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HLA class II antibodies at the time of kidney transplantation and cardiovascular outcome: a retrospective cohort study

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Abbreviations and acronyms

95%CI: 95% confidence interval; ACS: acute coronary syndrome; aHR: adjusted hazard ratio; BMI: body mass index; BPAR: biopsy-proven acute rejection; CAD: coronary artery disease; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration equation; CMV: cytomegalovirus; CVA: cerebrovascular accident; DC: dendritic cell; DCD: donation after circulatory death; DNA: deoxyribonucleic acid; DSA: donor-specific antibodies; eGFR: estimated glomerular filtration rate; ELISA: enzyme-linked immunosorbent assay; HDL-cholesterol: high-density lipoprotein cholesterol; HLA: human leucocyte antigen; IFN-ɣ: interferon gamma; IQR: interquartile range; KTR: kidney transplant recipient; LDL-cholesterol: low-density lipoprotein cholesterol; MACCE: major adverse cardiac and cerebrovascular event; MFI: mean fluorescence intensity; MHC: major histocompatibility complex; PAD: peripheral artery disease; (c)PRA: (calculated) panel reactive antibodies; mTOR: mammalian target of rapamycin; non-STEMI: non-ST-segment elevation myocardial infarction; OxLDL: oxidized LDL-cholesterol; PCR: polymerase chain reaction; PCR-SSO: sequence-

specific oligohybridisation primed PCR; PCR-SSP: sequence-specific primer PCR; SLE: systemic lupus erythematosus; STEMI: ST-segment elevation myocardial infarction; TIA: transient ischemic attack; Tfh cells: follicular helper T cells; TNF: tumor necrosis factor.

ABSTRACT

Background. The negative role of HLA class II DSA on graft outcome is well-recognized. However, the potentially negative cardiovascular effects of preformed HLA class II antibodies and donor HLA in kidney transplant recipients (KTR) remain unestablished.

Methods. We conducted a single-centre, retrospective cohort study including 1115 KTR (2003-2016) with up to 4449 person-years of follow-up after transplantation and a median follow-up time of 5.1 years (IQR: 2.7-7.6). We evaluated the unadjusted and multivariable-adjusted association between pretransplant HLA class I and II antibodies, as well as HLA-DR1 donor/recipient genotype and the primary (MACCE or all-cause mortality) and secondary (MACCE or cardiovascular mortality) outcome.

Results. In a multivariate Cox proportional hazard model, HLA class II antibodies prior to transplantation were associated with increased adjusted hazard ratio (aHR) for MACCE or all-cause mortality (aHR 1.71 [1.13-2.60]; *p*=0.012) even after adjustment for time-varying covariates graft loss (aHR 1.68 [1.08-2.62]; *p*=0.022) and biopsy-proven acute rejection (aHR 1.71 [1.13-2.60]; *p*=0.012). HLA class II antibodies were also associated with increased aHR for the secondary outcome, MACCE or cardiovascular mortality (aHR 1.92 [1.12-3.30]; *p*=0.018). We investigated the effect of donor and recipient HLA-DR1 on these outcome parameters and demonstrated that KTR with HLA-DR1 positive donors had an increased aHR for MACCE with all-cause (aHR1.45 [1.09-1.94], *p*=0.012) and cardiovascular mortality (aHR1.49 [1.00-2.22], *p*=0.05).

Conclusions. Prior sensitization against HLA class II antigens is associated with unfavourable long-term cardiovascular outcome in KTR independent of graft loss or rejection. Recipients of a HLA-DR1 donor also have an impaired cardiovascular outcome.

Introduction

In the general population, the immune system is involved in the pathogenesis of hypertension, atherosclerosis, coronary artery disease (CAD) and hence cardiovascular disease (CVD).[1-3] Emerging evidence suggests that the same might hold true in transplant recipients.[4-6] Atherosclerosis is the leading cause of CVD and inflammatory components participate at each stage of atherogenesis.[2,7-9] Genes involved in inflammation are associated with progression of CAD in rheumatoid arthritis, inflammatory polyarthritis, systemic lupus erythematosus (SLE), type 1 diabetes and in heart transplant recipients.[9-12] The increased risk of cardiovascular events in auto-immune disease suggests that autoimmunity and CVD are intertwined.

The major histocompatibility complex (MHC) is involved in both the recognition of foreign antigens and the development of autoimmune diseases.[13,14] HLA class II is up-regulated in autoimmune disease and atherosclerosis where it is expressed on dendritic, smooth-muscle and inflammatory cells within the atherosclerotic plaque.[15,16] It seems plausible that pro-inflammatory HLA antibodies could cause endothelial dysfunction and affect cardiovascular outcome.[5,6,17] Also certain HLA class II antigens, in particular HLA-DR1, are associated with increased cardiovascular risk in different populations probably through endothelial dysfunction and pro-inflammatory characteristics.[2,10-12,15,18-21] In lung transplant recipients, specific HLA-DR donor serotypes significantly affect lung graft outcome.[22]

Considering that the presence of certain HLA class II molecules (HLA-DR1) and HLA class II antibodies are associated with increased cardiovascular risk, we speculated that this might also hold true for kidney transplant recipients (KTR) independent of graft function. Our primary objective was to assess and compare the long-term outcome in KTR with versus without preformed especially HLA class II or class I antibodies. Our second objective was to assess the same outcome parameters in KTR according to recipient and donor HLA-DR1 genotype.

Materials and Methods

Summary

We conducted a single-centre, retrospective cohort study including 1115 adult KTR who underwent transplantation between January 1, 2003 and December 31, 2016 with follow-up until loss to follow-up or November 30, 2017. Using Cox proportional hazard regression modelling we evaluated the unadjusted and

multivariable-adjusted association between quarterly determined pretransplant HLA class I and II antibodies, as well as HLA-DR1 donor/recipient genotype and the primary (MACCE or all-cause mortality) and secondary (MACCE or cardiovascular) outcome.

Setting

A preexisting database containing data on all adult (aged ≥18 years) KTR who underwent transplantation at Ghent University Hospital (Ghent, Belgium) since 2003 was used to obtain relevant transplant baseline characteristics. We censored patients at time of death, loss to follow-up or November 30, 2017.

Study participants

We conducted a single-centre cohort study including 1115 adult KTR who underwent transplantation between January 1, 2003 and December 31, 2016. Details about immunosuppressive and prophylactic drugs are described in the "Supporting Information" section. In patients with previous kidney transplantation, we analysed the outcome of the last transplantation. The study was approved by the local ethics committee (B670201731678) and is in agreement with the principles of the Declaration of Helsinki.

Exposure and outcome definition

Primary exposure

The primary exposure was the presence of circulating HLA class I or II antibodies determined at quarterly intervals in waitlisted patients. Anti-HLA antibody screening was performed with an ELISA method (Lifecodes) from 2003 to 2008 and later with a Recombinant Single Antigen Bead assay on a Luminex platform (Lifecodes/Immucor). From 2012 on, anti-HLA antibody screening was considered positive if the mean fluorescence intensity (MFI) was ≥500. We define antigens as unacceptable and preclude transplantation when donor-specific antibodies (DSA) have MFI >2000.

