ADVANCES IN THE DIAGNOSIS AND TREATMENT IN KIDNEY TRANSPLANTATION

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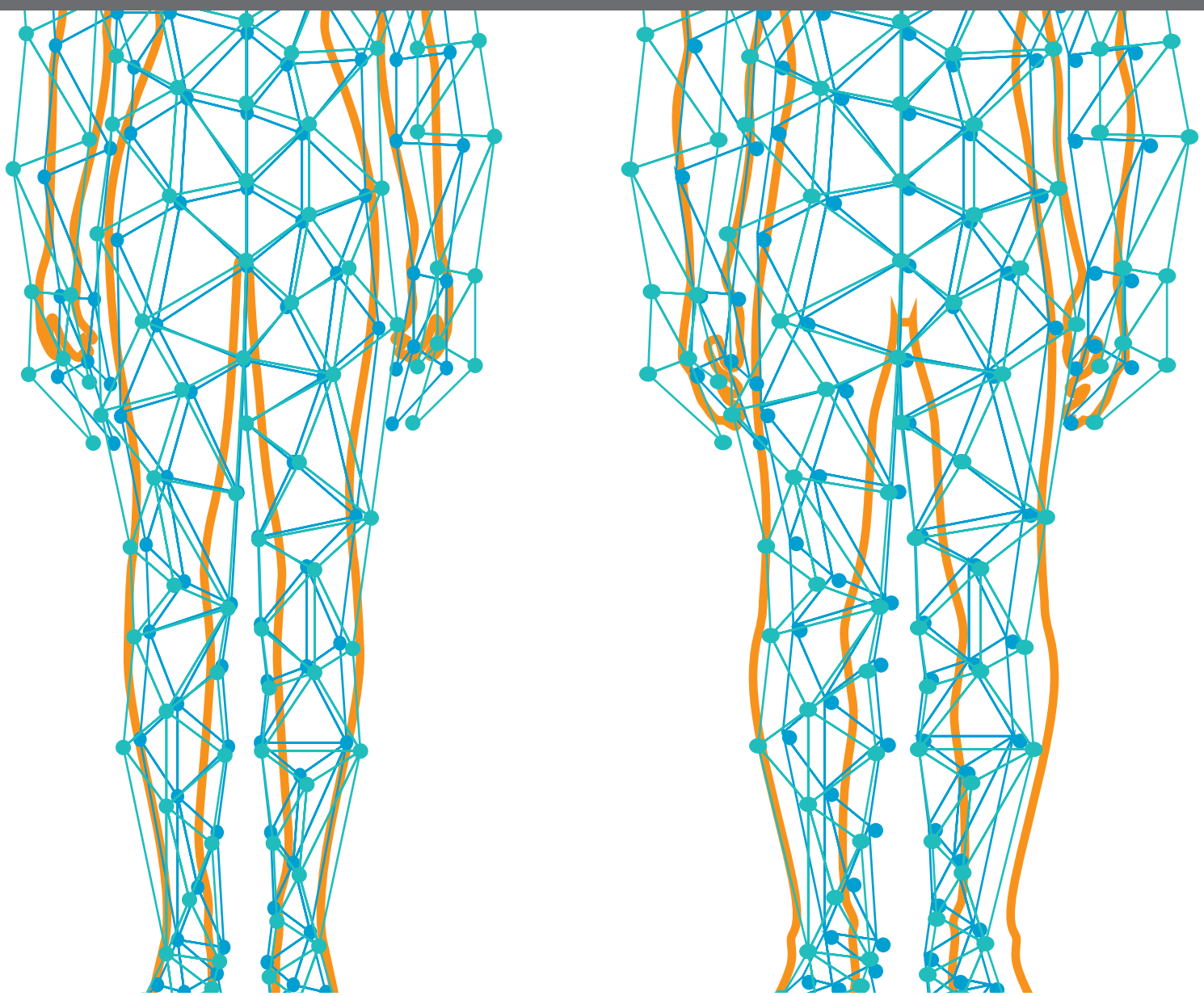

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Editorial: Advances in the diagnosis and treatment in kidney transplantation

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Editorial on the Research Topic
Advances in the diagnosis and treatment in kidney transplantation

Introduction

Kidney transplantation significantly improves patient survival and quality of life in patients with chronic kidney disease (CKD) stage 5 and is thus considered as the current optimal therapy for this patient cohort. More than 21,000 kidney transplantations have been performed in the EU in 2019 (1). Even though advances have been made in treating kidney transplant recipients leading to an improved graft and patient survival despite of increased numbers of transplanted organs from extended criteria donors (2), further advancements are needed especially in the field of organ preservation/regeneration using machine perfusion, diagnostic evaluations, antibody-mediated rejections as well as improvement of cardiovascular disease and treatment of infections (Figure 1). We believe that these fields hold great potential to improve the quality of treatment of our patients after kidney transplantation leading to better patient and graft outcomes. In the Research Topic of Frontiers in Medicine "*Advances in the Diagnosis and Treatment in Kidney Transplantation*," a great number of publications within these fields have been published and will be discussed in this Editorial.

Strategies to overcome ischemia-reperfusion injury

Ischemia-reperfusion injury has been considered to be the inevitable consequence of every transplantation procedure. Both the advanced donor age which reflects the poor organ quality and longer ischemia time were shown to be associated with delayed graft function and inferior kidney grafts outcome. Therefore,

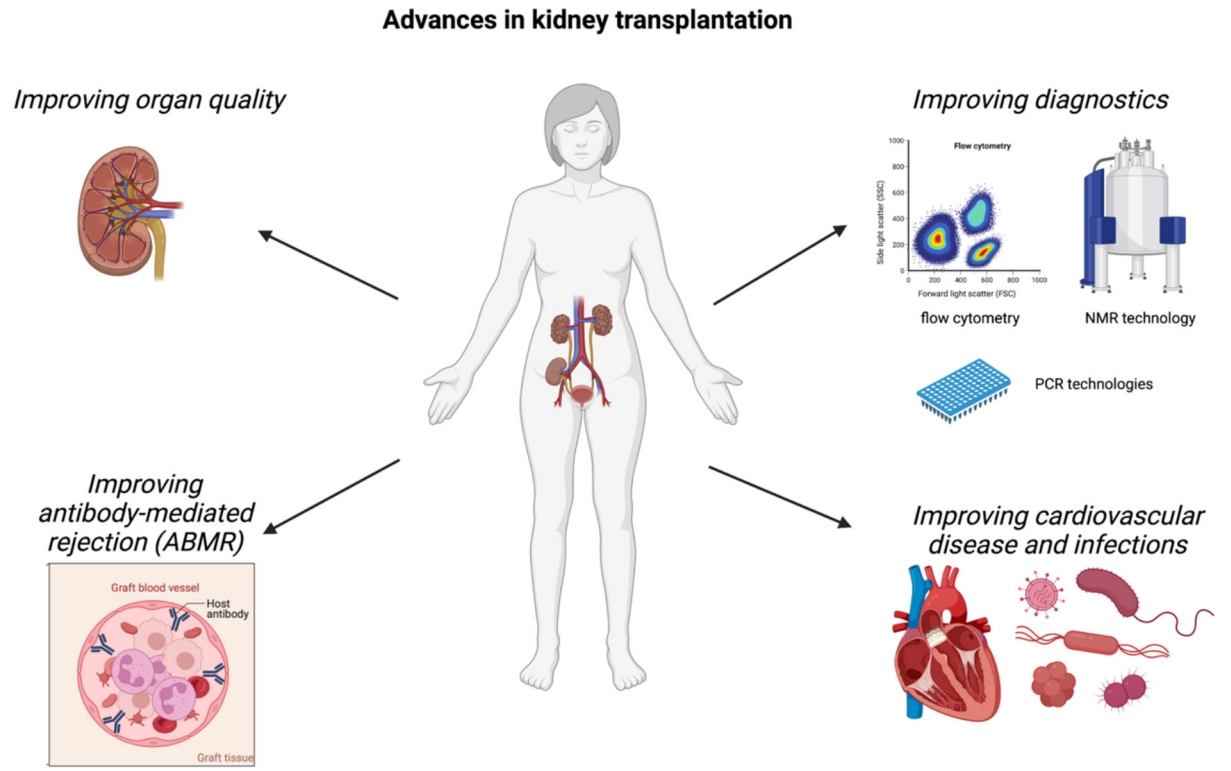

FIGURE 1
"Advances in the Diagnosis and Treatment in Kidney Transplantation." This Research Topic provides new evidence in improvements in different fields of kidney transplantation. Articles published focus on the improvement of organ quality using machine perfusion, of diagnostic possibilities to detect rejection and graft function, of antibody-mediated rejection (ABMR) as well as cardiovascular and infection outcomes Created with BioRender.com.

several attempts have been done to overcome such an obstacle to improve graft quality and prolong time of preservation. Among those Moers et al. as early as in 2009 presented the first randomized clinical trial on machine perfusion and found that hypothermic perfusion was associated with a reduced risk of delayed graft function and improved graft survival at the 1st year after transplantation (3). Since that time many transplant centers have introduced this method which has become a routine for at least grafts at risk for delayed graft function. Zulpaite et al. reviewed therapeutic potential and challenges of *ex-vivo* kidney machine perfusion techniques in this Research Topic. Furthermore, two original clinical studies and one experimental study have been published. Zeng et al. performed a small study comparing traditional antegrade perfusion technique with retrograde approach when they cannulated either renal artery or renal vein for hypothermic LifePort Kidney Transporter perfusion. Authors found that both approaches are associated with similar delayed graft function rates and eGFR at 6 months. Clearly, the study was underpowered and future studies are necessary to show this approach may have some advantages over the classical approach. Next, Weissenbacher et al. evaluated hemodynamic and metabolic parameters in normothermic kidney preservation. Normothermic machine preservation has been studied by several groups and this method is about to enter the clinical medicine. Normothermic machine perfusion may eliminate the effect of cold ischemia and allows evaluation of function and metabolic status of the graft and thus may help to decide whether the organ is still suitable for transplantation (4). Authors used 12 discarded human kidneys which underwent normothermic machine perfusion for 24 h. In eight cases, urine recirculation was used and in four case urine was replaced by Ringer's lactate. Several biomarkers and parameters of machine perfusion were assessed. Arterial flow, pH, NGAL and L-FABP correlated with donor creatinine and eGFR. Perfusate TNF-α was higher in kidneys with lower arterial flow. The cytokines IL-1β and GM-CSF decreased during 6 h. Kidneys with more urine output had also lower perfusate KIM-1 levels. These parameters may be considered as additional viability markers.

Finally, Xiang et al. showed in the mouse model of kidney cold storage that in kidney proximal tubules the p53, a tumor suppressor and stress response gene, is associated with kidney injury and graft dysfunction. They demonstrated that

pharmacological inhibition of p53 may reduce acute tubular injury, apoptosis and inflammation at 24 h after cold storage. These preclinical data support a role of p53 in the graft injury associated with ischemia-reperfusion injury and thus demonstrate the possible therapeutic potential of p53 inhibitors.

Diagnostics in kidney transplantation

During the last years diagnostic procedures have significantly improved. Especially in the field of transplantation, important progress has been made in the areas of biomarker search and omics technologies. These are not limited to kidney biopsies, but also include non-invasive investigations using serum or urine.

The use of omics technologies has a relevant impact to the further development of diagnostic possibilities, not only in terms of finding a diagnosis but also in making a prognosis, such as transplant survival.

The molecular microscope developed by Halloran et al. makes an interesting contribution to the diagnosis of rejection and injury in transplanted organs (5). Using indication biopsies from the INTERCOMEX study (6) the actual study by Halloran et al. was designed to extend previous investigations and to classify scenarios associated with parenchymal injury. By using archetypal analysis (AA) of scores for gene sets and classifiers previously identified in various injury states, six injury groups were defined. Two classes of early AKI could be separated showing differences in function, parenchymal dedifferentiation, response to injury, inflammation and graft survival. The most important predictors of function (estimated glomerular filtration rate) and graft loss were injury-based molecular scores, not rejection scores.

Zero-time biopsies reflect the state of a donor organ before transplantation. In contrast to other organ transplants zero biopsies in kidneys are not routinely performed. Vonbrunn et al. investigated in 26 zero-time biopsies gene expression profiles for different types of subsequent renal transplant complications. They could reveal significant differences between living and deceased donor kidneys mainly due to differences in the cold ischemia time. Genes encoding for immunoglobulins were differentially expressed in biopsies from transplants which later developed rejection corresponding to higher number of $CD20^+$ and $CD138^+$ cells. Acute renal failure before transplantation also had an influence on gene expression. Although transplant biopsies are still the gold standard in routine diagnostic, non-invasive diagnostic from urine or plasma can help to identify patients at risk for renal transplant rejection. Analysis of donor derived cell-free DNA (dd-cfDNA) has been shown to have the potential to detect cases of rejection episodes, with patients with rejection having higher plasma dd-cfDNA levels than patients with stable grafts without rejection (7). In their study Jerič Kokelj et al. describe a novel method using droplet digital PCR analysis to quantify dd-cfDNA in kidney transplant patients. By using a novel pilot set of assays targeting single nucleotide polymorphisms they found that droplet digital PCR is suitable for analysis of kidney transplant patients' plasma but recommend prior genotyping of donor DNA and performing reliable preamplification of cfDNA. As long as the transplant patient has sufficient urine output it is possible to use it for diagnostic investigations. Oblak et al. could correlate the estimated protein excretion rate in the 1st year after kidney transplantation with ABMR, vascular TCMR and *de novo* DSA, although not surprisingly the test validity was higher in ABMR compared to TCMR (AUC 0.95 vs. 0.68). Previously Banas et al. developed a novel, non-invasive method to detect graft rejection *via* a characteristic constellation of urine metabolites by NMR spectroscopy (8). In the following prospective international PARASOL study Banas et al. collected 1,230 urine samples and matched them to the corresponding kidney transplant biopsies. The clinical characteristics of subjects recruited, indicate a patient cohort typical for European renal transplantation. A typical shift from T-cellular early rejections episodes to later antibody mediated allograft damage over time after renal transplantation further strengthens the usefulness of the cohort for the evaluation of novel biomarkers for allograft damage. Living-donor kidney transplant recipients undergoing desensitization for Human Leukocyte Antigen (HLA)-incompatibility have a high risk of developing antibody-mediated rejection (ABMR). The purpose of the study by Cucchiari et al. was to evaluate if residual B cell activity after desensitization could be estimated by the presence of circulating B cell-derived extracellular vesicles. In studies on patients before and after desensitization and controls they could demonstrate a significant drop in B cell-derived extracellular vesicles after desensitization and that this paralleled the reduction in $CD19^+$ cells in lymph nodes, while in peripheral blood B cells, this change was almost undetectable.

To unravel the finding why kidney transplant recipients show impaired immune responses to SARS-CoV-2 infection and a reduced efficacy of SARS-CoV-2 vaccination compared to dialysis patients, Schuller et al. investigated peripheral blood B cell composition before and after kidney transplantation. They could detect persistent and profound compositional changes within the B cell compartment. Low Transitional B cells, 1 year after KT, may account for the low serological response to SARS-CoV-2 vaccination in KTRs compared to dialysis patients.

Advances in antibody-mediated rejection

In the last two decades, antibody-mediated rejection (ABMR) has become a major research focus in transplant medicine, and, today, our knowledge about molecular mechanisms and the manifold phenotypic presentation of this rejection type has improved substantially. Nevertheless, there are large numbers of unanswered questions, some of them

now addressed in the context of our present Research Topic. One is the burden of recipient sensitization—a dominant risk factor of ABMR occurrence. Individualized risk assessment in this context may be of decisive importance to adequately guide organ allocation and tailor the composition or intensity of desensitization protocols (9, 10). There is still a need for immunological variables that accurately predict allograft outcomes. In a retrospective cohort study including 108 donor-specific antibody (DSA)-positive deceased donor transplant recipients subjected to desensitization with anti-thymocyte globulin, plasmapheresis, intravenous immunoglobulin (IVIG) and/or rituximab, Osickova et al. found particularly high ABMR rates among recipients with a positive pre-transplant flow cytometric crossmatch (76 vs. 19% among crossmatch-negative recipients), demonstrating a 5-fold increased risk in multivariable analysis. These interesting results are of considerable clinical relevance, as they strongly support the systematic use of flow crossmatch testing in the context of DSA-positive deceased donor kidney transplantation. Moreover, Kälble et al. evaluated allograft outcomes in 38 DSA and/or crossmatch-positive recipients of a living donor transplant following an individualized algorithm of peri-transplant apheresis. Treatment, which included rituximab and/or thymoglobulin, was tailored according to careful immunological risk stratification, among others, based on single bead assay results and/or soluble CD30 (sCD30) monitoring. Patient and graft survival rates were found to be similar to those observed among standard-risk recipients, without differences regarding rejection rates. An interesting observation was that, following transplantation, 56% of the recipients had lost their DSA. ABMR rates in these patients were only 6%, but 60% in recipients with persistent and *de novo* DSA. In a retrospective study of 287 patients subjected to standard immunosuppression, Drasch et al. evaluated the impact of preformed DSA and sCD30 levels on renal allograft outcomes. In their study, graft survival was significantly lower in DSA-positive as compared to DSA-negative patients. While DSA-positive patients with increased levels of sCD30 had adverse 3-year graft survival, sCD30 levels were not associated with ABMR frequency, DSA persistence and long-term survival. Senn et al. studied a distinct risk constellation—husband-to-wife transplantations with mutual children—which may be complicated by the persistence of alloreactive T and B cells triggered by paternal HLA antigens. Analyzing 25 such transplants in comparison to women with prior pregnancies who received a kidney from other donor sources, they found numerically higher incidences of ABMR and inferior death-censored graft survival, despite the use of T cell-depleting induction therapy. Interestingly, in this cohort, DSA status, number of pregnancies, or the number of HLA-mismatches were not predictive for rejection or graft loss.

Treatment of ABMR has remained a major challenge, and evidence for efficacy of currently available anti-rejection treatments is considerably low, especially in chronic rejection. One promising concept of ABMR treatment may be the use of antibodies directed against interleukin-6 (IL-6) or its receptor (IL-6R). Noble et al. present a single-center study including 40 renal allograft recipients who all received the anti-IL-6R antibody tocilizumab for chronic active ABMR. Six patients lost their graft within 12 months, but many patients showed stable eGFR, and there was no change in microvascular inflammation scores and the extent of interstitial fibrosis and tubular atrophy. Transplant glomerulopathy scores, however, increased. Even though limited by its uncontrolled retrospective design, this study may suggest that tocilizumab is able to stabilize the decline in renal function and histological rejection lesions, at least in some of the treated patients. There is now accumulating evidence for a role of DSA-negative ABMR, but there is not much known about its pathophysiology or responsiveness to rejection treatment. The latter issue was addressed by Koslik et al. Evaluating 102 renal allograft recipients diagnosed with ABMR, among them 61 with detectable DSA, the authors studied the impact of multi-compound treatment, primarily based on apheresis and intravenous immunoglobulin (IVIG) on allograft outcomes. In case of persistent ABMR increase in maintenance immunosuppression or long-term application of IVIG was used. While late rejection diagnosis and positive C4d staining turned out to be independent risk factors for allograft failure, DSA status did not relate to graft survival. An interesting finding was that DSA-positive recipients showed significantly better allograft survival after long-term IVIG than patients with DSA-negative ABMR. Conversely, the latter exhibited better responses to intensified maintenance immunosuppression. Moreover, there are individual histological and molecular phenotypes of ABMR that may respond differently to treatment. In this context, Sazpinar et al. provide a detailed evaluation of 16 patients presenting with pure chronic active ABMR. The authors evaluated the expression of predefined rejection-related transcripts using NanoString™ technology and evaluated biopsy results in relation to clinical outcomes. In this preliminary small study, treatment responsiveness of ABMR was associated with the extent of microvascular inflammation and transcriptome changes in NK cell and endothelial cell associated genes. Cell therapies treating late ABMR are emerging including the transfer of mesenchymal stem cells (MSC). Still, these therapies might be accompanied by serious side-effects in individual patients as described by Večerić-Haler et al. They describe a single patient case with late ABMR treated with autologous MSC within a study and the patient developed life-threatening symptoms mimicking capillary leakage syndrome, which only resolved after explantation of the kidney graft. The authors speculate that Parvovirus B19 might have mediated the life-threatening condition. These data point to the fact that viral infections might be transferred *via* cell therapies which result in life-threatening complications for the patient.

