 1.
Baseline characteristics according to sex

Variable	Male	Female	P
	(n = 109)	(n = 51)	
Age at HTx ^a	52.5 ± 12.2	48.1 ± 12.2	0.034
Months of follow-up ^b	30.0 (18.3-52.8)	22.0 (11.0-43.5)	0.106
MCSO prior to HTx, n (%)	50 (45.9)	15 (29.4)	0.048
Time from MCSO to HTx, mo ^b	8.5 (5.0-17.3)	11 (5.0-16.0)	0.852
Re- HTx, n (%)	3 (2.8)	2 (3.9)	0.692
Ischemic CMP, n (%)	37 (33.9)	8 (15.7)	0.017
% PRA class I prior HTx ^b	6 (0-22)	11 (1-48)	0.007
% PRA class II prior HTx ^b	20 (0.5-46)	28 (0-46)	0.386
DSA I, n (%)	17 (19.8)	7 (19.4)	0.967
DSA II, n (%)	39 (45.3)	18 (50.0)	0.639

^a Data are presented as arithmetic mean ± standard deviation.

^b Data are presented as median with interquartile range [med (IQR)].
CMP, cardiomyopathy.

performed using IBM SPSS statistical software (SPSS for Windows, release 21.0, SPSS, Chicago, IL).

RESULTS

Patient Characteristics

The study cohort included 160 patients (32% women) and the median follow-up was 30 (38) months. Baseline patient characteristics according to sex category are given in Table 1. Female recipients were younger at the time of HTx compared with men (48.1 ± 12.2 years vs 52.5 ± 12.2 years; $P = 0.034$), with lower frequency of ischemic cardiomyopathy and less likely to get mechanical circulatory support devices (MCSO) as a bridge to HTx compared with men ($P < 0.05$). Women also had higher levels of anti-HLA I antibodies pre-HTx compared with men presented as a percentage PRA, reflecting the frequency of donors considered incompatible for the patient based on the identified antibodies (11% vs 6%, respectively, $P = 0.007$).

Posttransplant Immunosuppression

The maintenance immunosuppression therapy posttransplant included CNIs (tacrolimus or cyclosporine,) mycophenolate mofetil or azathioprine, and steroids (prednisone). During the study follow-up period, CNIs were replaced with sirolimus in 85 (53%) patients, and prednisone dose was completely discontinued in 140 (88%) patients by the end of the third posttransplant year. There were no significant differences in the incidence of sirolimus-based therapy and steroids withdrawal between female and male recipients ($P > 0.2$).

TABLE 2.
Multivariable cox regression model for prediction of antibody-mediated rejection in heart transplant patients

Variable	P	HR	95% CI for HR
Age at HTx, y	0.128	0.974	0.941-1.008
Female sex	0.017	3.211	1.232-8.370
MCSO before HTx	0.825	0.900	0.355-2.286
Ischemic CMP	0.962	0.972	0.304-3.109
% PRA class I before HTx	0.179	1.011	0.995-1.028

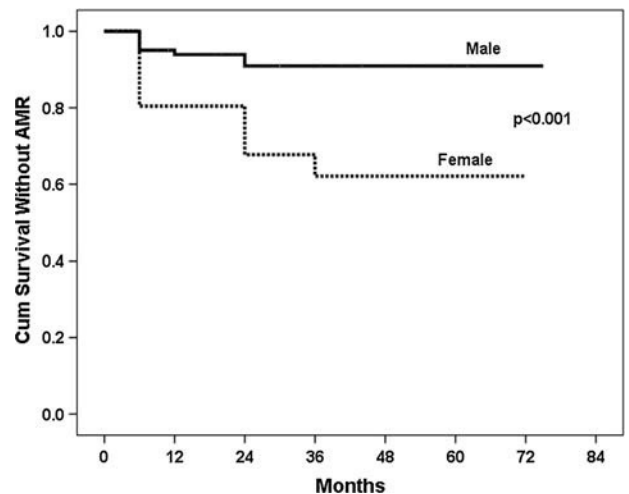


FIGURE 1. Distribution of antibody-mediated rejection over time according to sex.