Secondary exposure

Routine HLA class II typing of recipient and donor for HLA-DR1 was performed by DNA typing methods using sequence-specific oligo-hybridisation primed polymerase chain reaction (PCR-SSO) for patient and retrospective organ donor typing; and sequence-specific primer PCR (PCR-SSP) for donor typing.

Primary outcome

The primary outcome of interest was time to composite end-point of major adverse cardiac and cerebrovascular events (MACCE), defined by acute coronary syndrome (ACS) including ST-segment elevation (STEMI), non-ST-segment elevation myocardial infarction (non-STEMI), unstable angina or coronary revascularization, transient ischemic attack (TIA) and cerebrovascular accident (CVA) or all-cause mortality.

Secondary outcome

The secondary outcome was time to MACCE or cardiovascular death. Cardiovascular death was defined as death attributable to ACS, heart failure or sudden cardiac death.

Covariates

We extracted patient characteristics and outcome data from a compiled preexisting database which registers an extensive list of covariates which are described in the "Supporting Information" section.

We recorded the time to biopsy-proven acute rejection (BPAR), defined as acute T-cell mediated or antibody-mediated rejection as interpreted by the pathologist according to Banff classification. We also recorded time to graft loss, defined as the permanent (>30 days) need for renal replacement therapy. We also registered the estimated glomerular filtration rate (eGFR) using the abbreviated four-variable Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI) at three months, one, three and five years.[23]

Statistical analysis

We summarized descriptive statistics as mean ± standard deviation (SD) for normally distributed continuous variables or median with interquartile range (IQR) for skewed distributions. We performed pairwise deletion to handle missing values; however, apart from eGFR and cholesterol measurements, less than 1% of covariate data were missing.

We created a graphical representation of causal effects between variables by the causal directed acyclic graph (DAG) approach in order to visualize the relationships between exposure and outcome of interest as well as associated variables (Figure S1, SDC, http://links.lww.com/TP/B783). We plotted Kaplan-Meier survival curves for primary and secondary outcomes in patients with versus without HLA class I or II antibodies and compared them by log-rank test. We used Cox proportional hazard regression to evaluate the unadjusted and

multivariable-adjusted association between HLA class I and II antibodies and the primary and secondary outcome. We assessed the assumption of proportionality for each covariate by constructing log cumulative hazard plots and by visually examining the Kaplan-Meier plots.

We designed a series of models including an unadjusted (Model 1) and multivariable-adjusted (Models 2 to 4) Cox proportional hazard regression model with backward elimination to relate the primary exposure (the presence of HLA class II antibodies) with the primary and secondary outcome of interest. Model 2 included covariates associated with graft outcome based on existing literature and covariates with *P* value of <0.1 in the univariate analysis (Supplemental Data, SDC, http://links.lww.com/TP/B783): recipient age, recipient sex, panel reactive antibodies (PRA), retransplantation, smoking status, polyclonal induction, baseline CVD and diabetes mellitus. In Model 3 and 4 we used the covariates of Model 2, but respectively adjusted for BPAR and graft loss as time-dependent covariate. We also conducted additional analyses by stratifying for year of transplantation (2003-2007 versus 2008-2016), since the sensitive Luminex-based identification of HLA antibodies was only used after 2007.

We conducted additional secondary analyses to ascertain the robustness of our findings and analysed whether the presence of HLA class I or II antibodies would have different effects on MACCE with all-cause or cardiovascular mortality in specific subgroups: recipient age ≤ or >60 years, BPAR (yes/no), graft loss (yes/no) and retransplantation (yes/no). For each of these covariates, we analysed a potential statistical interaction with HLA antibody positivity.

In an attempt to eliminate the effect of DSA, we performed separate analyses by excluding patients with DSA at time of transplantation in patients with preformed HLA class II antibodies. To explore the association between anti-HLA sensitization (calculated PRA, cPRA) and the primary outcome, we assessed the correlation by constructing scatter plots and calculating the Pearson (r) correlation coefficient.

We also evaluated the unadjusted association between donor and recipient HLA-DR1 status and the same outcome parameters in the overall study population (Model 5). Although the allocation of HLA-DR1 donor kidney is at random, apart from allocation to HLA-DR1 positive recipients, we performed an additional multivariate-adjusted Cox proportional hazard regression model (Model 6) including covariates associated with graft outcome and covariates with *P* value <0.1 in the univariate analysis (Supplemental Data, SDC,

http://links.lww.com/TP/B783): recipient HLA-DR1 status, recipient age, recipient sex, smoking status, polyclonal induction, baseline CVD and diabetes mellitus. In Model 7 and 8 we used the covariates of Model 6, but respectively adjusted for BPAR and graft loss as time-dependent covariate.

Unadjusted and adjusted hazard ratios (aHR) were assessed with 95% confidence interval (95%CI). A two-sided *P* value <0.05 was considered to indicate statistical significance for all analyses. Statistical analyses were performed with SPSS® (IBM SPSS Statistics for Windows, version 25.0; Armonk, NY: IBM Corp). The study was reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology statement.[24]

Results

Baseline characteristics

In 828 of 1115 patients the presence of circulating HLA class I or II antibodies was measured prior to or at the time of transplantation (Figure S2, SDC, http://links.lww.com/TP/B783). Baseline characteristics of the study cohort and patients with HLA class II antibodies are described in Table 1. For more detailed information of the study cohort according to HLA class I and/or class II status we refer to Table S1 (SDC, http://links.lww.com/TP/B783). The median age of the study participants was 54 years, 62% were male and 19% had diabetes mellitus at the time of transplantation. The total number of person-years of follow-up after transplantation was 4494 years and the median follow-up time was 5.1 years (IQR: 2.7-7.6).

Meanwhile, 78 patients (9.4%) developed adverse cardiac and cerebrovascular events. Eighty-four patients (10.1%) died during follow-up: infections (N=22; 26.2%), cancer (N=17; 20.2%), cardiac (N=6, 7.1%), other (N=9; 10.7%) and unknown (N=30; 35.7%). A total of 138 patients (16.7%) had a MACCE or died and 79 patients (9.5%) a MACCE or cardiovascular death.

Overall, 74 (8.9%) participants had pretransplant HLA class I antibodies, 116 (14%) HLA class II antibodies and 41 (5%) had combined class I and II antibodies. The clinical characteristics of patients with and without preformed HLA class II antibodies are depicted in Table 1. Patients with versus without HLA class II antibodies were more often female, had more previous transplantations, PRA > 0% and received more polyclonal induction.

In the patients with HLA class II antibodies (N=116), 68 (58.6%) patients had no DSA, 14 (12.1%) had DSA and in 34 (29.3%) we have no data of DSA at time of transplantation. We lack data on DSA at time of transplantation in patients transplanted before 2008.

BPAR occurred in 121 patients (14.8%) and graft loss in 60 patients (7.4%). The incidence of both did not differ between patients with or without HLA class I or II antibodies (respectively $P = 0.243$ and $P = 0.570$ for BPAR; $P = 0.881$ and $P = 0.609$ for graft loss). During follow-up, eGFR did not differ between participants with versus without HLA antibodies.