There is definitely a need for well-designed randomized prospective trials to clarify the efficacy of new treatment

approaches that are currently in the pipeline. Trial design in ABMR, however, is a challenge, partly because of the requirement of large patient numbers and long periods of follow-up to demonstrate meaningful differences in allograft survival. In a retrospective monocentric study, Borski et al. confirmed a strong impact of late ABMR on renal allograft survival, demonstrating a 93, 64, 53, and 15% unadjusted overall allograft survival at 1, 3, 5, and 10 years after index biopsy, respectively. In search of surrogate endpoints that allow for accurate prediction of graft survival, they found a strong predictive value of early eGFR decline. An eGFR loss of 1 ml/min/1.73 m^2 per year was thereby associated with a 10% (12-month slope) to up to 30% (24-month slope) increase in the risk for future allograft loss. These data strongly support the utility of calculating eGFR slope as a surrogate endpoint of graft failure in ABMR trials. Moreover, in search of biopsy findings that predict transplant outcomes after ABMR diagnosis, Piñeiro et al. studied a retrospective cohort of 90 patients treated for active ABMR, exploring the clinical relevance of persistent inflammation detected in follow-up biopsies. Following treatment with plasma exchange, IVIG and rituximab, microvascular inflammation persisted in 71% and tubulitis in 19% of the biopsies. Persistent inflammation, even despite not strictly meeting any of the Banff rejection categories, was found to strongly associate with graft failure. Retreatment of patients with persistent inflammation was thereby associated with a better prognosis than in untreated patients. Finally, Novotny et al. studied 72 renal allograft recipients exhibiting vascular rejection (intimal arteritis), in association with TCMR or ABMR features (microvascular inflammation). The authors found that intimal arteritis in conjunction with ABMR was a significant risk factor of transplant glomerulopathy in follow-up biopsies, regardless of DSA status. Among DSA-positive patients with intimal arteritis and microvascular inflammation, resolution of rejection was less frequent (27% as compared to 58 or 90% in patients with phenotypes of intimal arteritis associated with DSA-negative ABMR or TCMR, respectively).

Advances in cardiovascular disease and infections after kidney transplantation

Alloimmune responses are causative for kidney graft loss in a valuable number of our kidney transplant recipient. Still, cardiovascular events as well as infections are major causes of graft loss as well as death with a functioning graft after kidney transplantation (11). Thus, strategies to improve outcome after kidney transplantation in these areas are of critical importance to improve graft- and patient-survival. In the Research Topic Advances in kidney transplantation in Frontiers in Medicine published articles further add important new information in the field of cardiovascular disease and infections after kidney transplantation. Végh et al. showed nicely that blood pressure monitoring by 24-h measurements (ABPM) as well as vascular stiffness correlated with the GFR in the long term follow up of pediatric kidney transplantation recipients. Interestingly, there was no correlation of vascular stiffness and ABMR 2 years after transplantation, which points to the fact that CV health is of crucial importance for long-term function of grafts. Protection and/or of vascular calcification is an important step toward an improvement of cardiovascular survival in the chronic kidney disease population including kidney transplant recipients. Supplementation of magnesium has been proposed to be beneficial in treating vascular calcification in animal models (12). In line, low serum-magnesium levels have been associated with an increased cardiovascular risk and all-cause mortality in the general population (13). Contrarily, Lahav et al. show in their article in Frontiers in Medicine that serum-magnesium levels correlated inversely with all-cause and cardiovascular mortality in their cohort of kidney transplant recipients. Furthermore, magnesium supplementation did not show a benefit, but was rather associated with worse cardiovascular outcomes. Still, these outcomes might be biased by an improved graft function, which is associated with lower magnesium levels (14). Clearly interventional studies are needed to evaluate whether magnesium supplementation is beneficial in CKD patients as well as kidney transplant recipients. Since post-transplantation diabetes mellitus is one of the most important factors in increasing cardiovascular mortality in kidney transplantation recipients, studies addressing this issue are of outmost importance. Yin et al. published a study enrolling 105 kidney transplant recipients, who will be treated with placebo, metformin or empagliflozin. The two drugs will be tested for safety, effectivity, and tolerability in the kidney transplant population. The primary end point is the change in the visceral-to subcutaneous fat area evaluated by MRI as well as inflammatory parameters. This study critically addresses the problem of obesity and PTDM in the kidney transplantation cohort with the great opportunities of new therapeutic drugs such as SGLT-2 inhibitors, which have been shown to improve kidney function and cardiovascular mortality in the CKD cohort (15). Still, they also hold great potential in the kidney transplantation population, where only few studies exist evaluating these drugs (16, 17).

Infections are an important factor of graft and patient loss in the kidney transplantation population. Rare infections occur in our immune suppressed population such as shown in a recent case report of a patient developing malacoplakia due to the infection with a multi-resistant *E. coli* and *Cryptococcus albidus*. Both colonized in the transplanted kidney. Yan et al. further show that metagenome sequencing can be utilized as an additional diagnostic tool complementing pathogen detection especially in transplant recipients with unusual infections. In contrast, BK Polyoma virus infection is a frequent problem in kidney transplant recipients associated with an increased

probability for graft loss. The study by Omić et al. showed that kidney transplant recipients with an insufficient decrease in BK Polyoma virus titers in the blood have a significantly increased risk for graft loss. Thus, not only patients with an initially high BK viral load, but also patients without a rapid decrease in BK viral load need to be closely followed. Omic also show that leflunomide did not improve GFR in their patient cohort but had the ability to fully clear the virus in a greater number of patients. Nevertheless, the only sufficient way to limit BK polyoma virus infection currently seems to be the reduction of immunosuppression. The COVID-19 pandemia put our kidney transplant cohort into severe risk for developing critical COVID-19 due to chronic immunosuppression and coexisting conditions (18). Dedinská et al. add to this evidence by providing retrospective, multicenter analysis of 186 kidney transplantation patients with COVID-19. Obese and patients >59 years are of high risk to develop critical COVID-19 illness. Corticosteroids more than 7.5 mg/day as well as HLA-DQ2 seem to be protective factors in their cohort of patients but need to be confirmed in larger trials.

Finally, a study on sex differences between start of kidney replacement therapy and inclusion on the waiting list for kidney transplantation has been published in this Research Topic of Frontiers in Medicine. Hödlmoser et al. show compelling sex disparities in both the US as well as in Austria with a significant higher chance for men to be listed for kidney transplantation. Even though the differences decrease over time, the gap persists especially for older women. Thus, we need to further understand the causes of sex disparities to set up ways to overcome them.

Author contributions

KE, GB, MB, and OV: wrote, reviewed, and approved the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Association Between Exposure to Low Magnesium Blood Levels After Renal Transplantation and Cardiovascular Morbidity and Mortality

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Lahav I, Steinmetz T, Molcho M, Lev N, Agur T, Nesher E, Rozen-Zvi B and Rahamimov R (2021) The Association Between Exposure to Low Magnesium Blood Levels After Renal Transplantation and Cardiovascular Morbidity and Mortality. Front. Med. 8:690273. doi: 10.3389/fmed.2021.690273

Background: Serum magnesium levels are associated with cardiovascular disease and all-cause mortality in the general population and chronic kidney disease patients, but the association between serum magnesium levels and cardiovascular risk after kidney transplantation is not established. We sought to evaluate whether exposure to low serum magnesium levels after renal transplantation is related to cardiovascular morbidity and mortality.

Methods: We conducted a single center retrospective study that included all transplanted patients who had a functioning graft for at least 6 months after transplantation between January 2001 and December 2013. We calculated exposure to magnesium using time weighted average for serum magnesium levels, using all values available during the follow-up. Several statistical methods were used, including liner regression analysis, χ^2 test, and multivariate Cox proportional hazard model.

Results: Four hundred ninety-eight patients were included. Median follow-up was 5.26 years. High time weighted average of serum magnesium was associated with a hazard ratio of 1.94 for all-cause mortality and major cardiovascular outcome compared to low levels (95% CI 1.18–3.19, $p = 0.009$). The high quartile of time weighted average of serum magnesium was associated with death censored major cardiovascular outcome (hazard ratio 2.13, 95% CI 1.17–3.86, $p = 0.013$) in multivariate analysis.

Conclusions: Exposure to low serum magnesium levels in renal transplant recipients was associated with a lower risk for all-cause mortality and major cardiovascular outcome. These findings contrast the higher risk found in the general population.

Keywords: magnesium, renal transplantation, cardiovascular outcomes, mortality, renal function

INTRODUCTION

Magnesium (Mg) is the second most prevalent intracellular cation (1) and has a vital role in cellular and biological processes. Serum magnesium (sMg) levels depend on the balance between renal excretion and intestinal absorption; thus, hypomagnesemia results from gastrointestinal losses and renal losses due to medications such as loop or thiazide-type diuretics, calcineurin inhibitors and

cisplatin, hypercalcemia, volume expansion, recovering acute tubular necrosis, post-obstructive diuresis and inherited tubulopathies (e.g., Bartter and Gitelman syndromes) (2).

Data from epidemiological studies suggests that low sMg levels are associated with increased cardiovascular disease (CVD) and all-cause mortality in the general population (3, 4).

In chronic kidney disease (CKD) patients, there is an increasing body of evidence for an inverse association between sMg and insulin resistance, new onset diabetes (5), hypertension (6), atherosclerosis (7), inflammation (8), CVD (3, 9), vascular calcification (10), dyslipidemia (11), and renal function decline and mortality risk (12, 13).

Magnesium deficiency is common after kidney transplantation mainly due to the effect of calcineurin inhibitors (CNI) on tubular magnesium handling. CNI induce hypomagnesemia through downregulation of the renal expression of epidermal growth factor (EGF) and transient receptor potential channel melastatin 6 (TRMP6) in the distal collecting tubule, leading to Mg wasting (14). Hypomagnesemia is associated with new onset diabetes after transplantation (15, 16) and also with vascular stiffness and endothelial dysfunction (17), measured by carotid-femoral pulse wave velocity. The association between sMg levels and CVD in kidney transplant recipients has not been evaluated yet.

The aim of this study was to evaluate whether there is association between exposure to low magnesium blood levels after renal transplantation and cardiovascular morbidity and mortality.

MATERIALS AND METHODS

Study Design

This is a single-center retrospective cohort study. The study protocol was approved by the Rabin Medical Center (RMC) institutional review board (IRB).

Study Population

We searched the RMC kidney transplant registry between January 2001 and December 2013 and selected all transplanted patients who had a functioning graft for at least 6 months after transplantation. We collected sMg values and calculated time weighted average (TWA) for sMg levels using all values available since the renal transplantation and during the follow-up period. TWA was calculated by multiplying each sMg value by the time of exposure, summing all the values, and dividing them by the time interval between the first and last values. We evaluated cumulative TWA of sMg from time of transplantation for each time point, by calculating TWA between the time of transplantation to the end of each 6-month interval. As a result, the exposure variable for this study was the mean sMg (weighted for time of exposure) during all the follow-up time until the time of the event. Schematic description of TWA for sMg at each time point is depicted in **Figure 1**.

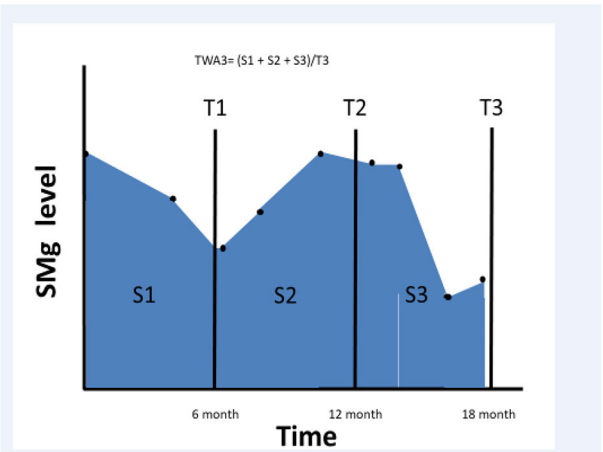

FIGURE 1 | Schematic description of sMg TWA calculation.

We excluded patients with less than six magnesium blood level values available, patients who were lost to follow-up, multiple organ transplant patients, and patients who experienced cardiovascular events during the first 6 months after the transplantation.

Endpoints

The primary endpoint was the composite outcome of all-cause mortality and major cardiovascular outcome (MACE) defined as non-fatal myocardial infarction (MI), non-fatal cerebrovascular accident (CVA), coronary revascularization, peripheral revascularization, and hospitalization due to acute coronary syndrome, congestive heart failure (CHF) exacerbation, or peripheral vascular disease (PVD).

Secondary endpoints included the separate components of the primary outcome, graft survival, and overall survival. The outcome of MACE was determined by two physicians (I.V. and M.M.). The physicians opened every medical file and did not rely on the written diagnosis. Whenever there was a doubt about the diagnosis, the physicians consult with a senior nephrologist (R.R. or B.R.Z.) in order to ascertain the MACE event. In any disagreement, another senior nephrologist (R.R. or B.R.Z.) was consulted.

Study Design

The time period from transplantation was divided into 6-month intervals. The primary exposure was TWA of sMg from the transplantation to the end of each interval. Each patient was assigned into a group according to the TWA of sMg at the beginning of each interval. As the TWA of sMg changed over time, each patient could be assigned to a different group at each time interval. Each outcome episode was attributed to the TWA

Abbreviations: BMI, body mass index; CHF, congestive heart failure; CI, Confidence Interval; CKD, chronic kidney disease; CNI, calcineurin inhibitors; CV, cardiovascular; CVA, cerebrovascular accident; CVD, cardiovascular disease; eGFR, estimate glomerular filtrate rate; HBA1C, hemoglobin A1C; HDL, High-density lipoprotein; HR, Hazard Ratio; IHD, Ischemic Heart Disease; IQR, interquartile range; LDL, low-density lipoprotein; MACE, major cardiovascular outcome; Mg, magnesium; MI, myocardial infarction; PPI, proton-pump inhibitors; PVD, peripheral vascular disease; WBC, white blood cells; sMg, serum magnesium; TWA, time weighted average.

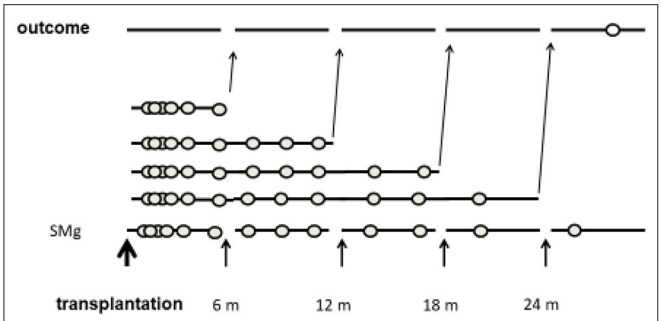

FIGURE 2 | Schematic description of study design.

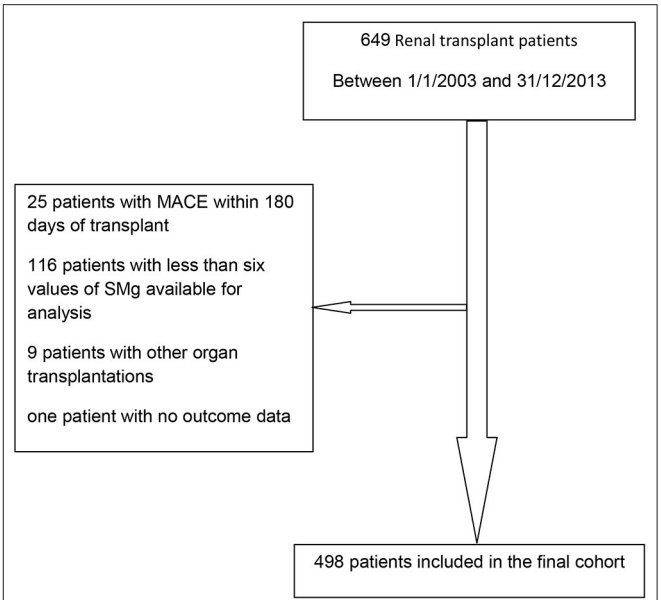

FIGURE 3 | Flow diagram of the recruitment and exclusion of patients in the study.

of SMg calculated from the transplantation to the end of the previous time interval, and analysis was done by time-varying manner. A schematic description of the study design is depicted in **Figure 2**.

Statistical Methods

Baseline patient characteristics are presented as means and standard deviations or median and interquartile range, as appropriate.

Data was collected from the patients' electronic health records. Recipient data included age, gender, baseline kidney disease, body mass index (BMI), history of hypertension, diabetes mellitus, ischemic heart disease, MI, coronary revascularization, CHF, valvular disease, PVD, CVA, past renal replacement therapy, and transplantations. Transplantation data included donor type (living or deceased), age and gender, panel reactive antibodies (PRA) status, human leukocyte antigen (HLA) mismatch, induction therapy, maintenance immunosuppression therapy, delayed graft function, hospitalization length, and episodes of acute rejections.

Laboratory data was collected at 6 months post-transplantation and included creatinine, urea, low-density lipoprotein (LDL) cholesterol, glucose, cholesterol, albumin, hemoglobin, white blood cells (WBC), platelets, and hemoglobin A1C (HBA1C). At 6 months, we also evaluated for development of hypertension and diabetes and recorded medications prescribed including immunosuppression medication (prograf, cyclosporine, mTOR inhibitors), magnesium supplements, blood pressure medications (ACE inhibitors, Ca channel blockers, Beta blockers), anti-aggregates, statins, and proton pump inhibitors. The protocol for magnesium supplementation at our clinic was magnesium citrate 100 mg thrice daily, and it was given at the discretion of the treating physician.

We used multiple imputations using liner regression analysis, with five repeats for missing data analysis. Mean and standard deviation described normally distributed variables, whereas median and range described non-normally distributed variables. For differences between groups, we used analysis of variance (ANOVA) for continuous variables and χ^2 test for discreet variables.

For modeling time to first event, we used time varying univariate and multivariate Cox proportional hazard model, with quartiles of TWA sMg as time dependent variable. We compared quartiles of sMg according to the TWA of sMg from the time of transplantation to each time point. The proportionality assumption was validated by evaluating each variable for interaction with time and assessing for the null hypothesis. For multivariate analysis, we used forward stepwise analysis with possible confounders using inclusion p-value of 0.05. We also forced into the model variables that were significantly associated with TWA of magnesium at 6 months post-transplantation. The variables in the multivariate analysis included recipient age and gender, BMI, history of heart disease, diabetes mellitus, smoking status, donor age, Mg supplementation, maintenance immunosuppression therapy, duration of dialysis, serum albumin, and eGFR. The full models of the multivariate analysis are presented in **Supplementary Tables 1, 3, 6**.