Antibody-Mediated Rejection

The median number of EMB per patient was 9 during the first 6 months of follow-up, 3 for the next 6 to 12 months, 3 and 2 for the second and third years posttransplant, respectively. There was no difference in the median number of EMB per patient between women and men recipients. Overall, 21 HTx recipients developed at least 1 episode of AMR (18 patients had pAMR = 1 and 3 patients had pAMR = 2). There was a statistically significant difference between men and women in the total incidence of AMR (defined as pAMR ≥ 1) during follow-up period post-HTx (7.5% vs 26.5%, respectively, $P = 0.001$) (Figure 2). Women had 4.1 times higher odds of having AMR during the follow-up period based on Cox regression analysis (95% confidence interval [CI], 1.7-9.9; $P = 0.002$). These odds were slightly attenuated in multivariate model after adjustment for all significant baseline characteristics: age at HTx, presence of MCSO prior to HTx, presence of ischemic cardiomyopathy, % and PRA class I prior to HTx, (hazard ratio [HR], 3.2; 95% CI, 1.2-8.4; $P = 0.017$) (Table 2).

66.7% of patients had a first AMR event in the first 6 months after HTx, whereas 33.3% developed AMR after this period (14 vs 7 patients, respectively). The distribution of AMR over time according to sex is shown in Figure 1. The Kaplan-Meier curve shows a statistically significant difference in time to first AMR event between the sexes (Log rank = 12.381; $P < 0.001$). Female sex was not significantly related to CR in our study population (HR, 1.7; 95% CI, 0.8-3.5; $P = 0.168$). The results of Cox-regression multivariate model for prediction of CR are presented in Table 3, whereas Kaplan Meier curve is shown in Figure 2.

TABLE 3.
Multivariable cox regression model for prediction of cellular rejection in heart transplant patients

Variable	P	HR	95% CI for HR
Age at HTx, y	0.027	0.969	0.942-0.996
Female sex	0.347	1.447	0.670-3.127
MCSO before HTx	0.811	0.912	0.426-1.949
Ischemic CMP	0.443	0.671	0.243-1.859
% PRA class I before HTx	0.819	1.002	0.986-1.019

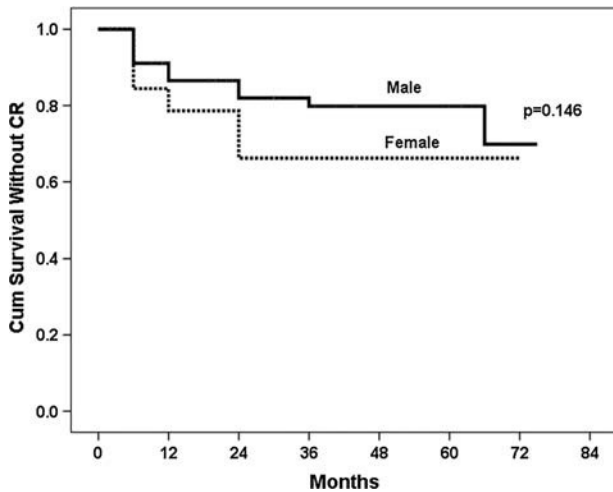


FIGURE 2. Distribution of cellular rejection over time according to sex.

Anti-HLA Class I Antibodies and AMR

Higher level of anti-HLA I antibodies presented as a PRA percentage before HTx was significantly associated with a history of pregnancy and to the total children number ($r = 0.229, P = 0.004$ and $r = 0.218, P = 0.047$, respectively) (Figures 3 and 4). There was also significant association between higher PRA I percentage before HTx and AMR during the first 6 months posttransplant calculated as a total incidence of AMR and as AMR score ($r = 0.197, P = 0.014$ and $r = 0.192, P = 0.017$).

Antibody-Mediated Rejection and CAV

Overall survival rates were not significantly different between women and men recipients during the follow-up period (90% vs 92%, $P = 0.5$). There was no significant difference in allograft function between male and female recipients. Although female recipients had significantly higher incidence of AMR during the follow-up period, there was not enough power to show statistical significant difference in moderate/severe or severe CAV incidence in female compared to male (16% vs 17%, $P = 0.86$; 7% vs 4%, $P = 0.39$, respectively). Adjusting for PRA levels did not alter these results. Nevertheless, in the entire cohort, history of any AMR event

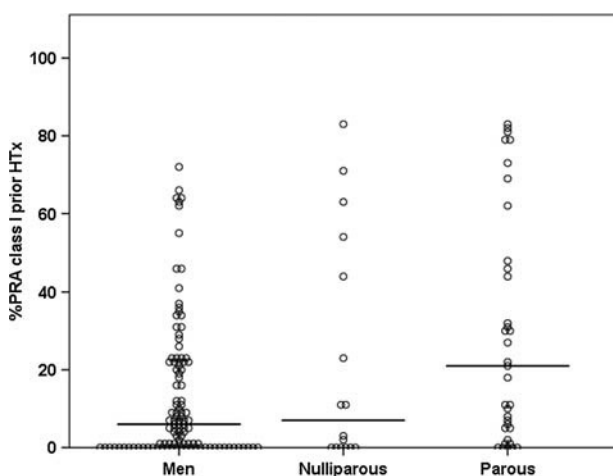


FIGURE 3. Association between history of pregnancies and PRA percentage prior to heart transplantation.