Primary outcome analyses

Participants with HLA class II antibodies had an estimated 65% higher hazard for MACCE or all-cause mortality than patients without HLA class II antibodies (95%CI of 1.09-2.49) (Figure 1a; Table 2: Model 1). The presence of HLA class II antibodies remained associated with an increased hazard for MACCE or all-cause mortality in the multivariate model (aHR 1.71 with 95%CI of 1.13 to 2.60) (Table 2: Model 2). This risk remained increased when BPAR or graft loss were included as time-dependent covariates (Table 2: Model 3 and 4).

The presence of HLA class I antibodies was not statistically associated with a higher hazard for MACCE or all-cause mortality (Table 2: Model 1 to 4).

Secondary outcome analyses

In patients with versus without HLA class II antibodies, the secondary outcome analysis also revealed a higher event rate for MACCE or cardiovascular mortality (Figure 1b) with an unadjusted HR of 1.78 [95%CI, 1.04 to 3.04] and a multivariate-adjusted HR of 1.92 [95%CI, 1.12 to 3.30] (Table 2: Model 1 and 2). Pretransplant HLA class II antibodies remained associated with MACCE or cardiovascular mortality when BPAR or graft loss were used as time-dependent covariates (Table 2: Model 3 and 4).

HLA class I antibodies were not significantly associated with MACCE nor cardiovascular mortality (Table 2: Model 1 to 4).

Additional analyses

In patients with versus without HLA class I or II antibodies, the unadjusted hazard ratio for all-cause mortality was not increased. However, the multivariable-adjusted hazard ratio for all-cause mortality in patients with HLA class II antibodies was increased with an aHR of 1.84 [95%CI, 1.05 to 3.22] (P = 0.033).

The increased risk for MACCE or all-cause mortality in patients with versus without preformed HLA class II antibodies was confirmed in subgroup analyses excluding patients with BPAR or graft loss (aHR 1.88 [95%CI, 1.22 to 2.92] and 1.98 [95%CI, 1.25 to 3.12] respectively). When excluding both groups the aHR increased even further to 2.20 [95%CI, 1.37 to 3.52] (Figure 2a).

The increased risk for MACCE or cardiovascular mortality in patients with HLA class II antibodies was also confirmed in subgroup analyses excluding patients with BPAR or graft loss (aHR of 1.97 [95%CI, 1.10 to 3.53] and 1.91 [95%CI, 1.08 to 3.40] respectively). When excluding both groups the aHR for MACCE or cardiovascular mortality increased even further to 2.09 [95%CI, 1.14-3.82] (Figure 2b).

When patients with HLA class II antibodies and preformed DSA were excluded from the primary and secondary analyses, we found identical but more pronounced results (Table S2, SDC, http://links.lww.com/TP/B783).

There was a clear correlation between the cPRA and the primary outcome of interest (Figure 3 and Figure S3, SDC, http://links.lww.com/TP/B783).

By performing separate sensitivity analyses across different subgroups, we confirmed these findings in patients independent of age (recipient age ≤ or > 60 years) and in patients with a first kidney transplantation. There were no significant interactions between HLA class II antibodies and BPAR, graft loss, recipient age or retransplantation (Table 3, Figure 4).

We also conducted additional analyses according to year of transplantation (2003-2007 versus 2008-2016) considering the Luminex-based identification of HLA antibodies since 2008. Our findings remained robust in KTR transplanted between 2008 and 2016 (Figure S4 and S5; Table S3, SDC, http://links.lww.com/TP/B783).

We performed separate sub-analyses in patients uniquely induced by basiliximab: the HR of MACCE or all-cause mortality was 1.65 (95%CI 1.09-2.49; p=0.019) in the univariate model and 1.71 (95%CI 1.13-2.60;

p=0.012) in the multivariate model. The HR of MACCE or cardiovascular mortality was 1.78 (95%CI 1.04-3.04; p=0.036) in the univariate model and 1.92 (95%CI 1.12-3.30; p=0.018).

Donor HLA class II antigens

Overall (N=1115), 238 (21.3%) participants were HLA-DR1 positive, 227 (20.4%) had a HLA-DR1 positive donor and 145 (13%) were HLA-DR1 donor and recipient positive.

The unadjusted HR for all-cause mortality for patients with versus without HLA-DR1 *donor* was not significantly increased (HR 1.41 [95%CI, 0.99 – 2.00], *P* = 0.056). However, KTR with versus without HLA-DR1 positive *donors* had more cardiovascular events (Figure 5a) with an unadjusted and adjusted HR for MACCE or all-cause mortality of 1.45 [95%CI, 1.09-1.94] and 1.72 [95%CI, 1.31-2.26] respectively (Table 4: Model 5 and 6). The risk for MACCE or all-cause mortality when either BPAR or graft loss were used as a time-dependent covariate remained increased (Table 4: Model 7 and 8). However, the presence of HLA-DR1 positivity in the *recipient* was not associated with an increased risk for MACCE or all-cause mortality. Furthermore, the interaction term between HLA-DR1 recipient and donor positivity was not significant (*P* = 0.432).

The risk for MACCE or cardiovascular mortality (Figure 5b) was also significantly increased in KTR with versus without HLA-DR1 positive *donor* after adjusting for other risk factors and when BPAR or graft loss were included as time-varying covariates (Table 4: Model 5 to 8). The interaction term between HLA-DR1 recipient and donor positivity was not significant (*P* = 0.345).

Discussion

We found an association between sensitization against HLA class II, but not HLA class I, antigens prior to kidney transplantation and unfavourable long-term cardiac and cerebrovascular outcome. The robustness of this association was ascertained after adjustment for confounders and through sensitivity analyses. Our study is the first to investigate a negative cardiovascular outcome in KTR with pretransplant HLA class II antibodies and this independent of graft outcome nor incidental rejection. Also, we are the first to demonstrate that *donor* HLA-DR1 might confer an increased cardiovascular risk in KTR.

Our results are in support of two recent studies. Sapir and co-workers demonstrated a dose-dependent relationship between PRA and both all-cause and cardiovascular mortality in wait-listed kidney transplant

candidates.[4] In the second study, performed by Loupy and co-workers, the presence of circulating DSA six months after renal transplantation in patients with severe and minimal arteriosclerosis was associated with respectively a 2.5- and 4.1-fold increase of major cardiovascular events.[5] Our results are of additional value because of the exploration of the role of *preexisting* HLA antibodies next to the extensive follow-up.