In order to further evaluate the risk of our primary and secondary outcomes, we used repeated measures generalized estimating equations (GEE). We used the cumulative TWA of magnesium level at the beginning of each time interval as repeated measures variable and the odds for an event during each interval as response variable using binary logistic model. The multivariable adjusted model included the same variables used for the time varying Cox analysis.

In order to increase the internal validity of our results, we performed several sensitivity analyses. We analyzed the association between sMg level and the primary outcome in all the patients in the cohort including patients with <6 sMg values and patients that had CV event during the first 6 months post-transplantation. We also analyzed the association between the highest sMg value and the primary outcome. Lastly, we evaluated the association between TWA of sMg at 1 year and the secondary

outcome of death censored CV events using competing risk analysis with non-CV death as the competing risk.

RESULTS

Between January 1, 2003 and December 31, 2013, 649 patients were transplanted; 498 were included in the final cohort. Reasons for exclusion were as follows: MACE within 180 days of transplant (18), less than six values of sMg available for analysis (116), other organ transplantations (9), and no outcome data (1). Flow diagram of the recruitment and exclusion of patients in the study is depicted in **Figure 3**.

The patients' characteristics according to TWA of magnesium concentration during the first 6 months post-transplantation are presented in **Table 1**. Low TWA of serum magnesium was associated with younger donor age, higher estimated glomerular filtrate rate (eGFR), treatment with magnesium supplementation, and non-smoker status.

Time Varying TWA of Blood Magnesium and the Primary Outcome

During a median follow-up time of 5.26 years [interquartile range (IQR) 4.21–7.37], 145 outcome events occurred including 98 events of MACE and 47 events of death.

We divided the patients into quartiles according to TWA of sMg at each time point. **Table 2** presents the association between time varying TWA of Mg levels and the primary composite outcome of MACE and overall death.

When compared to the lowest quartile, the highest quartile was associated with increased risk of the primary outcome [Hazard Ratio (HR) 1.94, 95% Confidence Interval (CI) 1.18–3.19, $p = 0.009$]. The results were not significantly changed by multivariate adjustment (HR 1.86, 95% CI 1.08–3.21, $p = 0.027$). We also analyzed the data by repeated measures GEE with comparable results [Odd Ratio (OR) 1.69, 95% CI 1.03–2.77, $p = 0.037$] and (OR 1.8, 95% CI 1.04–3.1, $p = 0.034$) for univariate and adjusted analysis, respectively (see **Supplementary Table 2**). Sensitivity analysis using the highest sMgl level gave similar results (HR 2.27, 95% CI 1.331–3.872, $p = 0.003$) and (HR 1.882, 95% CI 1.088–3.255, $p = 0.024$) for the highest quartile vs. the lowest quartile by univariate and multivariate analysis, respectively (see **Supplementary Table 4**).

Another sensitivity analysis included patients with <6 sMg levels (but at least one value) and patients that had CV events during the first 6 months post-transplantation. This analysis showed comparable results though, with numerically smaller effect size, when compared to the lowest quartile; the highest quartile was associated with increased risk of the primary outcome (HR 1.528, 95% CI 1.001–2.334, $p = 0.05$). The results were not significantly changed by multivariate adjustment (see **Supplementary Table 5**).

Comparable results were obtained when the secondary outcome of death censored MACE (with the exception of CV mortality) was evaluated. The high quartile of TWA of magnesium was associated with this outcome (HR 1.84, 95% CI 1.07–3.18, $p = 0.028$) and (HR 2.13, 95% CI 1.17–3.86, $p = 0.013$) for univariate and multivariate analysis, respectively. The results of association between sMg quartiles and death censored MACE and the full multivariate model are presented in **Table 3** and **Supplementary Table 6**, respectively.

When the association with death censored MACE was analyzed by competing risk analysis (with the competing risk of non-CV death), the highest quartile of TWA of Mg at 1 year was non-significantly associated with CVD by univariate analysis (HR 1.47, 95% CI 0.93–2.33, $p = 0.17$). However, by multivariate analysis, the association was significant (HR 1.88, 95% CI 1.14–3.11, $p = 0.038$) (see **Supplementary Table 7**).

In a similar manner, analysis by repeated measures GEE generated equivalent results (OR 1.69, 95% CI 0.97–2.94, $p = 0.062$) and (OR 2.15, 95% CI 1.15–4.02, $p = 0.017$) for univariate and adjusted analysis, respectively. As can be seen, the trend for increased odds for CV events was non-significant by univariate analysis but became significant after multivariate adjustment (see **Supplementary Table 8**).

Table 4 presents the association between time varying TWA of serum magnesium and the separate components of MACE.

Time Varying TWA of Blood Magnesium and All-Cause Mortality

During the follow-up, 77 patients died: 22 (28.6%) due to CVD, 30 (39%) due to infections, 7 (9.1%) due to malignancy, and 18 (23.4%) due to other or unknown cause. **Table 5** presents the association between quartiles of TWA of sMg levels during the follow-up and the secondary outcome of all-cause mortality. By univariate analysis, both the third and fourth quartiles were associated with increased mortality compared to the lowest quartile (HR 2.2, 95% CI 1.08–4.49, $p = 0.031$) and (HR 2.16, 95% CI 1.06–4.38, $p = 0.034$), respectively. By multivariate analysis, only the third quartile was significantly associated with mortality, while there was only a non-significant trend for the fourth quartile (**Table 5**). The full model of the multivariate analysis is presented in **Supplementary Table 6**.

By repeated measures GEE, univariate analysis the fourth quartile was associated with increased mortality compared to the lowest quartile with non-significant trend for the third quartile (OR 2.18, 95% CI 1.08–4.41, $p = 0.029$ and OR 1.9, 95% CI 0.93–3.89, $p = 0.081$, respectively). By multivariate analysis, the association was no longer significant for both quartiles compared to the lowest quartile (OR 1.74, 95% CI 0.85–3.55, $p = 0.127$ and OR 1.94, 95% CI 0.94–4.02, $p = 0.074$ for the third and fourth quartiles, respectively) (see **Supplementary Table 9**).

Association of Magnesium Supplementation at 6 Months and CV Outcomes

Of the 498 patients included in the study, 495 (99.4%) had information available regarding prescribed medications 6 months post-transplantation. Sixty-six of the 495 patients (13.3%) were treated with magnesium supplementation. **Table 6**

TABLE 1 | Baseline characteristics of the entire study population according to TWA quartiles of sMg during the first 6 months post-transplantation.

	Quartile 1 ($n = 123$)	Quartile 2 ($n = 121$)	Quartile 3 ($n = 122$)	Quartile 4 ($n = 127$)	*P*-value
Baseline variables					
Age (years)	47.1 ± 15.3	49.1 ± 14.4	47.9 ±14.5	47.7 ± 15	0.765
Gender (men)	50 (40.7%)	38 (31.4%)	40 (32.8%)	45 (35.4%)	
Dialysis duration (months)	35.6 ± 33.4	34.7 ± 40.2	30.3 ± 35.8	31.9 ± 32.9	0.633
Living donor	68 (55.3%)	64 (52.9%)	79 (64.8%)	76 (59.8%)	0.248
Donor age	40.7 ± 15.3	44.1 ± 14.3	45.5 ± 12.5	49.6 ± 13	<0.001
Systolic blood pressure	131.2 ± 19.4	129.3 ± 17.9	132.4 ± 18.5	130.1 ± 17.9	0.663
Creatinine at 6 months	1.26 ± 0.43	1.45 ± 0.67	1.45 ± 0.48	1.45 ± 0.48	0.005
eGFR at 6 months	66.74 ± 21.49	58.25 ± 18.38	57.47 ± 18.99	56.17 ± 19.68	<0.001
BMI	26.34 ± 5.9	25.89 ± 5.4	26.76 ± 5.3	25.37 ± 4.9	0.303
Triglycerides	178.3 ± 86.2	177.4 ± 130.1	171.7 ± 111.5	173.7 ± 86.8	0.957
LDL	92.5 ± 23.3	90.4 ± 25.9	91.8 ± 23.7	86.9 ± 27.9	0.331
Calcium	9.9 ± 0.76	9.8 ± 0.6	9.8 ± 0.6	9.8 ± 0.6	0.688
HDL	50.1 ± 14.3	49.8 ± 14.5	51.2 ± 13.7	51.2 ± 14.9	0.822
Phosphorus	3.2 ± 0.87	3.2 ± 0.76	3.2 ± 0.72	3.4 ± 0.74	0.099
Albumin	4.2 ± 0.36	4.3 ± 0.33	4.3 ± 0.35	4.3 ± 0.4	0.749
Cholesterol	177.7 ± 30.2	175 ± 33.2	176.9 ± 34.8	171.9 ± 35.5	0.535
Glucose	119.8 ± 48.1	112.8 ± 53	116.2 ± 45.4	117.9 ± 60.9	0.787
Mg at 6 months	1.55 ± 0.08	1.68 ± 0.09	1.77 ± 0.03	1.95 ± 0.14	<0.001
Mg at 1 year	1.6 ± 0.08	1.72 ± 0.09	1.82 ± 0.07	1.97 ± 0.15	<0.001
Gender	73 (59.3%)	83 (68.6%)	82 (67.2%)	82 (64.6%)	0.443
Diabetes before transplantation	35 (28.5%)	33 (27.3%)	34 (27.9%)	42 (33.1%)	0.735
Diabetes at 6 months	47 (38.2%)	41 (33.9%)	44 (36.1%)	52 (40.9%)	0.694
IHD before transplantation	17 (13.8%)	27 (22.3%)	31 (25.4%)	29 (22.8%)	0.132
Transplantation number ($n = 2$)	14 (11.4%)	10 (8.3%)	7 (5.7%)	18 (14.2%)	0.132
Delate graft function	21 (17.1%)	24 (19.8%)	26 (21.3%)	35 (27.6%)	0.222
Hypertension	80 (65%)	74 (61.2%)	77 (63.1%)	85 (66.9%)	0.593
Prograf	118 (95.9%)	113 (93.4%)	107 (87.7%)	116 (91.3%)	0.129
Cyclosporine	3 (2.4%)	7 (5.8%)	7 (5.7%)	6 (4.7%)	
mTOR inhibitors	2 (1.6%)	1 (0.8%)	8 (6.6%)	5 (3.9%)	
Anti-aggregates	96 (78%)	96 (80%)	104 (85.2%)	105 (82.7%)	0.493
PPI	93 (75.6%)	89 (73.6%)	88 (72.1%)	91 (71.7%)	0.714
ARB/ACE inhibitors	21 (17.1%)	21 (17.6%)	28 (23%)	33 (26%)	0.247
Mg supplement	32 (26.2%)	19 (15.8%)	9 (7.4%)	6 (4.7%)	<0.001
Calcium supplement	21 (17.1%)	18 (15%)	13 (10.7%)	18 (14.2%)	0.542
Phosphorus supplement	8 (6.5%)	4 (3.3%)	6 (4.9%)	5 (3.9%)	0.664
Statins	62 (50.4%)	54 (45%)	74 (60.7%)	62 (48.8%)	0.088
Beta-blockers	60 (48.8%)	49 (40.8%)	70 (57.4%)	57 (44.9%)	0.063
Calcium channel blockers	45 (36.6%)	40 (33.6%)	56 (45.9%)	56 (44.4%)	0.144
Acute coronary syndrome	11 (8.9%)	16 (13.2%)	7 (5.7%)	6 (4.7%)	0.065
Any smoking	30 (24.4%)	48 (39.7%)	47 (38.5%)	51 (40.2%)	0.027
Current smoking	11 (8.9%)	26 (21.5%)	17 (13.9%)	18 (14.2%)	0.05

describes the characteristics of patients treated with magnesium supplementation vs. the untreated patients.

By univariate Cox analysis, magnesium supplementation was not significantly associated with the primary outcome (HR 1.46, 95% CI 0.9–2.37, $p = 0.103$). However, by the multivariate model including TWA for sMg at 6 months, age, gender, BMI, ischemic heart disease (IHD) before transplantation, serum calcium, albumin and LDL, treatment with PPI, beta blockers, and inhibitors of the renin angiotensin, the association became significant (HR 2.07, 95% CI 1.23–3.46, $p = 0.006$).

DISCUSSION

Previous research has shown an association between sMg levels and renal and cardiometabolic outcomes in the general population and patients with CKD. Due to a lack of

TABLE 2 | Association between TWA quartiles of Mg level and the primary composite outcome of MACE and overall death by univariate and multivariate analysis.

	Univariate analysis			Multivariate analysis		
	Hazard ratio	**95% confidence interval**	***P*-value**	**Hazard ratio**	**95% confidence interval**	***P*-value**
Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
Quartile 2	1.51	0.89–2.56	0.124	1.86	1.08–3.18	0.024
Quartile 3	1.57	0.93–2.54	0.09	1.73	0.99–3.01	0.054
Quartile 4	1.94	1.18–3.19	0.009	1.86	1.08–3.21	0.027

TABLE 3 | Association between sMg quartiles and death censored MACE by univariate and multivariate analysis.

	Univariate analysis			Multivariate analysis		
	Hazard ratio	**95% confidence interval**	***P*-value**	**Hazard ratio**	**95% confidence interval**	***P*-value**
Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
Quartile 2	1.44	0.81–2.54	0.213	1.76	0.98–3.17	0.059
Quartile 3	1.09	0.59–2.2	0.781	1.38	0.72–2.73	0.332
Quartile 4	1.84	1.07–3.18	0.028	2.13	1.18–3.86	0.013

TABLE 4 | Association between quartiles of TWA of sMg and the separate components of MACE.

		Hazard ratio	**95% confidence interval**		***P*-value**
ACS	q1	ref	ref	ref	ref
	q2	0.782	0.324	1.889	0.585
	q3	0.528	0.195	1.429	0.209
	q4	0.858	0.364	2.021	0.726
CVA	q1	ref	ref	ref	ref
	q2	1.264	0.283	5.648	0.759
	q3	1.959	0.49	7.835	0.342
	q4	2.815	0.762	10.403	0.121
PVD	q1	ref	ref	ref	ref
	q2	1.56	0.731	3.331	0.25
	q3	0.898	0.381	2.115	0.805
	q4	1.583	0.753	3.327	0.225
CHF	q1	ref	ref	ref	ref
	q2	0.877	0.338	2.276	0.788
	q3	1.534	0.655	3.595	0.324
	q4	1.406	0.601	3.293	0.432
CV DEATH	q1	ref	ref	ref	ref
	q2	0.794	0.213	2.959	0.731
	q3	1.025	0.297	3.543	0.969
	q4	1.306	0.414	4.117	0.648

TABLE 5 | Association between TWA quartiles of Mg and all-cause mortality by univariate and multivariate analysis.

	Univariate analysis			Multivariate analysis		
	Hazard ratio	**95% confidence interval**	***P*-value**	**Hazard ratio**	**95% confidence interval**	***P*-value**
Quartile 1	Ref	Ref	Ref	Ref	Ref	Ref
Quartile 2	1.45	0.67–3.13	0.343	1.59	0.73–3.45	0.241
Quartile 3	2.2	1.08–4.49	0.031	2.37	1.14–3.91	0.021
Quartile 4	2.16	1.06–4.38	0.034	1.94	0.93–4.06	0.079

TABLE 6 | Baseline characteristics of patients treated with magnesium supplementation vs. untreated patients.

	No Mg supplement	Mg supplement	*P*-value
Number of patients	429	66	
Age	47.83 ± 14.79	49.00 ± 14.52	0.551
Gender (men)	278 (64.8%)	41 (62.1%)	0.672
Dialysis duration (months)	32.58 ± 36.49	37.09 ± 29.07	0.339
Donor age (years)	45.37 ± 13.93	43.52 ± 15.27	0.3
Systolic BP (mm Hg)	130.43 ± 18.95	131.23 ± 13.61	0.76
BMI (kg/m^2)	26.08 ± 5.45	25.81 ± 6.42	0.747
LDL cholesterol	90.19 ± 25.21	91.02 ± 21.61	0.807
HDL cholesterol	50.66 ± 14.67	49.66 ± 11.86	0.601
Albumin	4.26 ± 0.37	4.32 ± 0.34	0.215
Corrected calcium	9.62 ± 0.56	9.83 ± 0.69	0.007
Glucose	116.49 ± 51.75	118.15 ± 54.59	0.817
eGFR (ml/min/1.73 m^2)	58.53 ± 18.70	66.34 ± 13.42	0.003
Smoker status	61 (14.2%)	12 (18.2%)	0.398
History of IHD	93 (21.7%)	12 (18.2%)	0.518
Diabetes	159 (37.1%)	26 (39.4%)	0.716
Living donor	256 (59.7%)	32 (48.5%)	0.082
First transplantation	380 (88.6%)	64 (97.0%)	0.061
DGF	90 (17.1%)	16 (24.2%)	0.547
Hypertension	270 (63.2%)	49 (74.2%)	0.082
Prograf	392 (91.4%)	63 (95.5%)	0.395
Cyclosporine	23 (5.4%)	1 (1.5%)	
mTOR inhibitors	14 (3.3%)	2 (3.0%)	
Anti-aggregates	356 (83.0%)	48 (72.7%)	0.045
PPI	307 (71.6%)	56 (84.8%)	0.023
Ace inhibitors	97 (22.7%)	6 (9.1%)	0.018
Statins	217 (50.6%)	34 (51.5%)	0.888
Beta blockers	199 (46.4%)	39 (59.1%)	0.063
Ca channel blockers	173 (40.5%)	25 (37.9%)	0.684

studies examining this association in patients with kidney transplantation, we sought to examine this association. Our results indicate that renal transplant patients within the lowest quartile of exposure to sMg levels had a 1.5 lower risk for the composite outcome of all-cause mortality and MACE compared to patients with higher levels of exposure. In addition, magnesium supplements did not improve the prognosis of renal transplant patients and were also associated with negative outcomes by multivariate analysis.

Mg is essential for vital cellular functions and is required as a co-factor for many enzymatic reactions. Mg regulates ion channels that participate in neuromuscular excitability and cell permeability and immune response (1). In addition, Mg has anti-atherosclerosis, anti-inflammatory, and antioxidant properties. Recent studies demonstrated an association between dysmagnesemia and poor outcomes in the general population (3, 4), CKD patients (8, 13), dialysis patients (12), and heart failure patients (19). In a population-based study that included 4,203 individuals, low Mg levels were associated with higher all-cause mortality and cardiovascular mortality (20). Van laecke et al. conducted a study with 1,650 CKD patients and a follow-up time of 5.1 years. In the study, hypomagnesemia predicted mortality and renal function decline in CKD patients. Patients with low (<1.8 mg/dl) vs. high (>2.2 mg/dl) serum Mg had a 61% increased mortality risk (adjusted HR 1.613, 95% CI 1.113–2.338, $p < 0.0001$) (13).

A recent prospective multicenter study (CRIC study) that was conducted by Lavinia Negrea et al. included 3,867 patients with CKD. The study evaluated the association between sMg level and cardiovascular events and all-cause mortality. This was the largest study made in CKD patients with a long follow-up of 14.6 years. The authors conclude that sMg level <1.9 and >2.1 mg/dl was associated with increased risk for all-cause mortality. Low sMg level was associated with incident atrial fibrillation but not with composite CVD events. This study is added to the growing body of evidence that propose an association between high sMg level and cardiovascular outcomes (21).

Sakaguchi et al. in a study that included 142,555 HD patients showed a J-shaped association between serum magnesium and all-cause and cardiovascular mortality from the lowest to highest sextile, with significantly higher mortality in sextiles 1–3 and 6 (22). In contrast, Angkananard et al. (19) conducted a systematic

review and meta-analysis that included seven prospective studies with a total of 5,172 chronic heart failure (CHF) patients. The meta-analysis suggested that in CHF patients, hypermagnesemia with Mg >/= 1.05 mmol/liter (2.55 mg/dl) was associated with an increased risk of CV mortality and all-cause mortality. Due to the nature of this study, the effect of underlying conditions like CKD and advanced age was not ruled out. Hypomagnesemia was not found to be associated with CV mortality.