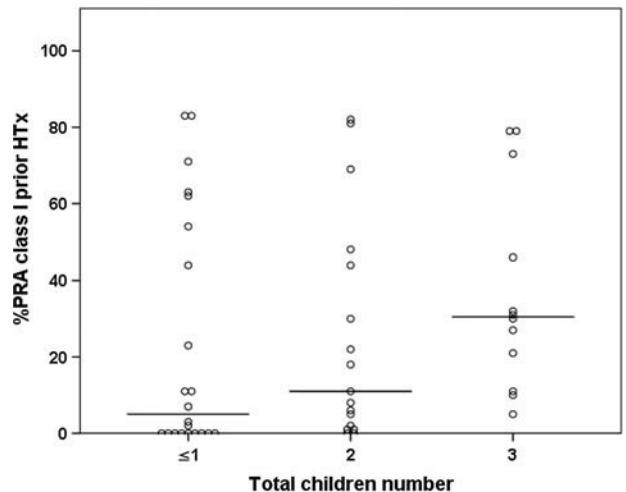


FIGURE 4. Association between total children number and PRA percentage prior to heart transplantation.

significantly increased the risk of developing severe CAV in the Cox regression analysis (HR, 7.0; 95% CI, 1.5-31.5; $P = 0.012$). By Kaplan-Meier analysis, history of AMR was associated with a significantly shorter time to severe CAV onset (log-rank test, $P = 0.003$) (Figure 5). There was no difference in Sirolimus use between patients with and without moderate/severe CAV, but it seems that Sirolimus had a protective effect with respect to any degree of CAV (including mild, moderate, and severe CAV). By the end of the third year of follow-up, 90% of patients without sirolimus versus 62% of patients treated with sirolimus developed any degree of CAV ($P = 0.04$). Sirolimus use did not have effect on pAMR or overall survival ($P > 0.05$ for both).

DISCUSSION

Our study has 2 primary findings. First, we identified female recipients as a high-risk group with increased risk for AMR post HTx. Second, we demonstrated a significant association between any degree of AMR and increased risk of severe CAV onset. These findings have important clinical application in the transplantation field. The evolution after the

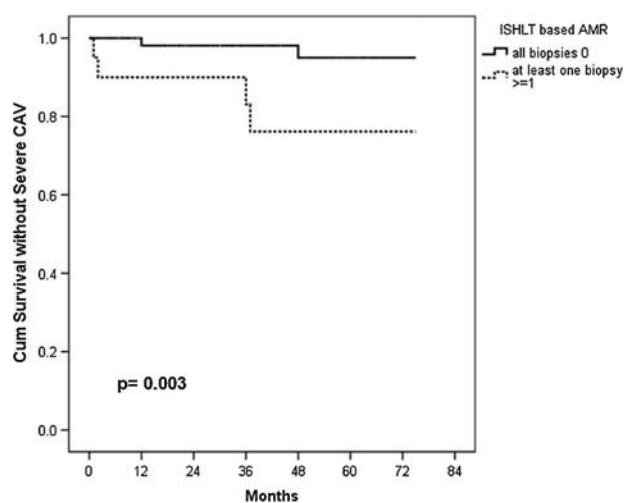


FIGURE 5. Severe CAV-free survival according to AMR.

development of more effective immunosuppressive agents that improved the posttransplant survival and allograft function has led to a new direction of individualization of the posttransplant approach in order to adjust the intensity of the immunosuppressive therapy and the frequency of rejection surveillance according to the recipient's profile. Our findings suggest that female recipients may benefit from more frequent EMB, coronary imaging studies, and targeted immunosuppressive therapy post-HTx to attenuate the development of CAV.