A systemic inflammatory state accelerating atherogenesis or destabilising vulnerable plaques may explain our findings. Accumulating evidence from experimental and observational studies points to a role of autoimmunity and inflammation in atherosclerosis and CVD.[2] The MHC alleles are involved in CVD of which HLA class II molecules are of particular interest considering their fundamental role in adaptive immunity.[11-13,15,20,25] They are expressed on dendritic cells (DC) within the atherosclerotic plaque and present extracellular peptide fragments of internalised oxidized low-density lipoprotein (OxLDL) to CD4+ T helper cells which secrete pro-inflammatory cyto- and chemokines.[15,26] HLA-DR is expressed on endothelial cells of coronary arteries and, especially in acute coronary syndromes, its expression on these cells and on $CD4^+$/$CD8^+$ T cells is upregulated.[26,27] HLA-DRB1 is considered to be "the notorious gene in the mosaic of auto-immunity" since it seems to determine the development of specific auto-antibodies and may lead to an abnormal immunological response to nonnoxious stimuli.[28] According to a meta-analysis, the HLA-DRB1*01 (*HLA-DR1*) allele is associated with an almost three times higher odds of antibody response after hepatitis B vaccination.[29] HLA-DRB1 is particularly associated with auto-immune diseases such as SLE and rheumatoid arthritis; both conditions which share an increased risk of cardiovascular disease.[30-32] In patients with SLE or antiphospholipid syndrome, autoantibodies were associated with accelerated atherosclerosis and cardiovascular events giving fuel to the hypothesis that HLA-antibodies are possibly reflecting exaggerated auto-immunity.[33]

Furthermore, by decreasing the endothelial secretion of the cytoprotective and anti-inflammatory glycoprotein thrombomodulin, HLA class II but not class I antibodies create a prothrombotic and proinflammatory state.[17] The expression of HLA class II is upregulated in many autoimmune diseases and this could contribute to the higher incidence of CVD.[10,11,15] Exposure to HLA class II rather than class I antibodies causes human allogeneic DC to preferentially differentiate naïve CD4+ T cells into follicular helper T (Tfh) cells.[34] Increased concentrations of circulating Tfh cells were found in patients with CAD and correlate with disease

activity in various autoimmune diseases.[35] The crosstalk between DC and T cells drives the inflammatory response not only within the graft,[34] but also in atherosclerotic plaques. Tfh cells expand in parallel with a loss of CD8+ T regulatory cells and promote atherosclerosis in murine and human aortas.[36]

Our finding that KTR who received their graft kidney from an HLA-DR1 positive *donor* have a higher cardiovascular risk is also supported by epidemiological studies. People with type 1 diabetes with HLA class II haplotypes DRB1*04-DQB1*03 have a higher incidence of cardiovascular events.[15] Also, in the general population and in heart transplant recipients, the haplotypes DRB1*01 and DRB1*04 were associated with a higher risk of CAD, myocardial infarction, a higher number of pro-inflammatory and cardiotoxic CD4+CD28null T cells in the peripheral blood, an increased high-sensitivity C-reactive protein, and increased production of interferon-ɣ upon stimulation.[7,12,19,20,37] In patients with rheumatoid arthritis, the HLA DRB1*04 genotype was associated with increased all-cause and cardiovascular mortality and with endothelial dysfunction without evidence of linkage disequilibrium with TNF-genes.[10,21] In contrast, some genome-wide association studies revealed no evidence of linkage of CAD to the HLA class II region,[25,38] possibly reflecting the multifactorial nature of human atherosclerosis.[2] It is likely that certain combinations of protective and harmful genes and nongenetic or epigenetic factors modify inflammation and promote atherogenesis.[2,12,13,20] In this respect, it would be interesting to further investigate the interplay between recipient and donor MHC in various study populations.

Our study has strengths and limitations. Our analysis with assessment of prespecified endpoints and almost without missing clinical data demonstrated a robust association across different subgroup analyses after broad adjustment for potential confounders. Considering the absence of an association between HLA class II antibodies and rejection or graft loss, our results refute that the difference in cardiovascular outcome is attributable to differences in kidney function or premature graft loss. Our observations are reminiscent of registry data in KTR where HLA class II antibodies in the absence of HLA class I antibodies were not associated with renal graft loss.[39] Furthermore, our finding of a negative outcome of KTR with HLA-DR1 positive donors has a low potential for selection bias considering HLA-DR1 serostatus in the recipient is the sole modifier of allocation of these grafts. Of note, HLA-DR1 recipient status was not associated with unfavourable cardiovascular outcome in our analysis.

A limitation of our retrospective analysis is that we did not screen DSA on fixed time points after transplantation. Pretransplant anti-HLA antibodies are associated with the presence of posttransplant antibodies including DSA.[40] We did not integrate DSA because of the small number of positive patients after transplantation in our analysis with inherent bias. Nevertheless, provided that HLA antibodies play a causal role in atherogenesis or in rupture of vulnerable plaques, we expect this to be especially true for DSA. In an attempt to overcome this limitation, we performed additional analyses excluding patients with DSA at time of transplantation, hereby finding an even more pronounced risk for MACCE and all-cause or cardiovascular mortality. Interestingly, by correlating the cPRA and the primary outcome of interest, we also found a clear correlation suggesting a dose dependent relationship between allosensitization and negative outcome. We could not correct for proteinuria and eGFR in our analyses, but we did demonstrate that there were no differences in eGFR assessed until 5 years posttransplantation. Considering the retrospective character of our study, we lack data on proteinuria in most of our study population and this could not be integrated as a time-dependent covariate in our multivariate model. As collinearity between proteinuria and rejection can be suspected and as early posttransplant proteinuria is mainly associated with donor characteristics we believe this is not an important flaw in our analysis. Although the ongoing exposure to immunosuppressive drugs could also not be measured accurately, we did confirm our association of HLA class II antibodies and increased cardiovascular risk in first KTR as well as in patients receiving basiliximab induction.

Most of our patients with class II antibodies had DR-antibodies (with or without DP or DQ antibodies). It was however not possible to differentiate between the individual antibodies. Also, in the paper by Loupy et al. this was a potential flaw which can and should be addressed in future prospective evaluations.[5] Certainly, it can be speculated that increased granularity in the data by further separation of HLA antibodies into different categories will possibly decrease the likelihood of finding an association and most likely we will need registry data to address this important issue more thoroughly.

Other weaknesses are the potential for unrecognized confounders, the lack of longitudinal analysis and qualitative assessment of HLA antibodies after transplantation and the lower sensitivity of the detection method in the earlier years of registration. If anything, we believe the latter has rather attenuated than strengthened the association demonstrated by our subanalyses according to year of transplantation.

We believe our findings could translate into a better risk stratification in KTR. They might also shed a new light on the pathophysiology of atherosclerosis, in which emerging data indicate that anti-inflammatory strategies might improve survival.[41] Another interesting concept would be to examine the relationship between HLA class II antibodies and cardiovascular events/mortality in nontransplanted waitlisted dialysis patients. This hypothesis should however be tested in a large (and preferably multicenter) cohort as concerns of lack of power seem imminent.