Cheungpasitporn et al. (23) evaluated the association of dysmagnesemia and different outcomes in 65,974 hospitalized patients. Hypermagnesemia (>2.3 mg/dl) in comparison to hypomagnesemia (Mg < 1.7 mg/dl) was a stronger predictor for poor outcomes including hospital mortality.

Several studies suggested a link between post-renal transplantation hypomagnesemia and new-onset diabetes (15, 16). Huang et al. conducted a retrospective study with 948 renal transplant recipients and observed 182 new-onset diabetes events. Multivariate analysis showed an increased risk between hypomagnesemia [<0.74 mmol/liter (1.8 mg/dl)] and new-onset diabetes (HR 1.24 per 0.1 mmol/liter decrease, 95% CI 1.05–1.46, $p = 0.01$) (15). Osorio et al. (24), performed a retrospective study with 589 renal transplant patients. Patients who received CNI had lower levels of Mg, and particularly, patients in the tacrolimus group had the lowest Mg level and the highest incidence of NODAT, but there was no relationship between Mg levels and occurrence of NODAT. Santos et al. (25) published a retrospective cohort study with 205 kidney recipients. The study did not find an association between hypomagnesemia and NODAT at a follow-up period of 1 year. Our study was not designed for evaluation of diabetes; however, no association between TWA of sMg at 6 months and glucose levels at that time point was observed.

Our study showed that hypomagnesemia post-transplantation was associated with a lower risk for all-cause mortality and MACE. Cheddani et al. (18) compared mortality risk in kidney transplant recipients beyond 1 year after successful transplantation vs. eGFR-matched CKD patients. In multivariable analysis, kidney transplant recipients had a 2.7-fold greater risk of mortality. Cardiovascular death rates in kidney transplant recipients (29.0%) approximated those of CKD patients (22.5%), whereas death rates due to infections were higher in kidney transplant recipients (19.4 vs. 10.0%). Our results demonstrate a higher mortality rate due to infections as mentioned above. It should be noted that renal transplant recipients within the lower quadrant of sMg at 6 months had better renal function compared to patients with higher levels of Mg. Although multivariate analysis including eGFR at 6 months did not change the association between low sMg and reduced risk of MACE, the difference in renal function might still explain the lower risk, as eGFR might poorly represent the full spectrum of kidney function. In addition, other unmeasured confounders might also play an important role in this association.

The difference between our results and other reports in different populations might be that after kidney transplantation sMg is mainly affected by immunosuppressive medications and kidney function (26), while in the general population, it is usually associated with a healthier diet. As a result, low sMg in the general population indicates poor nutrition, while in patients after kidney transplantation, it might indicate better kidney function and adequate immunosuppression.

In addition, our results strongly suggest that Mg supplementation was associated with higher all-cause mortality and MACE. As patients with low sMg, the main indication for Mg supplementation had a lower rate of adverse outcomes; these results do not support the routine use of Mg supplementation after kidney transplantation. It should be noted that very limited prospective interventional trials with patient-centered outcomes evaluating Mg supplementation are available. Such trials are urgently needed in order to make evidence-based decisions regarding the need for Mg supplementation.

This study has several important strengths. The use of all available sMg levels and time varying exposure produces a very precise evaluation of the exposure. In addition, the outcome analysis was based on detailed reports, each read by the researchers. Furthermore, the median follow-up time of more than 5 years enables adequate evaluation. In addition, we used several statistical methods to evaluate the risk of our primary and secondary outcomes and to increase the internal validity of our results.

Our study also has several limitations. Being a single center treating mostly a Caucasian population, the generalizability is limited. Also, despite extensive multivariate analysis, the possibility of residual confounding is still significant. Possible confounders might include kidney function and medications given over time as our analysis included creatinine and medications only at a fixed time point at 6 months post-transplant. Furthermore, eGFR is only rough estimation of true kidney function, and therefore, adjustment for kidney function might not be complete. Another possible confounder is nutrition status that might influence both sMg and CVD. In addition, the sample size, which is adequate for evaluation of the composite primary outcome, did not enable good evaluation of subgroups and the separate components of MACE. Also, the number of cardiovascular events in the study was small. In addition, evaluation of Mg supplementation was done only for one time point and dosing was not available. Detection bias is another possible limitation as high sMg was associated with reduced kidney function, which makes coronary angiography and the detection of CAD less likely.

CONCLUSIONS

This study found an association between low exposure to sMg and better outcomes, which might reflect the confounding effect of better kidney function and adequate immunosuppression. Mg supplementation at 6 months post-transplantation was associated with an increased risk of all-cause mortality and MACE. This is the first study investigating the association between Mg levels and outcomes such as all-cause mortality and MACE in renal recipients. More studies, especially randomized controlled trials, are needed to further evaluate this important issue.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IL: participated in research design, performance of the research, data analysis and in the writing of the paper. TS: participated in performance of the research, interpretation of data for the work and in the writing of the paper. BR-Z: participated in research design, in the performance of the research, in data analysis and in in the revising of the paper. RR: participated in research design, in interpretation of data for the work and in the revising of the paper. MM: participated in research design, in the performance of the research and in the revising of the paper. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.690273/full#supplementary-material

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CASE REPORT
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Case Report: Capillary Leak Syndrome With Kidney Transplant Failure Following Autologous Mesenchymal Stem Cell Therapy

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Mesenchymal stem cells (MSCs) have attracted great interest in the field of kidney transplantation due to their immunomodulatory and reparative properties. In registered clinical trials, MSCs have been used before, at the time of, or early after transplantation and have been reported to be well-tolerated with no serious safety concerns. No results are available on the use of MSCs in the late post-transplant period. Here, we present a case report of a severe systemic complication mimicking capillary leak syndrome with ultimate kidney transplant failure after autologous transplantation of MSCs used as rescue treatment of late antibody-mediated kidney allograft rejection.

Keywords: mesenchymal stem cell, kidney graft, kidney transplant, kidney graft failure, autologous stem cell, stem cell transplant, kidney graft rejection, capillary leak syndrome

INTRODUCTION

Mesenchymal stem cells (MSCs) have attracted much attention due to their immunomodulatory properties that can help in alloimmune diseases. Several factors, including immunosuppressive factors, growth factors, extracellular vesicles, and chemokines, contribute to the immunosuppressive mechanisms of MSCs (1). Pilot studies in clinical research with MSCs in kidney transplant recipients (KTRs) aimed to reduce immunosuppressive therapy, induce immune tolerance, treat T-cell rejection, and prevent delayed graft function (2–11). To date, the administration of MSCs in clinical transplantation has been shown to be safe and feasible without serious safety concerns being reported.

Here, we present a case report of a serious adverse reaction in KTR after autologous transplantation of MSCs from bone marrow, which was used as a rescue treatment for resistant antibody-mediated rejection (AMR). The patient was included in the study protocol (ClinicalTrials.gov, number NCT03585855), which was subsequently discontinued due to safety concerns.

CASE PRESENTATION

A 26-year-old man with a history of acute lymphoblastic leukemia (ALL) in childhood and end-stage kidney failure due to IgA nephropathy received a deceased donor kidney transplant at the age of 21, mismatched for 3 HLA antigens (one mismatch in HLA-A, HLA-B, and HLA-DR). Two years after kidney transplantation (KTx), an indication biopsy for an increase in serum creatinine (sCr) levels showed a mixed T-cell rejection (Banff 4/IB) and acute AMR with positive donor-specific antibodies (DSA; anti-HLA DQB1 and DQA1) while receiving triple maintenance immunosuppressive regimen (tacrolimus/mycophenolate mofetil/steroid). The rejection was treated with high-dose steroids, antithymocyte globulin, plasmapheresis, intravenous immunoglobulins (IVIg), and rituximab. After 3 years of stable kidney function with sCr in the range between 150 and 180 μmol/L, we observed a progressive deterioration of kidney function with a sCr value of 240 μmol/L and a 24-h proteinuria of 3.4 g/day before entering the study protocol. The kidney biopsy revealed chronic active AMR (**Figures 1A,B**).

Due to the history of childhood ALL, which was treated with a combination chemotherapy (vincristine, doxorubicine, methotrexate, cyclophosphamide, cytarabine), we first performed a bone marrow aspiration, which showed non-specific reactive changes. The treatment protocols were approved by National Ethic Committee (approval no. 0120-215/2018/4). The written informed consent was obtained from the patient. After completion of the standard of care therapy (including corticosteroids, membrane plasmapheresis, and IVIg), the patient received MSCs therapy according to the study protocol, consisting of 3 x 10^6 cells/kg, applied at 1-week intervals. Details of the MSCs cultures and criteria for bench release are described in the Supplementary documentation (**Supplementary Appendix 1 and Table 1**).

After the first dose of MSCs the patient reported short term nausea. One week later after the second dose of MSCs nausea, blepharitis and diarrhea developed, with their remission after 24 h. This was associated with a slight deterioration in kidney function, which we attributed to prerenal causes (**Figure 2A**). When the patient was referred for the third dose of MSCs (2 weeks after the first dose), the symptoms had completely disappeared. The evening after the third dose of MSCs was administered, the patient was admitted to the emergency department due to abdominal cramps, vomiting, and diarrhea. A further deterioration of the kidney function was observed (sCr of 390 μmol/L), accompanied by newly developed ascites and abdominal lymphadenopathy.

Although indicators of inflammation remained normal throughout the course of treatment, the initial clinical presentation was suspicious for infection. Therefore, the manufacturing process of MSCs was reevaluated and contamination by infectious agents during cultivation or before release was ruled out. An expanded diagnostic investigation (**Supplementary Appendix and Table 2**) ruled out common and opportunistic infections, including Herpes

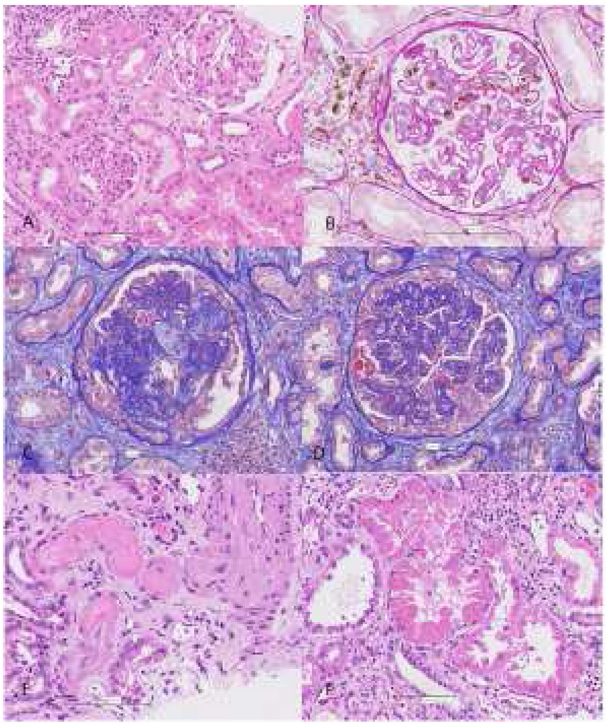

FIGURE 1 | Before application of MSCs (March 2019): chronic active C4d negative antibody-mediated rejection with preserved tubules **(A)**, focal glomerulitis and peritubular capillaritis **(B)**. According to Banff 2017 criteria, transplant kidney biopsy was consistent with chronic active C4d negative antibody mediated rejection: 12% glomerulitis with double contour formation (g1, cg3) in glomeruli, diffuse severe peritubular capillaritis (ptc3), chronic active vascular rejection (v1, cv3) in interlobular arteries without signs of thrombotic microangiopathy, diffuse more than 50% interstitial fibrosis and tubular atrophy with mild mononuclear cell inflammation consisting of lymphocytes, macrophages, and rare plasma cells (i-IFTA 3, ci3, ct3). There was no associated tubulitis. Peritubular capillary basement membrane multilamellation (ptcbm3) was seen by electron microscopy. There were no deposits in tubular basement membrane. Immunofluorescence revealed IgA deposits indicating IgA nephropathy recurrence. 60% of glomeruli were globally sclerotic. 1 of 25 glomeruli showed pseudocrescent formation without PAS positive droplets in podocytes. After application of MSCs (July 2019): glomerular TMA with mesangiolysis **(C)**, diffuse pseudocrescent formation in glomeruli with marked podocytes injury **(D)**, vascular TMA **(E)** and huge tubular injury with resorptive droplets consisted with mottled lysosomes on EM **(F)**. Glomeruli showed advanced double contour formation and segmental sclerosis without apparent glomerulitis, but mesangiolysis was seen in some glomeruli. 20% of glomeruli showed pseudocrescent formation with PAS positive droplets "in podocytes indicating huge podocytes injury". Peritubular capillaritis was mild and focal. Tubulitis was absent. Tubules show signs of severe tubular injury (attenuation of tubular epithelium with coarse vacuolization, loss of brush border, loss of nuclei) and were filled with large PAS-positive droplets in line mottled lysosomes on electron microscopy. Electron optic dense deposits in tubular basement membrane were detected by EM. Amount of interstitial fibrosis, tubular atrophy, and interstitial inflammation was similar as in previous biopsy. In fibrotic areas, there was mild mononuclear cell inflammation consisting of lymphocytes 75%, macrophages 15%, and plasma cells 10%. No CD105+, CD73+ nor CD90+ cells were found. In small arteries and arterioles, thrombotic microangiopathy with obliteration of vascular lumens, fragmentation of erythrocytes, and fibrinoid necrosis was present. Peritubular capillary basement membrane multilamellation (ptcbm3) was similar as in previous biopsy.

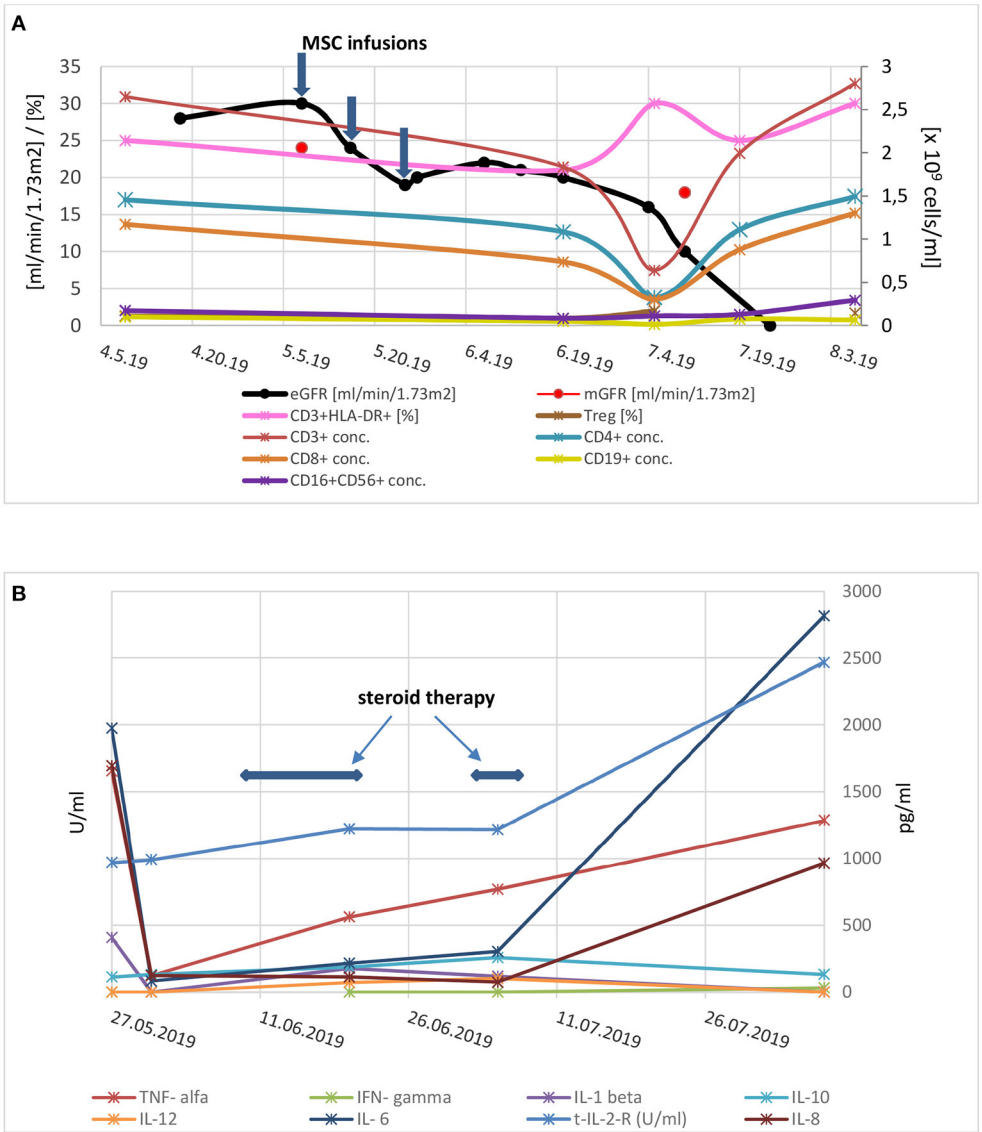

FIGURE 2 | (A) Kidney graft function and serum concentrations of lymphocyte populations before and after application of mesenchymal stem cells. Three consecutive applications of mesenchymal stem cells are marked with arrows. eGFR, estimated glomerular filtration rate; mGFR, measured glomerular filtration rate (by Cr-EDTA); conc, concentration; Treg, regulatory T cells. **(B)** Cytokine concentrations after mesenchymal stem cells application (in U/mL for soluble interleukin 2 receptor and in pg/mL for other cytokines). Time frame of corticosteroid treatment is marked with arrows; Legend: TNF, tumor necrosis factor; IL, interleukin; s-IL-2-R, soluble interleukin 2 receptor; IFN, interferon.

viruses 6, 7, and 8, CMV, HIV, and EBV viremia, bacteremia, gastrointestinal infections, and the possibility of intestinal bacterial overgrowth syndrome. Endoscopic biopsies of the colon and duodenum showed mucosal edema without inflammatory cell infiltration or apoptotic bodies. Given the normal concentrations of complement components and absence of fetal bovine albumin in the cell medium (platelet lysate was used instead), a serum disease-like syndrome did not seem likely. A capillary leak variant syndrome, associated with sudden capillary hyperpermeability, resulting edemas and hypoalbuminemia, was considered. Therefore, antihistamines and methylprednisolone were administered at a dosage of 1.5 mg per kg body weight. After administration of high-dose steroids, the symptoms disappeared, and the dose was reduced during the next 10 days. At this point, sCr stabilized in the range of 300 μmol/l.

Two weeks later (i.e., 5 weeks after the last dose of MSCs), there was a recurrence of abdominal symptoms (nausea, diarrhea, ascites), this time followed by resistant hypertension, a further deterioration of kidney function (sCr of 380 μmol/l), an

increase in proteinuria (7.3 g per day), and signs of hemolysis and pancytopenia. Tacrolimus trough levels were very low (<2 ng/ml), despite an increase of the dosage and assured adherence to therapy. The abdominal CT scan showed ascites, thickening of the intestinal wall (especially the jejunum), lymphadenopathy and an enlarged spleen. Transcriptomic analysis by the molecular microscope diagnostic system (MMDx) (12) of the subsequent kidney biopsy showed persistence of AMR, although the molecular classifiers of inflammation and AMR were lower compared to the biopsy before application of MSCs (inflammation score of 1.06 vs. 3.19, and AMR score of 0.63 vs. 0.94, respectively; **Figure 3**). In addition, the MFI levels of DSA decreased and we had no reason to assume a further deterioration of AMR.