Female sex has been shown in registry data reports to be a risk factor for rejection post-HTx,¹² but thus far there is limited data on the difference in rejection incidence between women and men, especially in the era of MCS as a bridge to HTx and the changes in immunosuppression therapy. Our findings reinforce the existing data on the significant association between higher level of allosensitization in women before HTx and the risk of AMR posttransplant. We showed that women had significantly higher incidence of AMR post-HTx compared with men, and that the sex differences in AMR appears early during the first 6 months posttransplant and remain consistent for at least 3 years of follow up.

Allosensitization has been also found to increase the risk of developing CAV.^{7,9,11,15} Cardiac allograft vasculopathy causes diffuse concentric stenosis of the coronary arteries due to intimal expansion and adventitial sclerosis, and is a major cause of allograft failure and mortality after HTx. This pathologic process is related to antibody and complement mediated injury, which are the main components of AMR, and especially antibodies against donor HLA class I which are commonly expressed in endothelial cells.

The diagnosis of AMR has been standardized only since 2011 when the ISHLT proposed criteria for common definition and standardized diagnostic scale of AMR.¹⁶ The reported incidence of AMR prior to the publication of this report varies in different studies depending on the patient cohort and the diagnostic methods used, which were mainly based on clinical manifestation of allograft dysfunction. The 2011 AMR diagnostic criteria are based on the combination of histopathologic and immunopathologic findings regardless of the clinical manifestations and introduced for the first time the entity of subclinical-asymptomatic AMR according to EMB findings without signs of allograft dysfunction.

Several studies have raised the question whether the presence of asymptomatic AMR is clinically relevant and have shown that this form of rejection still carries a higher likelihood of CAV and mortality.^{17,18} Wu et al¹⁷ reported that asymptomatic AMR was associated with greater development of CAV, which was similar in frequency to recipients with clinical manifestation of AMR required medical therapy.¹⁷ In the present study, we also showed a significant association between the history of any degree of AMR and onset of severe CAV. Our findings highlight the importance of pathology based diagnosis of AMR according to the recent ISHLT criteria.

Whether maintenance immunosuppression should be altered due to asymptomatic AMR events remains untested and subject to debate. In our institution we have used a strategy of close observation and follow-up for evidence of graft dysfunction among these patients, and in selected cases we have held or slowed down steroid tapers for several months. Our main strategy is to take more aggressive measures to

prevent CAV by using immunosuppressive agents, such as sirolimus, that have demonstrated benefit in delaying the onset or slowing the progression of CAV in recipient with high risk for AMR.¹⁹⁻²¹ Prospective, randomized studies to treat asymptomatic AMR events will be needed to demonstrate any benefit of treatment to improve outcome.

The use of MCS as a bridge to HTx has been reported as a risk factor for allosensitization resulting in an increased PRA.^{22,23} In the present cohort, female recipients were less likely to get MCS as a bridge to HTx compared to men, and there was a significant association between history of pregnancies and total children number to PRA I levels, suggesting that multiparity is the main reason for allosensitization among women. Although MCS should be used in female patients with advanced heart failure as a bridge to HTx based on the same clinical indications as in male patients, it is unclear whether this approach may result in a greater degree of allosensitization compared with male patients.

The higher incidence of AMR in women observed in this study and the association with CAV did not translate to worse survival posttransplant compared to men. One possible explanation is the small cohort with lack of statistical power to demonstrate significant changes between the 2 groups. Similarly, previous studies comparing male and female survival post-HTx have described comparable long-term survival.²⁴⁻²⁷ The women in most of these studies, including the present cohort, were significantly younger compared with men at the time of transplantation, and the survival analysis was not corrected to the natural longer predicted survival of women compared with men.

We acknowledge some notable limitations in our study. First, this is an observational study with retrospective analysis of prospectively collected data. Second, the study was conducted in a single tertiary medical center; hence, there may be patient selection bias. Third, due to the retrospective nature of the study, there are no data regarding DSA post-HTx as well as level of DSA at the time of AMR events. Fourth, the small patients' cohort may lack the statistical power to identify significant sex related differences in the incidence of CAV which may subsequently cause significant differences in long-term survival between female and male recipients.

In conclusion, multiparous women are at higher risk for allosensitization before HTx which is associated with higher incidence of AMR post-HTx compared with men, and subsequently increases their risk for CAV. Female recipients may benefit from closer surveillance to identify AMR at an earlier stage post-HTx, as well as targeted immunosuppressive therapy to attenuate the development of CAV. Prospective larger studies will be needed to demonstrate whether effective therapy to reduce allosensitization pretransplant and to treat asymptomatic AMR events posttransplant will improve outcome among female HTx recipients.

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