Though speculative, our findings could support an active role for micro-chimerism, which is a common but neglected phenomenon in KTR,[42,43] in the pathophysiology of posttransplant CVD. Micro-chimerism has been associated with both graft acceptance and increased acute rejection after kidney transplantation.[36,44] Disparities in its clinical effect may result from inter-patient differences in the amount or subtypes of microchimeric cells.[43]

Our findings of a potential negative role of donor but not recipient HLA-DR1 serostatus might embody a negative immunological constellation. In lung transplant recipients, specific HLA-DR donor serotypes are associated with an increased risk of bronchiolitis obliterans syndrome, with established role of chronic immunological injury.[22] Interestingly, in bone marrow transplant patients, smooth muscle cells of donor origin were found in coronary atherosclerotic plaques and not in the healthy vessel wall,[45] providing a link between chimerism, inflammation and CAD. Moreover, recent data indicated an increased mortality among male patients who received transfusions from ever-pregnant donors.[46] Also, multiparity especially with different fathers, is associated with increased cardiovascular mortality, providing indirect evidence for a role of alloimmunity.[47] In a large registry study in over 100,000 solid organ transplant recipients, patient survival was inferior in those with a female donor.[48] Endothelial damage by HLA antibodies may lead to a pro-inflammatory phenotype and de novo expression of endothelial neo-antigens causing immune activation. Unfortunately, we lack data on pregnancy history in our female kidney donors. In a recent review, Kinder et al. describe the immunological implications of pregnancy-induced micro-chimerism. Apart from vertical transmission of maternal cells into the fetus, fetal cells can be detected in maternal blood. These micro-chimeric cells may be target of or trigger a fetal or maternal alloimmune response. The transfer and persistence of microchimeric cells is influenced by genetic and external factors. However, it is unknown

whether micro-chimeric cells randomly seed recipient tissues or are actively recruited by chemo-attractants ("tissue-specific homing") to specific fetal or maternal organs.[49]

One can speculate that transplantation leads to an increased "chimeric state" by which certain (endothelial, dendritic, smooth muscle, …) donor cells migrate to certain recipient tissues (i.e. coronary endothelium) leading to a chronic inflammatory state promoting atherosclerosis and hence affecting long-term cardiovascular outcome.

Conclusion

Prior sensitization against HLA class II antigens is associated with unfavourable long-term cardiovascular outcome in KTR independent of effects on graft function or rejection. Transplant recipients with a HLA-DR1 positive kidney donor also face an impaired cardiovascular outcome. Our analysis emphasizes that previous and future immunization after transplantation could exert intrinsic cardiotoxic effects which herald further investigation.

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Figure 1:

Kaplan-Meier survival curves for (a) MACCE or all-cause mortality and (b) MACCE or cardiovascular mortality in kidney transplant recipients with or without HLA class II antibodies at time of transplantation.

HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular event, indicating acute coronary syndrome, coronary revascularization, transient ischemic attack or cerebrovascular accident

Figure 2:

Kaplan-Meier survival curves when excluding patients with BPAR and graft loss for (a) MACCE or all-cause mortality and (b) MACCE or cardiovascular mortality in kidney transplant recipients with or without HLA class II antibodies at time of transplantation.

BPAR: Biopsy-proven acute rejection; HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular event, indicating acute coronary syndrome, coronary revascularization, transient ischemic attack or cerebrovascular accident

Figure 3:

Correlation between cPRA and time to MACCE or all-cause mortality in patients with HLA class II antibodies. Patients without PRA were excluded in this analysis.

cPRA: calculated panel reactive antibodies; MACCE: major adverse cardiac and cerebrovascular event, indicating acute coronary syndrome, coronary revascularization, transient ischemic attack or cerebrovascular accident

Figure 4:

Forest plot demonstrating the adjusted hazard ratio for MACCE or all-cause mortality in kidney transplant recipients (KTR) with versus without preformed HLA class II antibodies according to subgroups. This model was adjusted for recipient age, recipient sex, PRA, smoking status, retransplantation, polyclonal induction, baseline diabetes mellitus and cardiovascular disease unless the covariate was used as a subgroup.

BPAR: biopsy-proven acute rejection; HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular event, indicating acute coronary syndrome, coronary revascularization, transient ischemic attack or cerebrovascular accident

Figure 5:

Kaplan-Meier survival curves for (a) MACCE or all-cause mortality and (b) MACCE or cardiovascular mortality in kidney transplant recipients with or without an HLA-DR1 donor.

HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular event, indicating acute coronary syndrome, coronary revascularization, transient ischemic attack or cerebrovascular accident

TABLES

Table 1. Baseline characteristics of the study cohort

Characteristic	Overall (N = 828)	No HLA class II antibodies (N = 712)	HLA class II antibodies (N = 116)	P value
Age recipient (years; median (IQR))	54 (44-62)	54 (44-62)	55 (47-62)	0.272
Sex recipient (men, %)	513 (62.0%)	464 (65.2%)	49 (42.2%)	**<0.001**
Non-Caucasian race (%)	43 (5.2%)	36 (5.1%)	7 (6%)	0.660
Age donor (years, median (IQR))	47 (36-55)	47 (35-55)	49 (37-56)	0.449
Sex donor (men, %)	467 (56.4%)	394 (55.3%)	73 (62.9%)	0.126
BMI category (%)				
<18.5 kg/m²	37 (4.7%)	32 (4.5%)	5 (4.3%)	
18.5 ≤ BMI < 25 kg/m²	354 (42.9%)	304 (42.8%)	50 (43.5%)	0.349
25 ≤ BMI < 30 kg/m²	277 (33.6%)	240 (33.8%)	37 (32.2%)	
≥ 30 kg/m²	157 (19.0%)	134 (18.9%)	23 (20.0%)	
Cause of ESRD (%)				
Hypertension	17 (2.1%)	14 (2.0%)	3 (2.6%)	
GN	56 (6.8%)	45 (6.3%)	11 (9.5%)	
Reflux	36 (4.3%)	33 (4.6%)	3 (2.6%)	
PKD	142 (17.1%)	120 (16.9%)	22 (19.0%)	0.080
IgA nephropathy	81 (9.8%)	77 (10.8%)	4 (3.4%)	
Diabetes	120 (14.5%)	109 (15.3%)	11 (9.5%)	
Other/unknown	376 (45.2%)	314 (44.1%)	62 (53.4%)	
Dialysis vintage (days, median (IQR))	917 (530-1425)	907 (516-1391)	1050 (596-1511)	0.208
Waiting time (days, median (IQR))	506 (202-1001)	480 (187-968)	641 (338-1107)	0.057
Dialysis modality (%)				
HD	567 (68.5%)	489 (68.7%)	78 (67.2%)	
PD	173 (20.9%)	142 (19.9%)	31 (26.7%)	0.086
Preemptive	88 (10.6%)	81 (11.4%)	7 (6.0%)	
Retransplantation (%)				
First	768 (92.8%)	679 (95.4%)	89 (76.7%)	**<0.001**
Second	56 (6.8%)	32 (4.5%)	24 (20.7%)	**<0.001**
Third	4 (0.5%)	1 (0.1%)	3 (2.6%)	**<0.001**
Combined transplantation (%)				
Only kidney	783 (94.6%)	672 (94.4%)	111 (95.7%)	
Kidney-Pancreas	27 (3.3%)	26 (3.7%)	1 (0.9%)	
Kidney-Liver	15 (1.8%)	12 (1.7%)	3 (2.6%)	0.287