Cytokine assessment after MSCs treatment (at the time of onset of symptoms) showed increased levels of IL-2R and IL-8, while other cytokines, including TNF-α and IL-6, were within near normal ranges and not consistent with a cytokine storm (**Figure 2B**, for details see **Supplementary Table 2**). Comparative analysis of T and B lymphocyte subsets before and after MSC therapy showed a transient decrease in all lymphocyte subsets with an increase in the ratio of activated T lymphocytes that occurred concomitantly with worsening of symptoms and the appearance of pancytopenia (**Figure 2A**). Bone marrow examination was consistent with trilineage dysplasia and a secondary myelodysplastic syndrome attributable to potentiated immunosuppressive therapy. It is more likely, however, that the changes in the bone marrow were of reactive origin. Morphologically, there was no evidence of parvovirus B19 infection of the bone marrow.

The kidney allograft failed 2 months after MSCs transplantation and hemodialysis was initiated. After administration of granulocyte colony stimulating factor (G-CSF) and reintroduction of methylprednisolone at a dose of 1 mg/kg, leukopenia improved minimally, and gastrointestinal symptoms worsened after each application of GCS-F. Therefore, the steroids and GCS-F were discontinued. The patient's general condition gradually deteriorated with progressive malaise, vomiting/diarrhea, generalized edema, and laboratory signs of hypoalbuminemia and agranulocytosis. The clinic's expert council made the empirical decision to end a life-threatening situation by removing the failed kidney transplant. After the transplant nephrectomy, the cell counts recovered almost instantly (**Figure 2A**). At this time, parvovirus B19 viremia was also detected. After treatment with high-dose IVIg, the parvovirus B19 infection resolved. Retrospectively, parvovirus B19 was detected by PCR in both the preimplantation kidney biopsy (6 years before MSC therapy) and in the kidney graft after MSC therapy. The patient is currently on chronic hemodialysis, remains aviremic and no further MSCs associated adverse events were observed during the 24-month follow-up period. Patohistologic analysis of the explanted kidney was consistent with advanced AMR, glomerular and vascular thrombotic microangiopathy (TMA), and extensive tubular injury (**Figures 1C-F**).

DISCUSSION

Here, we describe a case report of a serious adverse event associated with autologous bone marrow MSCs in KTR as part of a rescue therapy protocol for chronic AMR. To briefly summarize: following the use of autologous MSCs, the patient developed symptoms and signs of systemic disease, including malaise, nausea, vomiting, blepharitis, diarrhea, ascites, splenomegaly, resistant hypertension, hemolytic anemia, nephrotic range proteinuria with rapid deterioration of kidney function, and pancytopenia. Symptoms developed progressively, responded only initially to steroid treatment, worsened after GCS-F and resolved almost immediately after explantation of the failing kidney. Pathohistological findings in biopsied organs after MSCs therapy included: (i) mucosal edema of the colon and duodenum; (ii) MDS-like changes in bone marrow; (iii) new onset of TMA and severe tubular injury in the kidney allograft, which overlaid the previously observed AMR. To the best of our knowledge, these undesirable outcomes have not yet been reported following transplantation of the MSC line.

So far, 10 clinical studies in KTRs using MSCs have been completed. In six of these studies autologous or donor derived MSCs were injected intravenously or intraarterially before or during surgery or up to 6 months after KTx, in a maximum of two doses up to 10 x 10^6/kg (2–6, 8). Vanikar et al. (7) co-infused donor adipose derived MSCs together with hematopoietic stem cells in portal circulation prior to living donor kidney transplantation. Two recent studies reported on the use of human umbilical cord derived MSCs applied before or during transplant surgery (9) and third-party MSCs up to day 5 after transplantation (10). No serious adverse reactions have been reported. However, the pilot study of the Remuzzi group reported negative effects when autologous MSCs were used in two KTRs (2). They reported a temporary decrease in kidney function with the histological picture characterized by a low number of infiltrating $CD4^+$ and $CD8^+$ T cells, B cells and monocytes, but a high number of neutrophils with increased deposition of complement component C3. A considerable number of $CD105^+$ $CD44^+$ double positive cells (markers co-expressed by MSCs) were also detected in the biopsy, suggesting the recruitment of systemically infused MSCs within the kidney allograft. After administration of steroids, kidney function improved in both patients. In contrast, no adverse effects were reported when MSCs were administered before KTx (4). These clinical cases differ from ours, because the histological findings in our patient's transplanted kidney showed neither infiltration of neutrophils nor infiltration of MSCs ($CD105^+$ $CD44^+$were absent) or complement deposition. It is important to note that none of the patients in the study by Perico et al. (2) showed multi-organ dysfunction.

Given the systemic clinical presentation and patient history, probable causes such as common infections, contamination of MSC product, progression of AMR, recurrence of ALL and adverse effects of immunosuppressive therapy were initially ruled out. Unfortunately, we were not aware of active parvo B19 infection at the beginning because we performed blood PCR

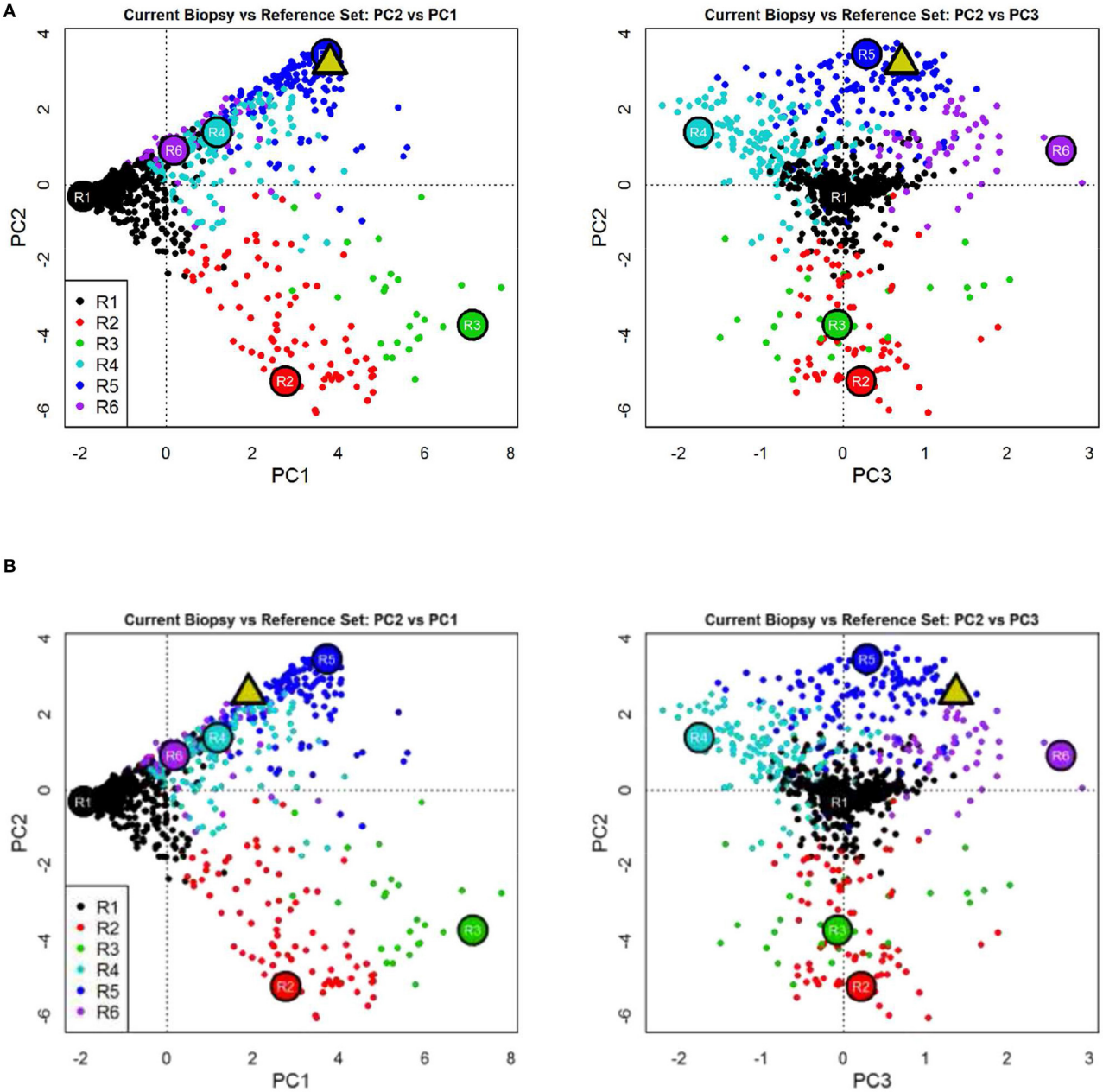

FIGURE 3 | Molecular microscope diagnostic report (MMDx) for kidney transplant biopsy before **(A)** and after **(B)** application of mesenchymal stem cells showing fully developed antibody-mediated rejection with extensive microvascular inflammation, chronic glomerulopathy, and atrophy-fibrosis molecular features.

only when the clinical picture of pancytopenia progressed. Later, we confirmed parvo B19 in the preimplantation kidney biopsy, suggesting possible donor transmission, so parvo B19 was present in the transplanted kidney long before MSC therapy. Infections with parvo B19 may be associated with significant morbidity in immunocompromised patients (13). The most frequent manifestations of parvo B19 infection in KTRs are cytopenia's with the dominance of anemia. Collapsing glomerulopathy and TMA have been reported in 10% of cases with dysfunction or failure of the kidney transplant (14). In addition, as reported by Sundin et al. (15) MSCs are among the cells that express the B19 receptor (P-antigen/globoside) and a co-receptor and harbor the parvovirus B19, which can impair their clinical utility. Subsequent analysis of the patient's historical sera and a pretransplant biopsy of the donated kidney revealed that the virus was transmitted to the recipient via a donated organ, and that the parvo B19 viremia was present shortly before MSCs transplantation. Although MSCs did not induce viremia, the

cumulative effect of additional immunosuppression inhibited the B-cell response, which may have had an enhancing effect on the replication of parvovirus B19.

The pleiotropic clinical picture with gastrointestinal involvement, massive transudation of plasma into the third space and pancytopenia strongly suggested early complications that would otherwise be expected after hematopoietic stem cell transplantation, in particular capillary leak syndrome (16). In addition, the continuous clinical picture observed in this case showed some similarities with other entities such as engraftment syndrome (17, 18), hemophagocytic lymphohistiocytosis (19), autologous GVHD (20), and POEMS syndrome. However, strict criteria for making one of the mentioned diagnoses were not met. Therefore, it is highly probable that endothelial damage due to the action of various and difficult-to-define transplant-related factors played an important role in its development. The progressive endothelial damage led to a prothrombotic and proinflammatory state in the patient, which eventually resulted in capillary occlusion. Dysregulation at the level of the complement system and/or the possible presence of antibodies, either donor- or recipient-specific, or as yet unrecognized inflammatory triggers could play a significant and crucial role in terminal endothelial damage. In animal studies, Koch et al. (21), who administered isogenic MSCs to rats at the time of allogeneic KTx, reported similar adverse effects. All animals developed severe kidney failure, 20% of which developed TMA, and 75% had to be sacrificed within 30 days. These life-threatening events were evident despite decreased T- and B-cell kidney allograft infiltration, reduced interstitial inflammation and downregulated inflammatory genes.

Many other studies in animal models of KTx emphasized the importance of the timing of MSCs infusion and provided evidence for improved outcomes when MSCs transplantation was performed prior to KTx [for details see review by Casiraghi et al. (22)], with higher renoprotective and anti-inflammatory effects when used prior to the development of a potent inflammatory microenvironment. However, subsequent studies have shown that peri-transplant infusion of syngeneic MSCs can lead to severe kidney failure associated with tissue injury, increased expression of pro-inflammatory cytokines, B-cell infiltration and C4d deposition in the allograft, regardless of the immunosuppressive protocol used (23).

CONCLUSION

This case report describes the first clinical case of acute kidney transplant failure with life-threatening systemic adverse effects after autologous MSCs transplantation to reverse late AMR. The scenario described suggests important safety concerns related to the use of MSCs in the long-term period following solid organ transplantation when the allograft is already injured by chronic active rejection.

Considering the absence of similar adverse effects in the two patients who followed the same study protocol (follow-up periods of 24 and 20 months after MSCs application; unpublished results), the individual susceptibility of the patient may also have played a role in the development of adverse events. Nevertheless, for safety reasons, we would strongly recommend further preclinical research before proceeding with clinical trials to accurately assess the benefits and risks of MSC therapy in the context of solid organ transplantation. The current report also highlights the importance of monitoring for possible transmission of parvovirus B19 through MSCs products or donor organs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Medical Ethics Committee. Štefanova 5 1000 Ljubljana. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ŽV-H and MA: study design, performance of the research, data analysis, and drafting of the manuscript. NK: pathohistological diagnosis, performance of the research, and manuscript revision. PH: MMDX analysis and critical revision. MS, SZ, and TU: study conception, performance of the research, and critical revision. UŠ, PP, and KH: performance of the research, analysis and interpretation of data, and critical revision. GM, MO, AA, AI, and JB: acquisition and interpretation of data, and critical revision. All authors have approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.708744/full#supplementary-material

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ORIGINAL RESEARCH
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Outcome of Husband-to-Wife Kidney Transplantation With Mutual Children: Single Center Experience Using T Cell-Depleting Induction and Review of the Literature

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Few data on husband-to-wife transplantations with mutual children (H2W) exist in the current era. We investigated the outcome of H2W transplantations ($n = 25$) treated with T cell-depleting induction compared to women with prior pregnancies also receiving their first HLA-mismatched kidney transplant, but from a different donor source: (i) other living donor ($n = 52$) and (ii) deceased donor ($n = 120$). Seventy-four percent of the women had ≥2 pregnancies; median follow-up time was 5 years. Death-censored allograft survival was significantly lower in the H2W group compared to the other two groups ($p = 0.03$). Three of four graft losses in the H2W group were due to rejection. 5-year patient survival in the H2W group was high and similar compared to the other living donor group (100 vs. 98%; $p = 0.28$). The incidence of (sub)clinical antibody-mediated rejection was higher in the H2W group (36 vs. 20 vs. 18%) ($p = 0.10$). The frequency of infections was similar among the three groups. No immunological parameter was predictive for rejection or graft loss in H2W transplantations. In conclusion, H2W transplantation is a valuable option, but associated with a higher risk for allograft loss due to rejection despite T cell-depleting induction. Further research is required for better risk prediction on an individual patient level.

Keywords: husband-to-wife transplantation, kidney transplantation, allograft rejection, infection, pregnancy-induced sensitization

INTRODUCTION

Pregnancy is an important reason for HLA-directed sensitization. Using sensitive single antigen bead assays on the Luminex platform, child-specific HLA-directed antibodies are detected immediately after delivery in about 20–30% of women after one pregnancy and almost 50% after three or more pregnancies (1). This suggests that repeated exposures to the same HLA molecules increases the likelihood of a detectable humoral immune response (2, 3). Furthermore, it is well-known that pregnancy-induced HLA antibodies can diminish over time, while alloreactive T- and B cells still persist (4).

Many women evaluated for kidney transplantation had prior pregnancies more than 10–20 years ago (5, 6). As sera dating back to the immediate time after delivery are very rarely available, sensitization cannot be excluded, even if no HLA antibodies are detectable in current sera. This is a major diagnostic challenge in husband-to-wife transplantations with mutual children (H2W), because there will be a 20–50% chance of prior husband-specific sensitization depending on the number of pregnancies. Indeed, several case reports/series demonstrated that severe early rejection can occur in H2W transplantations despite the absence of detectable HLA antibodies prior to transplantation (7–10).

Although H2W transplantations carry an unpredictable risk of rejection, some women do not have other options as living donors. In addition, the benefit of a preemptive/timely H2W transplantation compared to a yearlong dialysis period and subsequent deceased donor transplantation has to be considered. At our center, we regard H2W transplantations as immunological risk and treat these women with T cell-depleting induction. This therapy has been shown to reduce the incidence and severity of rejection in patients with donor-specific HLA antibodies (DSA) (11). So far, outcomes of H2W transplantations receiving T cell-depleting induction have not been described.

The aim of this study was to investigate pertinent outcomes of H2W transplantations treated with T cell-depleting induction in comparison to other options for women with prior pregnancies receiving their first HLA-mismatched kidney transplant (i.e., other living donor or deceased donor transplantation).

PATIENTS AND METHODS

Patient Population

The ethics committee of Northwestern and Central Switzerland approved this retrospective study (www.eknz.ch; project ID 2021-00584). The pregnancy history is documented in all women evaluated for kidney transplantation. In case of spousal donors, we carefully evaluated during the living donor evaluation process, if the spouse was indeed the biological father of the children (i.e., separate interviews of the couple by a physician and a dedicated psychologist).

For this study, we assessed all kidney transplantations in women performed at the University Hospital Basel from January 1st, 2005 until January 31st, 2020 for eligibility ($n = 368$). One-hundred and seventy-one of 368 transplantations (46%) were excluded for the following reasons: no previous pregnancies ($n = 85$), previous transplantation(s) ($n = 56$), induction protocol violation ($n = 19$; detailed in the immunosuppression section), HLA-identical living donor transplantation ($n = 8$), child-to-mother transplantation ($n = 2$), and unknown pregnancy status ($n = 1$). The remaining 197 women all had their first HLA-mismatched kidney transplantation and previous pregnancies. According to the kidney donor source and the detailed pregnancy history the transplantations were divided into three groups: (i) H2W ($n = 25$), (ii) other living donor ($n = 52$), (iii) deceased donor ($n = 120$).

Living and Deceased Donor Selection Process

HLA antibody analysis was performed by single antigen beads on the Luminex platform using a cutoff of 500 MFI, and DSA were determined by a virtual cross-match approach as previously reported (5, 12).

All willing and medically eligible living donors are usually evaluated regarding histocompatibility. Priority is given to donors without DSA constellation. Husbands having mutual children with the recipient were accepted as donors, if no DSA constellation was present. If DSA were present, transplantation was pursued after discussion with the couple regarding other options, and if considered as immunologically feasible (negative T- and B-cell CDC-cross-matches, and usually no more than three DSA at $\leq$2 loci and cumulative MFI$<$10000). Other living donors with DSA were accepted using the same criteria.

For deceased donor selection, priority is given to DSA negative donors according to the algorithm of the national donor allocation program (13). DSA were accepted in patients with high cPRA, if regarded as immunologically feasible (negative T- and B-cell CDC-crossmatches) (12).

Immunosuppression

H2W transplantations were considered as immunological risk and received an induction therapy consisting of a polyclonal anti T cell globulin (ATG; Gravalon 9 mg/kg bw prior to reperfusion of the allograft and 3 mg/kg bw on day 1–4 or Thymoglobulin 4 days 1.5 mg/kg bw). In case of circulating DSA, intravenous immunoglobulins (IvIg) were additionally given (5 days 0.4 g/kg bw). Maintenance immunosuppression consisted of tacrolimus (Tac), mycophenolate (MPA) and prednisone. Target tacrolimus trough levels were 10–12 ng/ml for the first month, 8–10 ng/ml for months two to three, 6–8 ng/ml for months four to six, and 4–8 ng/ml thereafter. Steroids were tapered to 0.1 mg/kg body weight by month three post-transplant.

For all other transplantations, the induction therapy was selected based on the presence/absence of DSA. Patients without DSA received an induction therapy with basiliximab (20 mg on day 0 and 4) and triple therapy with Tac-MPA-P or a steroid-free regimen consisting of Tac-MPA and a mTOR-inhibitor. In case of a rejection-free clinical course, immunosuppression was modified and reduced within the first 6 months to establish a dual Tac-MPA therapy on the long-term. Target trough levels of tacrolimus were identical to the levels described above. Patients with DSA received an induction therapy with ATG and IvIg and maintenance immunosuppression consisting of Tac-MPA-P. Target trough levels of tacrolimus were identical to the levels described above. Steroids were tapered to 0.1 mg/kg body weight by month three post-transplant and maintained at this level.

All ABO-blood group incompatible (ABOi) transplant recipients received a single dose of rituximab 4 weeks prior to transplantation and immunadsorption depending on the anti-blood group titers.

TABLE 1 | Baseline characteristics.

Parameter	Husband-to-wife ($n = 25$)	Other living donor ($n = 52$)	Deceased donor ($n = 120$)	*p*
Recipient age	56 (47–64)	49 (39–57)	59 (53–65)	<0.0001
Renal disease				
ADPKD	8 (32%)	16 (31%)	28 (23%)	0.48
Diabetic nephropathy	1 (4%)	7 (13%)	13 (11%)	
Glomerulonephritis	6 (24%)	15 (29%)	33 (28%)	
Interstitial nephropathy	-	3 (6%)	8 (7%)	
Vascular nephropathy	2 (8%)	3 (6%)	13 (11%)	
Other nephropathies	4 (16%)	6 (12%)	8 (6%)	
Unknown nephropathy	4 (16%)	2 (3%)	17 (14%)	
Renal replacement therapy				
Preemptive transplantation	12 (48%)	22 (42%)	5 (4%)	<0.0001
Dialysis vintage time [years]	0.8 (0.2–1.8)	0.7 (0.2–1.4)	2.6 (1.6–4.3)	<0.0001
Donor age	60 (51–66)	52 (46–59)	53 (25–66)	0.15
Number of pregnancies				
One	4 (16%)	23 (44%)	23 (19%)	0.004
Two	13 (52%)	19 (37%)	50 (42%)	
≥3	8 (32%)	10 (19%)	47 (39%)	
Blood transfusions (yes/no/unknown)	29%/54%/17%	24%/69%/8%	40%/42%/18%	0.03
cPRA (A/B/DR/DQ) [%]	1 (0–48)	20 (0–42)	23 (0–78)	0.03
ABO incompatible	2 (8%)	10 (19%)	n.a.	n.a.
DSA present	9 (36%)	8 (15%)	28 (23%)	0.13
DSA characteristics	$n = 9$	$n = 8$	$n = 28$	
Number	2 (1–3)	2 (1, 2)	1 (1, 2)	0.48
Class (I/II/I+II)	3/3/3	1/3/4	10/13/5	0.40
Cumulative MFI	1,571 (889–7,023)	1,533 (1,014–4,269)	1,425 (597–4,646)	0.80
HLA mismatches (A/B/DR/DQ)	6 (5–7)	4 (3–6)	5 (4–6)	0.004
CMV risk constellation				
High risk (D+/R–)	4 (16%)	7 (14%)	15 (13%)	0.71
Intermediate risk (R+)	14 (56%)	37 (71%)	80 (67%)	
Low risk (D–/R–)	7 (28%)	8 (15%)	25 (21%)	
Prophylaxis with valganciclovir	18 (72%)	21 (40%)	41 (34%)	0.002
Induction therapy				
ATG ± Ivlg	25 (100%)	8 (15%)	28 (23%)	<0.0001
Basiliximab	-	44 (85%)	92 (77%)	
Maintenance immunosuppression				
Tac-MPA-P	25 (100%)	40 (77%)	114 (95%)	0.0007
Tac-MPA-mTOR	-	11 (21%)	4 (3%)	
Other*	-	1 (2%)	2 (2%)	

ADPKD, autosomal polycystic kidney disease; DSA, donor-specific HLA-antibodies; ATG, anti T-cell globulin; Ivlg, intravenous immunoglobulins; Tac, tacrolimus; MPA, mycophenolic acid; mTOR, mTOR inhibitors; P, prednisone; n.a., not applicable.

**No Tac-based immunosuppression.*

As mentioned above, 19 transplantations were excluded from the study, because the used induction deviated from the protocol. Most of these violations occurred in deceased donor transplantations due to retrospective DSA status corrections after extending the donor HLA typing (i.e., additional loci or high resolution typing) (14). Only one patient in the H2W group was excluded. This patient received an ABOi transplant without DSA and the induction was restricted to rituximab without additional ATG due to her frailty.

Medication as Infection Prophylaxis

All patients received prophylaxis with trimethoprim/sulfamethoxazole (160/800 mg three times per week) against pneumocystis jirovecii infection for 6 months. The CMV prevention strategy at our center has been described previously (15). Briefly, high-risk patients (D+/R–) received prophylaxis with oral valganciclovir (Valcyte, Roche) 450 mg twice daily adjusted for renal function. Intermediate-risk patients (R+) received prophylaxis

TABLE 2 | Major outcomes.

Parameter	Husband-to-wife (*n* = 25)	Other living donor (*n* = 52)	Deceased donor (*n* = 120)	*p*
Reason for graft failure	***n* = 4**	***n* = 2**	***n* = 5**	
Rejection	3	1	2	n.a.
Recurrent GN	1	–	–	
Vascular/surgical	–	1	–	
Other	–	–	3	
Cause of death	***n* = 0**	***n* = 4**	***n* = 33**	
Cardiovascular	–	–	6	n.a.
Malignancy	–	1	5	
Infection	–	1	11	
Other	–	1	4	
Unknown	–	1	7	
Estimated GFR				
At 1 year	54 (39–68)	58 (47–70)	54 (38–70)	0.24
At 3 years	50 (37–62)	61 (46–75)	54 (32–72)	0.16
At 5 years	58 (38–70)	55 (43–75)	51 (33–83)	0.60
Urine protein/creatinine ratio [mg/mmol]				
At 1 year	10 (8–19)	13 (8–18)	13 (8–24)	0.50
At 3 years	11 (6–22)	11 (8–15)	13 (9–23)	0.20
At 5 years	16 (8–20)	9 (5–23)	13 (9–23)	0.25
Post-transplant DSA screening	***n* = 18**	***n* = 34**	***n* = 73**	
No *de novo* DSA	10 (56%)	26 (76%)	44 (60%)	0.27
De novo DSA	1 (6%)	2 (6%)	10 (14%)	
Persisting pre-transplant DSA	3 (16%)	1 (3%)	11 (15%)	
Disappearing pre-transplant DSA	4 (22%)	5 (15%)	8 (11%)	

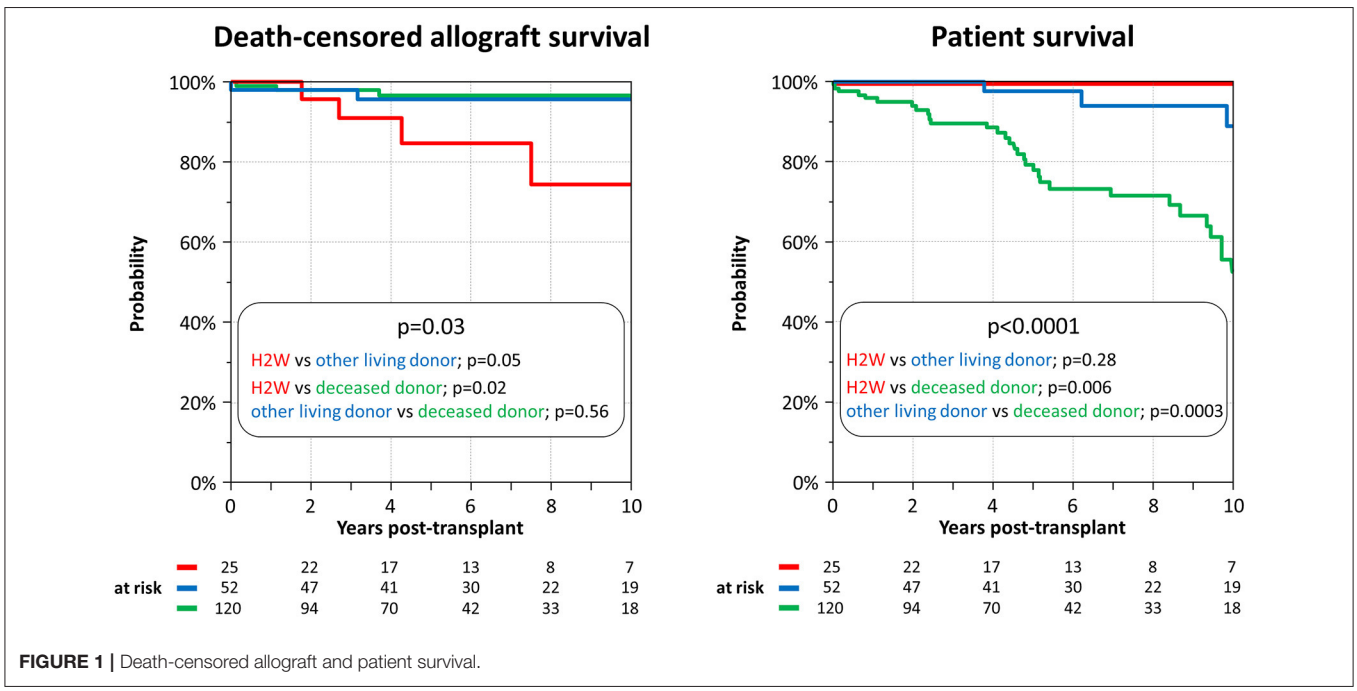

FIGURE 1 | Death-censored allograft and patient survival.

with valganciclovir, if they had an induction therapy with ATG or were ABO-incompatible. All other intermediate-risk patients were managed by regular monitoring and deferred therapy. Low-risk patients (D–/R–) received no prophylaxis and had no regular screening. Prophylaxis was given for a minimum of 3 months and prolonged, if

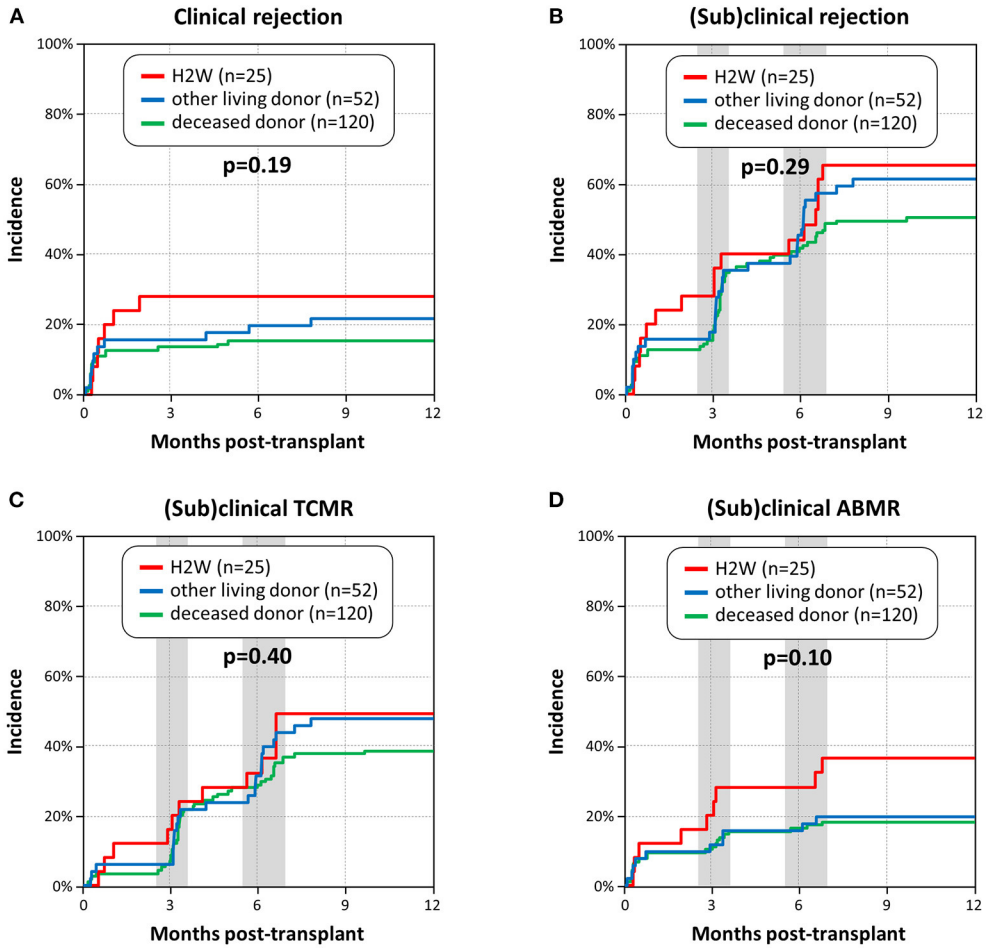

FIGURE 2 | Incidence of rejection within the first year post-transplant. **(A)** Incidence of clinical rejection. **(B)** Incidence of (sub)clinical rejection. **(C)** Incidence of (sub)clinical T cell-mediated rejection (TCMR). **(D)** Incidence of (sub)clinical antibody-mediated rejection (ABMR), including mixed rejection. The gray background areas represent the two time frames, in which surveillance biopsies at 3 and 6 months were performed.

immunosuppression was still considered as high (e.g., recent rejection therapy).

Assessment and Treatment of Allograft Rejection

Patients were monitored by surveillance biopsies at 3 and 6 months post-transplant. Clinically indicated allograft biopsies were performed when serum creatinine increased by >20% from baseline. Findings were graded according to the Banff 2015 classification (16). Mixed rejection were grouped to antibody-mediated rejection (ABMR). Clinical and subclinical rejection episodes were treated according to the phenotype and severity. Clinical T cell-mediated rejection (TCMR) were mainly treated with i.v. steroid pulses (3-5*500 mg methylprednisolone) and a steroid taper. Subclinical TCMR were mainly treated with p.o. steroids (3*200 mg prednisone) and a steroid taper. Patients with clinical ABMR and mixed rejection episodes received ATG and in some cases in addition IvIg. Subclinical ABMR or mixed rejection were treated with i.v. or p.o. steroid pulses and a steroid taper. Borderline changes, which are by far the most frequent rejection phenotype in the current era of immunosuppression, were regarded and treated as TCMR (17).

Statistical Analysis

We used JMP software (SAS Institute Inc., Cary, NC) for statistical analysis. Categorical data are presented as counts and/or percentages and were analyzed by chi-square test or Fisher's exact test as appropriate. Continuous data are shown as median and interquartile ranges [IQR] and compared by Wilcoxon rank sum tests. For all tests, a (two-tailed) $p < 0.05$ was considered to indicate statistical significance. Time-to-event analyses were performed by the Kaplan-Meier method and compared by the log-rank test. Multivariate Cox regression analysis was used to investigate independent predictors for rejection and graft failure in H2W transplantations.

TABLE 3 | Infections occurring within the first year post-transplant.

Parameter	Husband-to-wife ($n = 25$)	Other living donor ($n = 52$)	Deceased donor ($n = 120$)	p
Polyomavirus BK				
No relevant viruria (i.e., no Decoy cells)	18 (72%)	34 (65%)	86 (72%)	0.29
Viruria, but no viremia	6 (24%)	13 (25%)	15 (13%)	
Viremia	1 (4%)	4 (8%)	17 (14%)	
Definitive BK-nephropathy	–	1 (2%)	2 (2%)	
CMV				
No CMV viremia	21 (84%)	43 (83%)	73 (61%)	0.01
Asymptomatic CMV viremia	3 (12%)	9 (17%)	27 (22%)	
CMV syndrome	–	–	12 (10%)	
Tissue-invasive CMV disease	1 (4%)	–	8 (7%)	
Other infections including lower UTI				
None	8 (32%)	18 (35%)	31 (26%)	0.24
Only one	6 (24%)	19 (37%)	32 (27%)	
≥2 infections	11 (44%)	15 (29%)	57 (47%)	
Other infections excluding lower UTI				
None	13 (52%)	29 (56%)	66 (55%)	0.22
Only one	5 (20%)	18 (34%)	29 (24%)	
≥2 infections	7 (28%)	5 (10%)	25 (21%)	

UTI, urinary tract infection.

RESULTS

Baseline Characteristics

Baseline characteristics of the three groups are summarized in **Table 1.** We observed significant differences regarding several parameters. Recipients in the deceased donor and H2W groups were older than in the other living donor group (59 vs. 56 vs. 49 years; $p < 0.0001$). In addition, women in the H2W and deceased donor groups had more often ≥2 pregnancies compared to the other living donor group. As expected, the latter group had less HLA mismatches than the other groups due to related in-family donors (i.e., parents, siblings). We observed no statistically significant differences regarding the frequency of DSA as well as their characteristics. CMV constellations were equally distributed among the three groups, but prophylaxis with valganciclovir was given more often in the H2W group due to universal use of ATG induction. Maintenance immunosuppression with Tac and MPA was used in 194/197 patients (98%).

Patient and Graft Survival

After a median follow-up time of 5.1 years (IQR 2.9–9.0), 11/197 allograft (5.6%) failed: four in the H2W group, two in the other living donor group, and five in the deceased donor group. Rejection accounted for 6/11 graft failures (55%), three of these occurred in the H2W group. One of these three patients had DSA (Cw4 and DR15 with cumulative MFI of 5759), the other two patients had no DSA. Details on the reasons for graft loss are summarized in **Table 2**. Death-censored allograft survival was significantly lower in the H2W group compared to the other two groups ($p = 0.03$). In detail, 1-year survival was still similar among the three groups (100 vs. 98 vs. 99%), but lower in the H2W group at five (85 vs. 96 vs. 97%) and 10 years (74 vs. 96 vs. 97%) (**Figure 1**).

Thirty-seven of 197 patients (19%) died. None in the H2W group, 4 in the other living donor group and 33 in the deceased donor group. Leading causes of death were infections ($n = 12$), cardiovascular diseases ($n = 6$), and malignancies ($n = 6$) (**Table 2**). Patient survival was significantly lower in the deceased donor group compared to the other two groups ($p < 0.0001$) (**Figure 1**). Notable, patient survival was similar in the H2W and the other living donor group ($p = 0.28$).

The median estimated glomerular filtration rate (eGFR) and proteinuria of functioning allograft were similar among the three groups at one, three and 5 years post-transplant (**Table 2**).

Allograft Rejection

Overall, the 197 patients had 81 clinical biopsies within the first year as well as 259 surveillance biopsies at 3 and 6 months, respectively. Only 37/197 patients (19%) had no allograft biopsy at all within the first year post-transplant. The frequency of clinical and surveillance biopsies was not different among the three groups ($p = 0.20$ and $p = 0.38$). Clinical biopsies beyond the first year post-transplant were obtained in 34/197 patients (17%), with similar frequency among the three groups ($p = 0.64$).

The 1-year incidence of clinical rejection was statistically not different among the three groups ($p = 0.19$), but numerically highest in the H2W group (28 vs. 22 vs. 15%). We observed similar incidences of (sub)clinical rejection and (sub)clinical TCMR ($p = 0.29$ and $p = 0.40$) among the three groups. Interestingly, the incidence of (sub)clinical ABMR was numerically around twice as high in the H2W group compared

to the other groups (36 vs. 20 vs. 18%), but this did not reach statistical significance ($p = 0.10$) (**Figure 2**).