Kidney-Heart	3 (0.4%)	2 (0.3%)	1 (0.9%)	
Preexisting conditions (%)				
Diabetes mellitus	155 (18.7%)	137 (19.2%)	18 (15.5%)	0.340
Cardiovascular disease	155 (18.7%)	134 (18.8%)	21 (18.1%)	0.854
Hypertension	758 (91.5%)	653 (91.7%)	105 (90.5%)	0.668
Baseline cholesterol (mg/dL, median (IQR))				
Total cholesterol[a]	180 (152-210)	181 (152-210)	178 (149-210)	0.363
HDL-cholesterol[b]	52 (41-66)	52 (41-66)	52 (38-66)	0.975
LDL-cholesterol[c]	93 (68-119)	93 (68-119)	90 (67-118)	0.920
Induction therapy (%)				
Basiliximab	760 (91.8%)	666 (93.5%)	94 (81.0%)	**<0.001**
Polyclonal induction	68 (8.2%)	46 (6.5%)	22 (19.0%)	
DCD donor (%)	91 (11.0%)	75 (10.5%)	16 (13.8%)	0.298
PRA > 0% (%)	90 (10.9%)	53 (7.4%)	37 (31.9%)	**<0.001**
HLA-A mismatches (%)				
0	250 (30.2%)	219 (30.8%)	31 (26.7%)	
1	458 (55.3%)	388 (54.5%)	70 (60.3%)	0.501
2	120 (14.5%)	105 (14.7%)	15 (12.9%)	
HLA-B mismatches (%)				
0	167 (20.2%)	148 (20.8%)	19 (16.4%)	
1	509 (61.5%)	432 (60.7%)	77 (66.4%)	0.454
2	152 (18.4%)	132 (18.5%)	20 (17.2%)	
HLA-DR mismatches (%)				
0	243 (29.3%)	205 (28.8%)	38 (32.8%)	
1	515 (62.2%)	441 (61.9%)	74 (63.8%)	0.101
2	70 (8.5%)	66 (9.3%)	4 (3.4%)	
Immunosuppressive regimen (%)				
Cyclosporine	103 (12.4%)	90 (12.6%)	13 (11.2%)	
Tacrolimus	700 (84.5%)	600 (84.3%)	100 (86.2%)	
mTOR inhibitors	15 (1.8%)	14 (2.0%)	1 (0.9%)	0.495
Other / combination	10 (1.2%)	8 (1.2%)	2 (1.7%)	
Smoking status (%)				
Never smoker	388 (46.9%)	325 (45.6%)	63 (54.3%)	
Former smoker	388 (46.9%)	342 (48.0%)	46 (39.7%)	0.212
Current smoker	52 (6.3%)	45 (6.3%)	7 (6.0%)	
Posttransplant malignancy (%)	58 (7.2%)	52 (7.5%)	6 (5.4%)	0.434
Cold ischemia time (minutes, median (IQR))	748 (490-948)	738 (471-948)	787 (584-959)	0.179
eGFR (mL/min, median (IQR))				

3 months[d]	52 (42-66)	52 (42-65)	53 (40-67)	0.663
Year 1[e]	56 (45-71)	56 (45-71)	59 (43-75)	0.678
Year 3[f]	57 (45-70)	57 (45-70)	64 (44-77)	0.253
Year 5[g]	58 (45-72)	56 (45-70)	63 (46-80)	0.289
PAD (%)	39 (4.7%)	34 (4.9%)	5 (4.5%)	0.602
Graft loss (%)	60 (7.2%)	53 (7.4%)	7 (6.0%)	0.609
BPAR (%)	121	102	19	
T-cell mediated rejection	95 (78.5%)	81 (79.4%)	14 (73.7%)	
Antibody-mediated rejection	22 (18.2%)	18 (17.6%)	4 (21.1%)	0.570
Missing value	4 (3.3%)	3 (2.9%)	1 (5.3%)	
All-cause mortality (%)	84 (10.1%)	68 (9.6%)	16 (13.8%)	0.160
MACCE or all-cause mortality (%)	138 (16.7%)	110 (15.5)	28 (24.1%)	**0.021**
MACCE or cardiovascular mortality (%)	79 (9.5%)	62 (9.0%)	17 (15.3%)	**0.037**

BMI: body mass index; BPAR: biopsy-proven acute rejection; DCD: donation after circulatory death; ESRD: end-stage renal disease; GN: glomerulonephritis; HD: hemodialysis; HDL: high-density lipoprotein; HLA: human leukocyte antigen; IQR: interquartile range; LDL: low-density lipoprotein; MACCE: major adverse cardiac and cerebrovascular events; PD: peritoneal dialysis; PKD: polycystic kidney disease; PRA: panel reactive antibodies; SD: standard deviation

Depicted are absolute numbers (percentages), mean ± standard deviation or median (interquartile range) depending upon the distribution.

Overall <1% of data were missing except for [a]:15,9%; [b]: 18.7%; [c]: 24.8%; [d]: 7.9%; [e]: 12.4%; [f]: 13.0%; [g]: 9.8%

Table 2. The hazard ratio for MACCE and all-cause or cardiovascular mortality in patients with versus without HLA class I or II antibodies by different modelling approaches.

Cox Model	MACCE or all-cause mortality		MACCE or cardiovascular mortality	
HLA class I antibodies	**HR [95% CI]**	**P value**	**HR [95% CI]**	**P value**
1	1.27 [0.72-2.25]	0.413	1.17 [0.54-2.54]	0.694
2	1.16 [0.56-2.40]	0.691	1.51 [0.68-3.33]	0.312
3	1.15 [0.56-2.39]	0.699	1.51 [0.68-3.33]	0.312
4	1.07 [0.51-2.23]	0.863	1.45 [0.65-3.23]	0.363
HLA class II antibodies	**HR [95% CI]**	**P value**	**HR [95% CI]**	**P value**
1	1.65 [1.09-2.49]	**0.019**	1.78 [1.04-3.04]	**0.036**
2	1.71 [1.13-2.60]	**0.012**	1.92 [1.12-3.30]	**0.018**
3	1.71 [1.13-2.60]	**0.012**	1.92 [1.12-3.30]	**0.018**
4	1.68 [1.08-2.62]	**0.022**	1.78 [1.02-3.10]	**0.044**

Data are presented as hazard ratio (HR) with 95% confidence interval
Model 1 included only the presence of HLA class I or II antibodies (unadjusted)
Model 2 included the multivariate-adjustment with recipient age, recipient sex, PRA, smoking status, retransplantation, polyclonal induction, baseline diabetes mellitus and cardiovascular disease
Model 3 included the same covariates as model 2 adding BPAR as time-dependent covariate
Model 4 included the same covariates as model 2 adding graft loss as time-dependent covariate
95%CI: 95% confidence interval; BPAR: biopsy-proven acute rejection; HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular events

Table 3. Secondary analyses for patients with versus without HLA class II antibodies stratified by certain subgroups.