Infections Within the First Year

Infections occurring within the first year post-transplant are summarized in **Table 3**. We observed no differences regarding frequency and severity of polyomavirus BK infection (BKV). Cytomegalovirus (CMV) infections were more frequent and more severe in the deceased donor group compared to the other groups ($p = 0.01$). However, they were equally often observed in the H2W and the other living donor group.

We recorded 345 infections other than BKV and CMV. The percentage of patients having no, one or ≥2 infections was not different among the three groups ($p = 0.24$). As expected, lower urinary tract infections (UTI) were the most frequent infection accounting for 54% of all cases, followed by upper airway infections (13%), gastrointestinal infections (11%), skin infections (9%), and upper UTI (i.e., pyelonephritis) with 7%. Fungal infections were rare (15/345; 4.3%). When excluding lower UTI, about 50% patients in the three groups experienced no infection within the first year post-transplant (**Table 3**).

Evolution of Pre-transplant DSA and Development of *de novo* DSA

Post-transplant screening for HLA antibodies was performed in 125/197 patients (63%), including 35/45 women having pre-transplant DSA (78%) and 90/152 women without pre-transplant DSA (59%). The frequency of screening was similar among the three groups (H2W 72%, other living donor 65%, deceased donor 61%; $p = 0.54$) and was performed at a median of 3.1 years post-transplant. Of 35 patients with pre-transplant DSA, 3 developed *de novo* DSA (9%), 15 had persisting DSA (43%), and in 17 cases DSA disappeared (48%). In those 90 women without pre-transplant DSA, 10 developed *de novo* DSA (11%). The frequency of *de novo* DSA, persisting or disappearing DSA was not different among the three groups ($p = 0.27$) (**Table 2**).

Predictors of Rejection and Graft Failure in H2W Transplantations

During the whole follow-up 16/25 H2W transplantations developed (sub)clinical rejection, 10/25 clinical rejection, and 4/25 allograft failed. DSA status (yes/no), number of pregnancies (1 vs. ≥2), and HLA-mismatches were not associated with any of the above mentioned events in univariate analysis and multivariate Cox-models (all $p \geq 0.09$).

Review of the Literature

We found 10 publications investigating H2W transplantations with prior pregnancies: 5 cohort studies, 4 detailed case reports, and one case series (**Table 4**).

Nine of ten reports were published between 1995 and 2008. In these studies, a Tac- or cyclosporine-based immunosuppression without induction therapy was used for most patients. All patients were considered as having no pre-transplant DSA by either cytotoxicity or flowcytometric crossmatches. The five cohort studies or case series published between 1995 and 2008 reported 1-year graft survival from 60 to 90%, which was mostly lower than in the comparison groups and lower than in our study (100%) (10, 19–22). All case reports described acute antibody-mediated or mixed rejection episodes within the first week post-transplant, which could be successfully reversed (7–9, 23).

The only more recent publication in 2020 by Kim et al. reported on 159 H2W transplantations using Tac-MPA-P immunosuppression and basiliximab induction (18). They observed a higher frequency of clinical rejection in the H2W group, but similar patient and graft survival compared to offspring-to-mother and other living unrelated donors.

DISCUSSION

The key observation is this study was that despite T cell-depleting induction, H2W transplantations have a higher risk for death-censored graft loss possibly mediated by a higher frequency of ABMR compared to other HLA-mismatched living or deceased donor transplantations in women with prior pregnancies. However, patient survival in H2W transplantation is excellent, and the universal use of T cell-depleting induction was not associated with a higher frequency or infections within the first year post-transplant.

Previous cohort studies published between 1995 and 2008 reported 1-year graft survival rates between 78 and 90% in H2W transplantations, which were mostly lower than in the comparison groups (19–22). Although the frequency of rejection was often not reported, we assume that most of these 10–20% early graft losses might be related to rejection, which were not prevented by the used immunosuppressive protocol. We speculate that ATG induction together with Tac-MPA-P maintenance immunosuppression contributed to the better 1-year graft survival (i.e., 100%) in our study. Support for this interpretation comes from a retrospective cohort study, which demonstrates the efficacy of ATG induction in allograft recipients with pre-existing donor-specific sensitization (11). Clearly, only a prospective randomized study in H2W transplantations comparing ATG vs. other induction (e.g., basiliximab) or no induction can provide more conclusive evidence, but it is very unlikely that such a study will ever be performed.

A recent study from South Korea reported excellent short and long-term graft survival in 159 H2W transplantations receiving basiliximab induction and Tac-MPA-P maintenance immunosuppression (18). We cannot explain these favorable results compared to reports from Europe and North America. One possibility might be that South Korea has a less heterogeneous population and hence less immunogenic HLA mismatches. Furthermore, the number of pregnancies was not reported in their study. A lower frequency of prior pregnancies will reduce the overall risk of a husband-specific sensitization, which can critically influence the outcome (1).

Our study as well as most of the referenced publications highlight that H2W transplantations are associated with a wide range of outcomes, ranging from early, severe rejection

TABLE 4 | Review of the literature.

References	Study type	H2W (*n*)	Number of pregnancies	Husband as biological father confirmed	Pre-transplant DSA assessed	With DSA	Induction	Maintenance immuno-suppression	Patient survival	Graft survival	Key observation
This manuscript	Cohort study	25	1 preg: 16% 2 preg: 52% ≥3 preg: 32%	Yes	Yes (Luminex SA)	36%	ATG ± IvIg	Tac-MPA-P	1 year: 100% 5 years: 100%	1 year: 100% 5 years: 85%	Death-censored graft survival lower in H2W compared with other living or deceased donors
Kim et al. (18)	Cohort study	159	At least one (no details given)	No	Yes (method not reported; likely solid-phase assays)	32%	ATG (4%) Basiliximab (96%)	Tac-MPA-P (79%)	5 years: 98%	5 years: 96%	No difference in patient and graft survival compared with offspring-to-mother ($n = 175$) and other living unrelated donors ($n = 56$), but higher rate of clinical rejection
Ghafari et al. (19)	Cohort study	9	2–5 (mean 2.6)	No	Yes (AHG-CDC-XM)	0%	None	CyA-MPA/AZA-P	1 year: 97% 5 years:78%	1 year: 78% 5 years: 69%	More rejection and lower graft survival compared to other living unrelated donors
Pretagostini et al. (20)*	Cohort study	33	At least one (no details given)	No	Yes (CDC-XM)	0%	Not reported	CyA-based	Not reported	1 year: 80% 5 years: 73%	Lower 1 year graft survival compared to W2H (90%) and other unrelated living donor (100%)
Gjertson et al. (21)	Cohort study	407	1 preg: 31% ≥2 preg: 69%	No	Yes (CDC-XM)	0%	Not reported	Not reported	Not reported	1 year: 90% 5 years: 78%	No difference in graft survival according to the number of pregnancies in H2W
Terasaki et al. (22)	Cohort study	75	At least one (no details given)	No	Not reported (likely CDC-XM)	n.a.	Not reported	Not reported	Not reported	1 year: 87% 3 years: 76%	Graft survival @3 years worse compared to H2W without any pregnancies (76% vs. 87%; $p = 0.40$)
Ortiz-Arroyo et al. (10)	Case series	5	At least one (no details given)	No	Yes (AHG-CDC-XM)	0%	Basiliximab (40%)	Triple therapy	Not reported	1 year: 60%	Two out of five patients developed acute ABMR and lost the graft
Matsuo et al. (9)	Case report	1	2	Yes	Yes (FCXM/FlowPRA)	No	Basiliximab	Tac-MPA-P	Good function at 6mt		Acute ABMR starting POD 3, reversed by plasmapheresis, steroids and rituximab

(Continued)

TABLE 4 | Continued

References	Study type	H2W (*n*)	Number of pregnancies	Husband as biological father confirmed	Pre-transplant DSA assessed	With DSA	Induction	Maintenance immuno-suppression	Patient survival	Graft survival	Key observation
Rosenberg et al. (8)	Case report	1	5	Yes	Yes (CDC-XM/FCXM)	No	Not reported	Tac-MPA-P	Good function at 2 year		Acute rejection (Banff IIA) starting POD 2, reversed by plasmapheresis
Habicht et al. (7)	Case report	1	3	Yes	Yes (CDC-XM/FCXM)	No	None	CyA-MPA-P	Good function at 1 year		Acute ABMR (only C4d positive) POD 7, reversed by steroids, ATG and immunoadsorption
Böhmig et al. (23)	Case report	1	5	Yes	Yes (CDC-XM/FCXM)	Yes**	None	CyA-MPA-P	Good function at 3mt		Acute mixed rejection first week, reversed by ATG and immunoadsorption

H2W, husband-to-wife transplantation with previous pregnancies; W2H, wife-to-husband transplantation; CDC-XM, complement-dependent cytotoxicity crossmatch; FCXM, flowcytometric crossmatch; POD, post-operative day.
**These authors previously reported outcomes of H2W from the same population with fewer cases (24, 25).*
***Retrospectively detected by FCXM.*

and graft loss to uneventful courses. Unfortunately, currently available parameters such as presence of DSA, number of pregnancies, and number of HLA mismatches seem not to be very predictive. Analyses on the molecular level of HLA disparities (i.e., eplet load and eplet immunogenicity) might provide better prediction, but such an evaluation would require a significantly larger number of cases (26–30). Another interesting approach could be the evaluation of memory B cell responses (31). With recent advances to detect memory B cells, it seems nowadays possible to obtain a more complete picture of the pre-transplant alloimmunization status in these women (32–35). Since the magnitude of memory B cell responses following previous HLA immunization such as pregnancies may differ, their detection might explain the higher proportion of early (sub)clinical rejection episodes in H2W transplants, as observed in our study and described before (7–10, 23).

How should we council pairs in evaluation for a H2W transplantation? If the husband is indeed the biological father of the mutual child/children, we recommend to use an appropriate immunosuppressive regimen including ATG induction and to discuss with the pair the increased risk for rejection as well as allograft failure. Alternative living donors having different HLA haplotypes than the biological father should be prioritized, especially in case of numerous pregnancies. If a broad and/or high husband-specific sensitization can be detected by single antigen bead assays and no alternative suitable living donor is available, a deceased donor transplantation or inclusion in a kidney-paired donation program avoiding all major DSA might be the best option (36). Avoidance of even all mismatched husband HLA antigens (despite lack of detectable husband-specific sensitization by the most sensitive techniques) by opting for deceased donor transplantation is probably the safest approach to prevent early severe rejection (37). However, this will often significantly reduce the pool of suitable donors. From a statistical point of view, a woman with two full-term pregnancies has a 40% risk to be sensitized against any HLA-A/B/C/DRB1 mismatches of the father (1). If a deceased donor shares only one HLA mismatch with the father, the risk of performing the transplantation in the presence of pre-existing sensitization will be much lower in most cases, but it still exists (37).

Despite a numerically higher incidence of ABMR in the H2W group compared to the other two groups, the frequency of detectable circulating DSA post-transplant was not different. Notably, post-transplant screening for DSA was not performed at the time of allograft biopsies, but mainly beyond the first year post-transplant after a median of 3.1 years. It is possible that the frequency of circulating DSA post-transplant is underestimated due to absorbance of circulating DSA in the allograft.

Patient survival in the deceased donor group was rather low, but death-censored allograft survival excellent (**Figure 1**). Notably, the women in this group were significantly older (median 59 years) and had longer dialysis vintage time (median 2.6 years) compared to the other groups. Therefore, they had very likely more relevant comorbidities and a poorer health condition, which were

responsible for the high mortality. Patient death is the leading cause of allograft loss in elderly patients, which will consequentially lead to a high death-censored allograft survival (38).

This study has certain limitations. First, as in all previous reports, H2W transplantations represent a real-life selection, and it is difficult to define appropriate control groups. To reduce biases, we decided to include all women with prior pregnancies, who received the first HLA-mismatched kidney transplant and grouped them according to the donor source. The defined groups (H2W, other living donor, and deceased donor) essentially delineate the three possible options for women with prior pregnancies. Second, several analyses have clear statistical limitations due to a low patient number in the H2W group ($n = 25$) and/or low event rates. In particular, the absence of significant predictors of rejection and allograft failure in H2W transplantations has to be interpreted with caution. Third, review of the literature revealed that important parameters such as number of pregnancies, definition and presence of DSA, and induction therapy are incompletely reported, making comparison between studies difficult.

In conclusion, H2W transplantation with mutual children is a valuable option, but is associated with a higher risk of allograft loss due to rejection despite T cell-depleting induction. Further research is required to better predict this risk on an individual patient level.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of Northwestern and Central Switzerland (www.eknz.ch); project ID 2021-00584. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LS and SS: designed study, performed research, analyzed data, and wrote the manuscript. LS, CW, GH, PA, PH-M, and IG: collected data. CW, GH, IG, PA, PH-M, JS, and MD: critically reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Malacoplakia Due to *E. coli* With *Cryptococcus albidus* Infection of a Transplanted Kidney in a Patient With Recurrent Urinary Tract Infection

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Background: Colonization of *Cryptococcus* rarely occurs in a graft. This study reports a case of malacoplakia and cryptococcoma caused by *E. coli* and *Cryptococcus albidus* in a transplanted kidney, with detailed pathology and metagenome sequencing analysis.

Case Presentation: We presented a case of cryptococcoma and malacoplakia in the genitourinary system including the transplant kidney, bladder, prostate, and seminal vesicles caused by *Cryptococcus albidus* and *Escherichia coli* in a renal-transplant recipient. Metagenome sequencing was conducted on a series of samples obtained from the patient at three different time points, which we termed Phase I (at the diagnosis of cryptococcoma), Phase II (during perioperative period of graftectomy, 3 months after the diagnosis), and Phase III (2 months after graftectomy). Sequencing study in the Phase I detected two and four sequences of *C. albidus* respectively in cerebrospinal fluid (CSF) and feces, with resistant *Escherichia coli 09-02E* presented in urine and renal mass. A 3-month antibiotic treatment yielded a smaller bladder lesion but an enlarged allograft lesion, leading to a nephrectomy. In the Phase II, two sequences of *C. albidus* were detected in CSF, while the *E. coli 09-02E* continued as before. In the Phase III, the lesions were generally reduced, with one *C. albidus* sequence in feces only.

Conclusions: The existence and clearance of *Cryptococcus* sequences in CSF without central nervous system symptoms may be related to the distribution of infection foci

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in vivo, the microbial load, and the body's immunity. Overall, this study highlights the need for enhanced vigilance against uncommon types of *Cryptococcus* infections in immunocompromised populations and increased concern about the potential correlation between *E. coli* and *Cryptococcus* infections.

Keywords: cryptococcoma, malacoplakia, *Cryptococcus albidus*, *Escherichia coli 09-02E*, transplanted kidney, metagenome sequencing

INTRODUCTION

As an opportunistic pathogenic fungus, *Cryptococcus* is the third most common invasive fungi in solid organ transplantation (SOT) (1). The prevalence of cryptococcosis in this population is 0.2 to 5.8%, with a total mortality rate ranging from 20 to 50% (1, 2). Human immunodeficiency virus-infected patients and SOT recipients are at the highest risk for *Cryptococcus* infection (3), with *Cryptococcus neoformans* and *Cryptococcus gattii* the most common causes of cryptococcosis. Once inhaled, *Cryptococcus* can disseminate to the whole body or colonize in host tissue through the bloodstream, resulting in diseases such as cryptococcal meningoencephalitis, pulmonary cryptococcosis and cryptococcal granuloma (2, 4, 5). However, *Cryptococcus albidus* infection is rare, with skin the most commonly involved organ and a mortality rate of around 28% (5/18) (6).

Only one case of localized cryptococcal lesion in transplant kidney has been reported so far (7). Herein we reported the first case of pathology- and metagenome sequencing-proven cryptococcoma caused by *C. albidus* of a transplanted kidney in a patient presenting with urinary tract infection (UTI) of *Escherichia coli* and BK polyomavirus viruria. This study sheds light on the correlation between drug-resistant *E. coli* and *Cryptococcus* infection. The results suggest that alteration of the immune microenvironment caused by a long-term infection, such as *E. coli* infection, may be the key reason for the colonization of *Cryptococcus* in uncommon sites of the body, like an allograft.

CASE DESCRIPTION

Clinical History

A 50-year-old male with end-stage renal disease received a left kidney transplant from a deceased male donor who died in a motor vehicle accident in March 2013. After renal transplantation, the patient received a maintenance immunosuppressive regimen consisted of tacrolimus (3.5 mg, bid), mycophenolate mofetil (360 mg, bid), and prednisone (4 mg, qd). At 11 months postoperatively, 1+ to 2+ proteinuria was found on a routine urine examination. The proteinuria was relieved after treatment with Tripterygium glycosides tablets (10 mg, bid). At 15 months postoperatively, the patient developed BKV viruria with a urinary viral load of 1.25×10^7 copies/mL (normal range for reference, <5,000 copies/mL). The viral load was undetectable after the dosage of tacrolimus was reduced to 1.5 mg BID and treating with immunoglobulin (infusion). In April 2016, the patient had a chronic rejection reaction and the 24-h urinary protein quantity increased to 1.13 g/24 h. To maintain the allograft function and suppress proteinuria, the corresponding treatment regimen was methylprednisolone (40 mg) combined with cyclophosphamide (0.2 g) intravenous drip for 3 days/month. After three courses of treatment, the 24-h urinary protein quantity decreased to 0.56 g/24 h. The patient has had recurrent symptoms of UTI such as frequent and urgent urination without obvious inducement since June 2016. Regular outpatient review of urinary examination revealed leukocytes fluctuating from 1+ to 3+. *E. coli* was detected in the midstream urine culture and intravenous cefoperazone sodium sulbactam (1.5 g, 1/12 h) was given for 1 week. In September 2018, the patients came to the hospital because of cough for 1 day. mNGS of the alveolar lavage fluid indicated pneumosporidiosis and blood tested positive for herpes simplex virus. The pulmonary infection resolved after treated with compound sulfamethoxazole tablets (480 mg, bid). Besides, serum creatinine decreased from 305 μmol/L to 245 μmol/L. However, BKV viruria relapsed with the urinary viral load fluctuated from 2.84×10^5 copies/mL to 3.81×10^7 copies/mL. The immunosuppressive regimen was adjusted to tacrolimus (1.5 mg, bid), mycophenolate sodium enteric-coated tablets (180 mg, bid), and prednisone (4 mg, qd). In November 2018, the patient's serum creatinine was 197 μmol/L, and color Doppler examination of the transplanted kidney and renal vessels showed no significant abnormalities. Regular color Doppler ultrasound examinations of the allograft and transplanted kidney vessels were performed every 6 months after transplantation, all showing neither significant abnormalities nor transplanted kidney masses until the current admission. The patient had not undergone an allograft puncture biopsy within 3 years after renal transplantation. His postoperative serum creatinine level was 190 μmol/L. And the postoperative glomerular filtrate rate (GFR) was summarized in **Figure 1**. The patient was admitted to hospital in May 2019 because of frequent and urgent urination. The clinical course of the patient is summarized in three phases according to the disease progression.