	HR [95% CI]	P value for HR	P value for interaction
MACCE with all-cause mortality			
BPAR	0.47 [0.10-2.23]	0.342	0.227
No BPAR	1.88 [1.22-2.92]	**0.005**	
Graft loss	0.14 [0.01-2.13]	0.159	0.404
No graft loss	1.98 [1.25-3.12]	**0.004**	
Recipient age ≤ 60 years	1.91 [1.05-3.49]	**0.034**	0.437
Recipient age > 60 years	1.69 [1.02-2.83]	**0.042**	
First transplantation	1.94 [1.22-3.09]	**0.005**	0.482
Second or more transplantation	1.60 [0.43-5.95]	0.487	
MACCE with cardiovascular mortality			
BPAR	0.88 [0.15-5.14]	0.888	0.632
No BPAR	1.97 [1.10-3.53]	**0.023**	
Graft loss	1.01 [0.03-29.71]	0.997	0.693
No graft loss	1.91 [1.08-3.40]	**0.027**	
Recipient age ≤ 60 years	1.94 [0.92-4.10]	0.081	0.603
Recipient age > 60 years	1.81 [0.82-4.02]	0.143	
First transplantation	1.99 [1.10-3.59]	**0.023**	0.794
Second or more transplantation	2.46 [0.35-17.22]	0.365	

Data are presented as hazard ratio (HR) with 95% confidence interval
95%CI: 95% confidence interval; BPAR: biopsy-proven acute rejection; HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular events

Table 4. The hazard ratio for MACCE and all-cause or cardiovascular mortality in patients with versus without an HLA- DRB1*01 donor by different subgroup-analyses.

Cox Model	MACCE or all-cause mortality		MACCE or cardiovascular mortality	
	HR [95% CI]	P value	HR [95% CI]	P value
5	1.45 [1.09-1.94]	**0.012**	1.49 [1.00-2.22]	0.050
6	1.72 [1.31-2.26]	**0.008**	1.59 [1.07-2.38]	**0.022**
7	1.48 [1.11-1.98]	**0.008**	1.59 [1.07-2.38]	**0.022**
8	1.49 [1.09-2.04]	**0.013**	1.62 [1.08-2.41]	**0.019**

Data are presented as hazard ratio (HR) with 95% confidence interval [95%CI]
Model 5 included only the presence of donor HLA-DRB1*01 (unadjusted)
Model 6 included the multivariate-adjustment with recipient age, recipient sex, diabetes mellitus, smoking status, polyclonal induction, baseline cardiovascular disease and the presence of recipient HLA- DRB1*01
Model 7 included the same covariates as model 6 adding BPAR as time-dependent covariate
Model 8 included the same covariates as model 6 adding graft loss as time-dependent covariate
95%CI: 95% confidence interval; BPAR: biopsy-proven acute rejection; HLA: human leukocyte antigen; MACCE: major adverse cardiac and cerebrovascular even

FIGURE 1

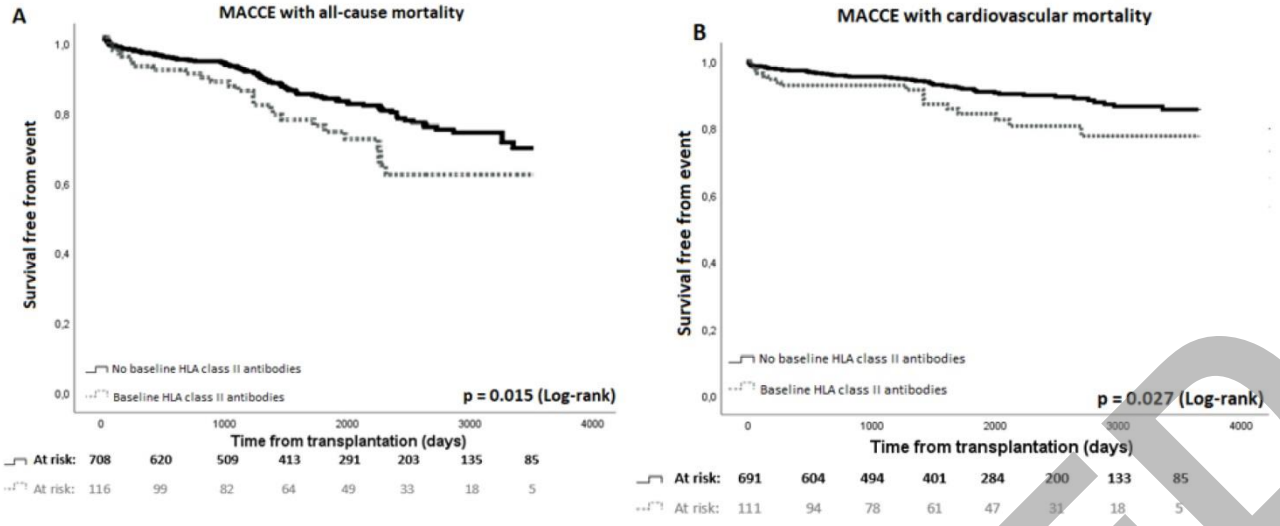

FIGURE 2

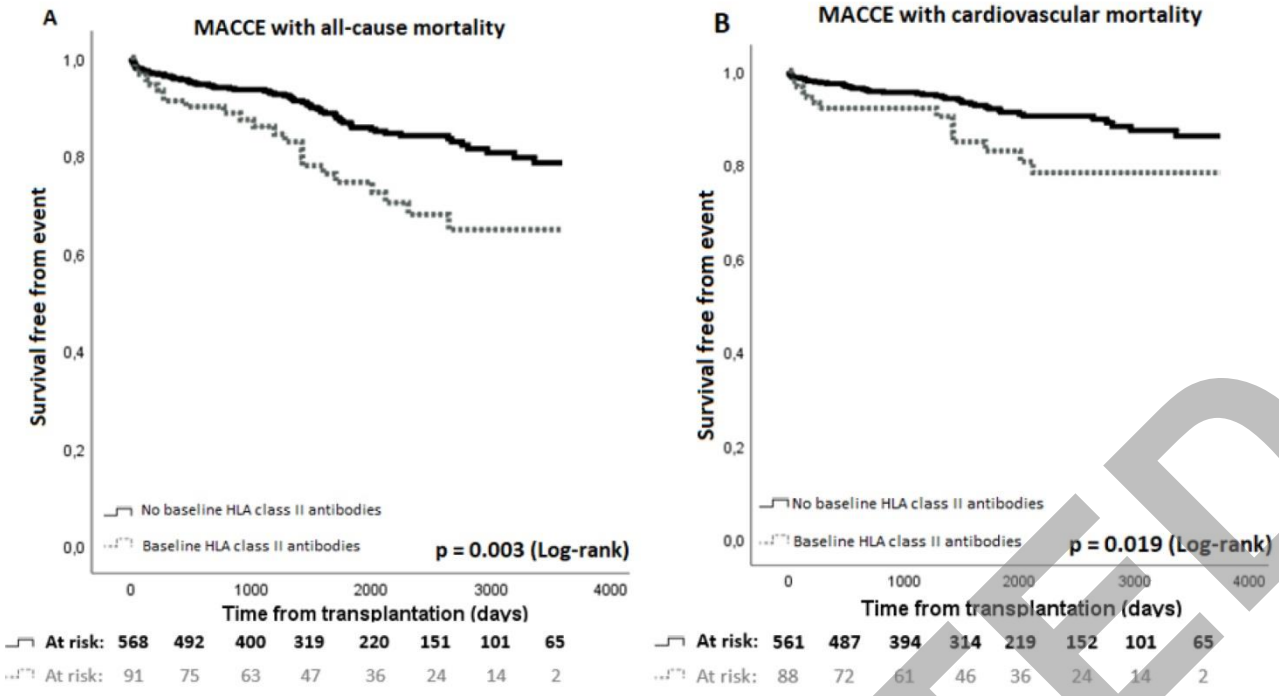

FIGURE 3

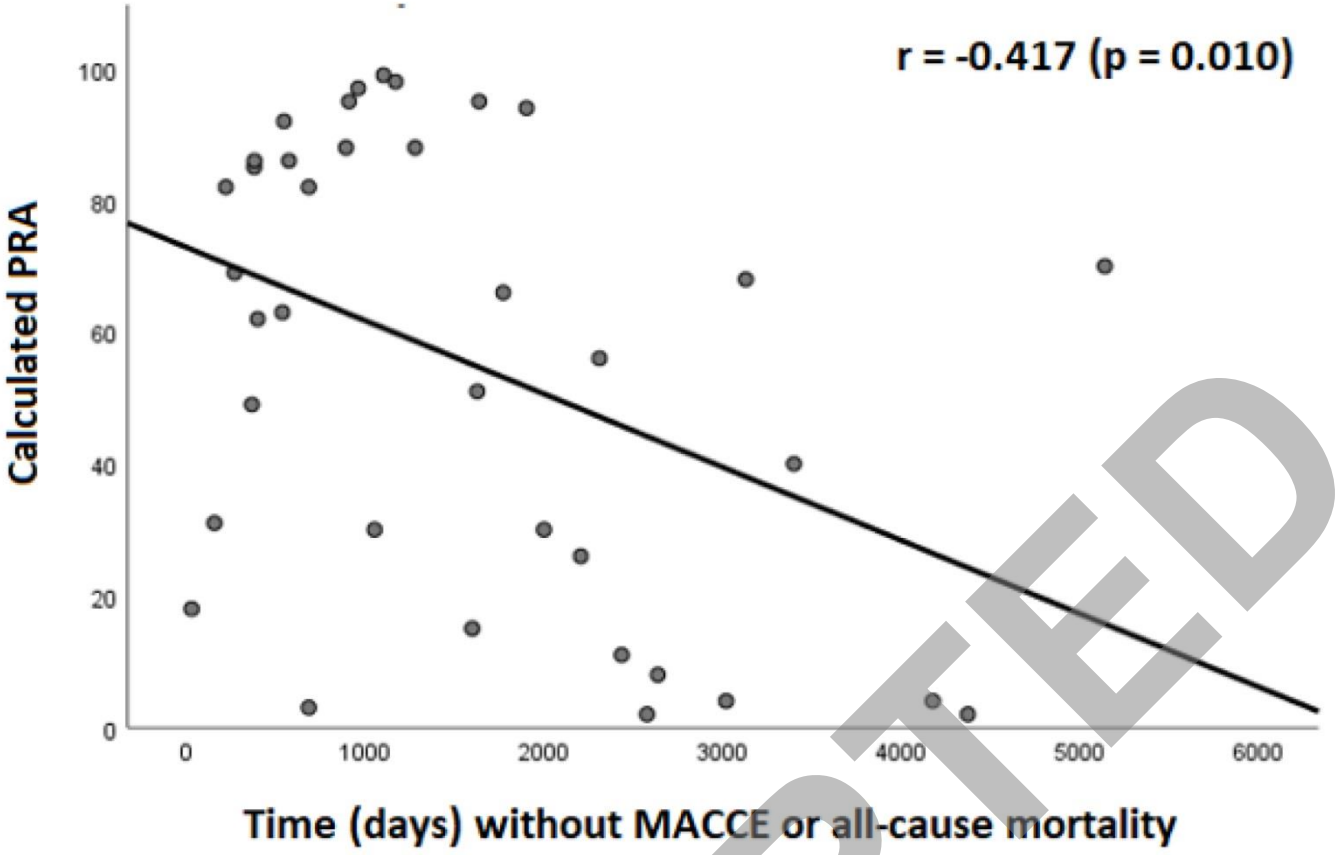

FIGURE 4

MACCE or all-cause mortality in KTRs with versus without HLA class II antibodies

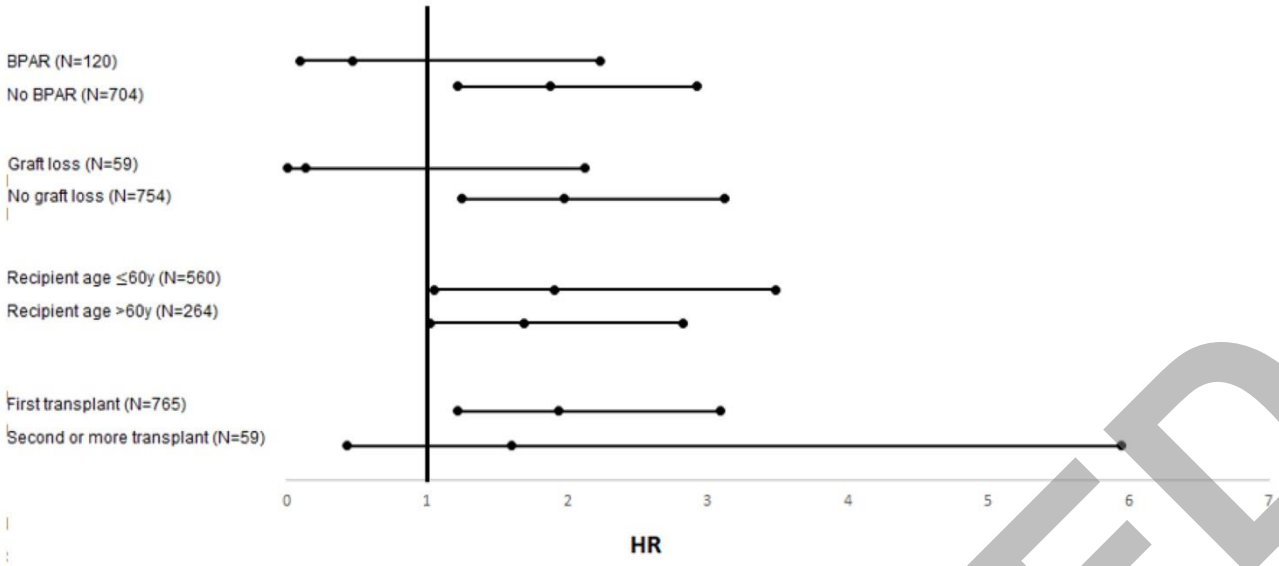

FIGURE 5