Phase I

In May 2019, the patient was admitted to hospital for recurrent frequent and urgent urination. On admission, his serum creatinine level was 229 μmol/L. Ultrasound and PET/CT showed solid space-occupying lesions in the upper pole of the transplanted kidney and bladder, prostate and seminal vesicles (**Figures 2A1–H1**). Biopsy of the graft kidney and bladder lesions revealed cryptococcal granulomas, with cystoscopy results provided in **Supplementary Figure 1**. Extended-spectrum β-lactamase positive *E. coli* was cultured from both renal graft tissue and midstream urine. To rule out systemic cryptococcosis,

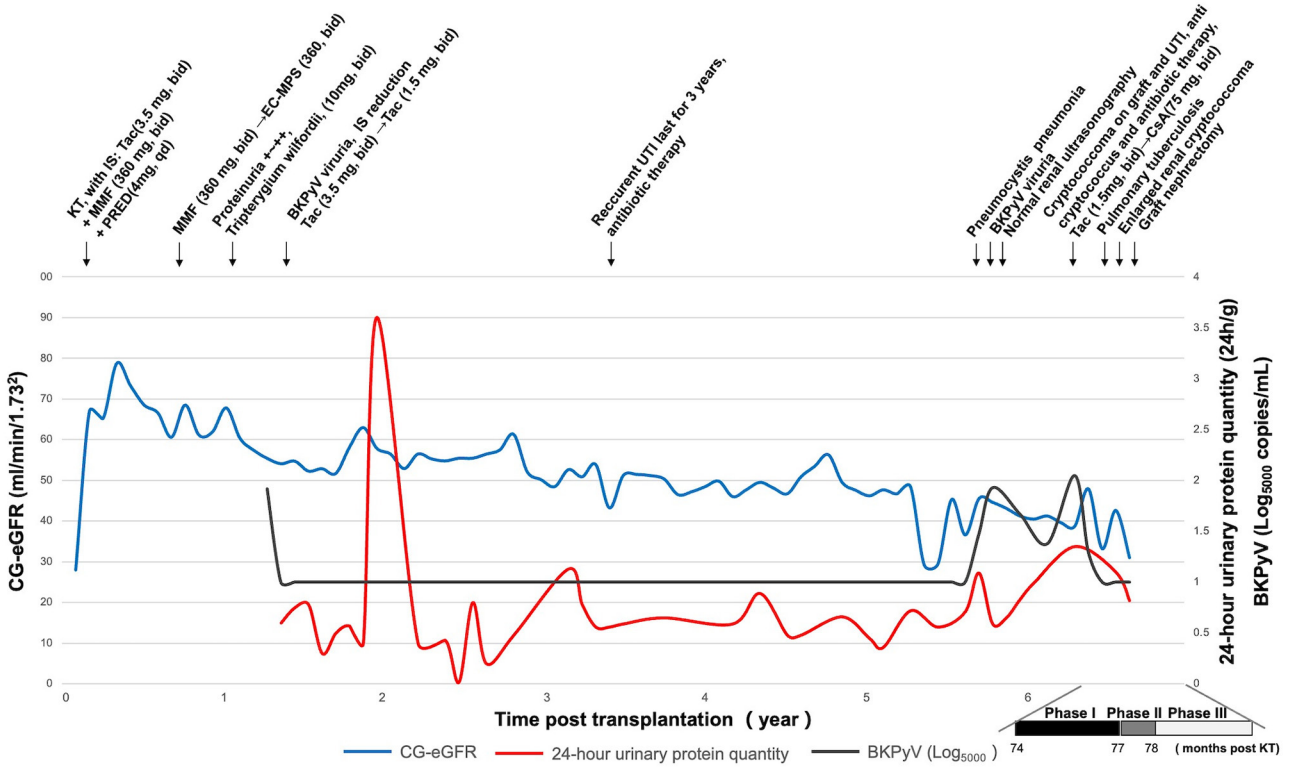

FIGURE 1 | Clinical history of the patient. CG-eGFR, estimated glomerular filtration rate by the Cockcroft-Gault; KT, kidney transplantation; IS, immunosuppressant; Tac, tacrolimus; MMF, mycophenolate mofetil; PRED, prednisone; EC-MPS, mycophenolate sodium enteric-coated tablets; CsA, cyclosporine; BKPyV, BK polyomavirus; UTI, urinary tract infection.

a lumbar puncture was performed to collect CSF and CSF opening pressure measured. Cell counts and biochemical parameters in CSF, and CSF opening pressure were normal. Ink staining and *Cryptococcus* antigen detection of CSF were both negative. No significant abnormalities were observed in brain MRI and chest CT scans. Based on the imaging and pathological findings, the patient was diagnosed with cryptococcoma and malacoplakia of the genitourinary system including transplanted kidney, bladder, prostate and seminal vesicles (**Figures 2A1–H1** and **Figure 3**), accompanied by an UTI of *E. coli*. The patient was treated with meropenem (0.5 g twice a day), and fluconazole (50 mg twice a day) combined with flucytosine (0.5 g twice a day). Meanwhile, immunosuppression was reduced by conversion from tacrolimus (1.5 mg twice a day) to cyclosporine (100 mg twice a day). Additionally, cyclosporine was later adjusted to 75 mg BID after use of the antifungal drug, fluconazole, which can affect the concentration of cyclosporine. The concentrations of immunosuppressants from renal transplantation to transplant nephrectomy were shown in **Supplementary Figure 4**. After 10 days' treatment, his renal function improved and the serum creatinine decreased from 229 to 185 μmol/L. In July 2019, his chest X-ray showed a soft tissue shadow in the left hilum, which was diagnosed as pulmonary tuberculosis by bronchoscopy; and the patient was thus treated with isoniazid (300 mg, once daily), ethambutol (750 mg, once daily) and levofloxacin (250 mg, once daily).

Phase II

Follow-up ultrasound and FDG-PET/CT examination in August 2019 showed that although the area of bladder lesion was significantly reduced from 4.7 × 4.1 ×5.5 cm to 3.8 × 2.3 × 3.6 cm, the lesion in the transplanted kidney was enlarged from 4.3 × 4.9 × 4.1 cm to 6.0 × 4.0 × 4.7 cm, with the cryptococcoma and malacoplakia in the upper pole of the transplanted kidney protruding into the adjacent Gerota's fascia (**Figures 2A2–H2**). Fluconazole was given at 100 mg BID against cryptococcal infection while the anti-Escherichia coli as well as the anti-tuberculosis regimen was maintained, as described in Phase I. The net immunosuppression status of an individual is influenced by the immunosuppression regimen and individual susceptibility and can be assessed by immunosuppressive drug concentrations, peripheral blood leukocyte counts, lymphocyte counts, and viral infection conditions. The peripheral blood leukocyte and lymphocyte counts of this case were lower compared to the average level during uninfected period (**Supplementary Figure 5**). The presence of pneumosporidiosis, herpes simplex virusemia, and BKV uremia were all suggestive of a low net immunosuppressive status. To stop the disease progression, the patient received a transplant nephrectomy 1 week later.

Phase III

After transplant nephrectomy, the patient's immunosuppressants were discontinued. He was on dialysis three times a week.

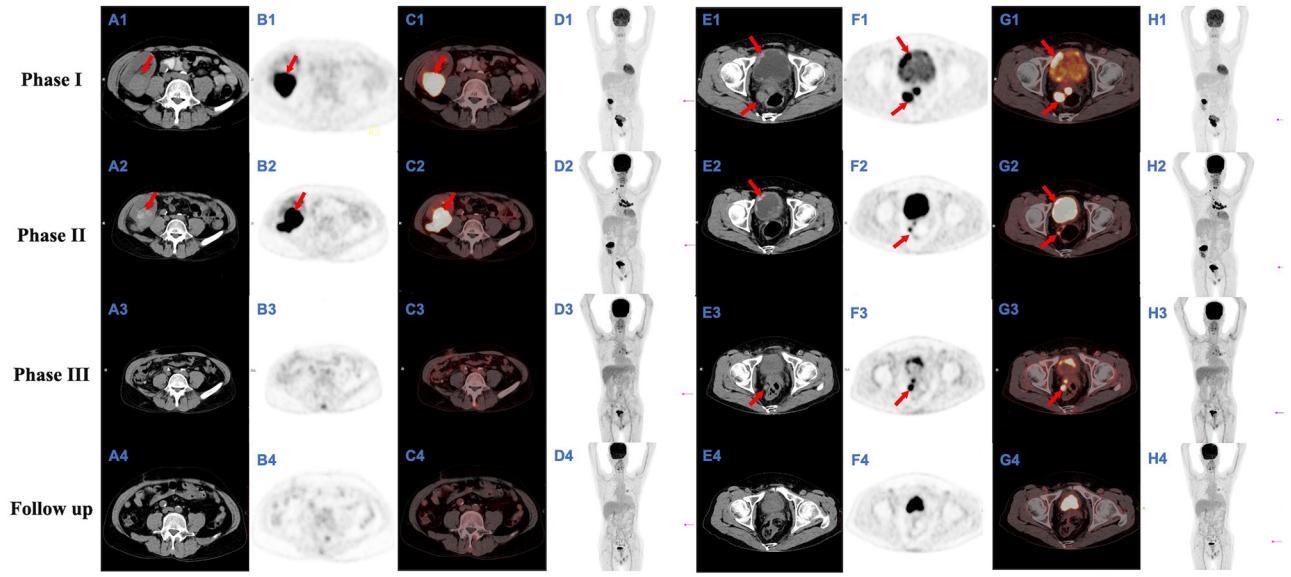

FIGURE 2 | FDG-FDG-PET/CT images. Full-body PET-maximum intensity projection (MIP) **(D1–4, H1–4)** show several space-occupying hypermetabolic lesions; they also indicate the positions (magenta arrows) of the slice shown (**A1–4, B1–4, C1–4**, and **E1–4, F1–4, G1–4**, respectively); an axial CT image (**A1–4** and **E1–4**, respectively), axial PET image (**B1–4** and **F1–4**, respectively), and axial FDG-PET/CT fusion image (**C1–4** and **G1–4**, respectively) from a single plane show a mass of hypermetabolic lesions in the transplanted kidney and bladder respectively; the largest of which (**A1–2**, **B1–2**, and **C1–2**, red arrows), ~4.3 × 4.9 × 4.1 cm and 6.0 × 4.0 × 4.7 cm in size, with maximal standardized uptake value (SUVmax) of 20.3 and 31.0 and average standardized uptake value (SUVave) of 8.8 and 17.4 respectively; the largest of which (**E1–3**, **F1–3**, and **G1–3**, red arrows), ~4.7 × 4.1 × 5.5 cm, 3.8 × 2.3 × 3.6 cm, and 2.8 × 1.4 × 2.5 cm in size, with SUVmax of 21.9, 21.4, and 6.4 and SUVave of 8.7, 8.9, and 4.3 respectively.

The anti-tuberculosis treatment was changed to isoniazid (300 mg, once daily), ethambutol (870 mg, once every 2 days) and levofloxacin (250 mg, once every 2 days), while the anti-*Cryptococcus* treatment was changed to fluconazole (100 mg, twice a day) and flucytosine (0.5 g, three times a day). After 2 months, a follow-up FDG-PET/CT showed that the lesions in the urogenital system and lung were significantly reduced compared with the last examination (**Figures 2A3–H3**). Six months post-transplant nephrectomy, the lesions in the genitourinary system were eventually eliminated (**Figures 2A4–H4**).

Pathology

Gross inspection of the transplanted kidney showed that the resected transplant volume was 11 × 9.0 × 5.0 cm with its capsule closely adherent to the surrounding fat. A solid yellow mass of 6.0 × 4.0 × 4.7 cm was found in the upper pole renal parenchyma without breaking through the renal capsule. Another 5.0 × 2.5 × 2.5 cm yellow mass was found in the hilar sinus fat (**Figure 3F**). The pathological changes of the transplanted kidney lesions were consistent with *Cryptococcus* infection (**Figures 3A–E**).

Metagenome Sequencing

Metagenome sequencing revealed that only two and four sequences of *C. albidus* were respectively detected in the CSF and feces specimens in Phase I. Two sequences of *C. albidus* were detected in the CSF specimen in Phase II. After transplantation nephrectomy, withdrawal of immunosuppressants and anticryptococcal therapy for 2 months, one sequence of *C. albidus* was detected in the feces specimen but no sequence in CSF in Phase III. All sequences were typed as *[Cryptococcus] albidus var. albidus strain NRRL Y-1402* (**Table 1**). The type of the *E. coli* detected in urine and granuloma of the allograft in Phase I and Phase II was *E. coli 09-02E* (**Table 1**).

Culture *in vitro*

In order to understand the interactions between *E. coli* and *Cryptococcus*, we cocultured the two microorganisms *in vivo*. After an 8-h co-culture of *E. coli 09-02E* filtrate and *Cryptococcus neoformans JEC21* (ATCC@96910) *in vitro*, the *Cryptococcus* counts in the control group (without *E. coli* filtrate) and the experimental group (adding 80, 160, 320, 640, and 1,280 μl *E. coli* filtrate, respectively) were respectively $2.46 \pm 0.52 \times 10^5$/mL, $2.30 \pm 0.57 \times 10^5$/mL, $2.83 \pm 0.72 \times 10^5$/mL, $3.13 \pm 0.76 \times 10^5$/mL, $3.09 \pm 0.61 \times 10^5$/mL, and $2.60 \pm 0.63 \times 10^5$/mL. Comparison between the control group and the experimental Group III (320 μl *E. coli* filtrate) and Group IV (640 μl *E. coli* filtrate) showed a statistically significant difference ($P < 0.05$) (**Supplementary Figure 2**), indicating that *E. coli* at these concentrations may stimulate cryptococcal growth.

DISCUSSION

This is the first sequencing study, to our knowledge, of malacoplakia and cryptococcoma of *E. coli* and *C. albidus* in the transplanted kidney. *Cryptococcus* usually attacks the

immunocompromised population, resulting in mostly systemic infection (8). Its colonization in the transplanted kidney is extremely rare, with only one case reported before (7). In this case, a 50-year-old male patient with a recurrent UTI of *E. coli* for 3 years developed cryptococcoma in the transplanted kidney on the 74th month after transplantation. The granuloma disseminated to urogenital organs such as the bladder, prostate, and seminal vesicle. Metagenome sequencing identified the *[Cryptococcus] albidus var. albidus strain NRRL Y-1402* as the culprit. And the drug-resistant *E. coli 09-02E*, first detected in feces from healthy Vietnamese people in 2018, and with unclear pathogenic mechanism and unique urinary system properties (9), arose after the long-term antibiotic use presumably due to selective pressure. Microbiological analysis of kidney transplant preservation fluids was performed prior to transplantation and showed negative results. The patient's recurrent UTI of *E. coli* began 3 years postoperatively, so the *E. coli* infection was considered non-donor-derived in this case.

Although co-infection of *Cryptococcus* and *E. coli* in the same lesion are rarely detected or reported, we speculated that there is an inevitable relationship between them: long-term repeated antibiotics use leads to dysregulation of bacterial flora drug-resistant strains (10), and promotes the emergence of drug-resistant *E. coli* and *Cryptococcus* colonization. In this case, *E. coli* might have invaded the transplanted kidney prior to *Cryptococcus*.

Pathological examination revealed granuloma and focal inflammatory cell infiltration and fibrosis in the interstitium. Microbiological culture and metogenomic sequencing results of the transplanted kidney tissues both showed a large number of *E. coli*. The α-hemolysase released by *E. coli* can cause renal injury and cicatrization, facilitate the formation of abscesses or granulomas, and block urine excretion in the collecting duct (11), which may be the biological causes of *Cryptococcus* retention. Studies have shown that *E. coli* infection alters the immune microenvironment of the infected foci, such as the inflammatory response induced by the activation of cytokines TNF-α, IL-1, IL-6, and IL-8 (11, 12). This immune microenvironment may be the fertile soil for *Cryptococcus* infection and colonization in *E. coli* infectious foci.

Interactions between fungi and bacteria are common (13). Our *in vitro* co-culture result showed that metabolites of *E. coli* at certain concentration may stimulate cryptococcal growth, suggesting correlated growth between *E. coli* and *Cryptococcus*. Urinary susceptible *E. coli* is an important co-factor of multiple

TABLE 1 | Metagenome sequencing results of Cryptococcus and *Escherichia coli* in patient' specimens.

Phase	Specimen	***Cryptococcus*** **(Sequence number, coverage)**	***Escherichia coli*** **(Sequence number, coverage)**
I	Blood	—*	—
	CSF	*[Cryptococcus] albidus var. albidus strain NRRL Y-1402* (2, 100.00%)	—
	Feces	*[Cryptococcus] albidus var. albidus strain NRRL Y-1402* (4, 100.00%)	*Escherichia coli strain AR_0006* (357,907, 100.00%)
	Urine	—	*Escherichia coli 09-02E* (110,960, 100%)
	Allograft tissue	—	*Escherichia coli 09-02E* (14,751, 100%)
	Blood	—	—
II	CSF	*[Cryptococcus] albidus var. albidus strain NRRL Y-1402* (2, 100.00%)	—
	Feces	—	*Escherichia coli strain 2012C-4221*n (786, 100%)
	Urine	—	*Escherichia coli 09-02E* (32,995, 100%)
	Sputum	—	—
	Allograft tissue (uninvolved)	—	—
	Allograft tissue (previous lesion)	—	—
	Allograft tissue (new-born lesion)	—	*Escherichia coli 09-02E* (12,361, 100%)
III	Blood	—	—
	CSF	—	—
	Feces	*[Cryptococcus] albidus var. albidus strain NRRL Y-1402* (1, 95.33%)	*Escherichia coli strain Ec-2Lar* (69,700, 100%)
	Sputum	—	—

**Undetected or not consistent with the filter criteria.*

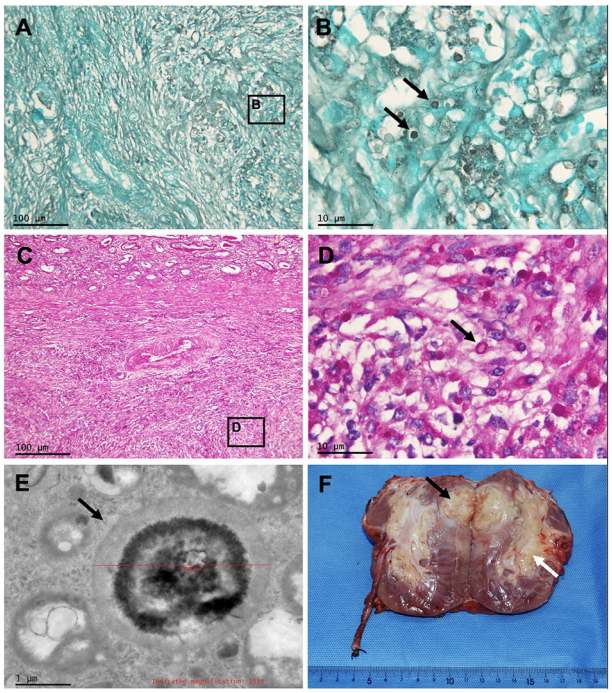

FIGURE 3 | Pathology of the masses of the transplanted kidney, and gross photograph of the allograft nephrectomy specimen. Periodic acid-Schiff staining and Grocott methenamine silver staining of renal mass at 100 × **(A,C)** and at 1,000 × **(B,D)** show granuloma caused by Cryptococcus (arrows). Electron microscopy of renal mass **(E)** shows the Cryptococcus (arrow). **(F)** Renal cryptococcoma (black arrow) and enlarged renal crptococcoma (white arrow).

stress factors involved in the generation of melanin which is a necessary pathogenic factor for *Cryptococcus*, assisting in the removal of oxygen free radicals and averting the onset of oxidative stress response (14). In UTI, the genes involved in the Cu^{1+} efflox system of *E. coli* are highly up-regulated, and the copper efflox of *E. coli* may be the source of the copper intake of *Cryptococcus* (15), which may facilitate the infection and colonization of *Cryptococcus*.

Cryptococcus infection within 30 days after transplantation is generally considered donor-derived, and the median time for non-donor-derived *Cryptococcus* infection is 16–21 months after transplantation (2, 16, 17). Combined with the fact that the patient had no history of pathogen exposure, we suspected that the *Cryptococcus* spores were accidentally inhaled into the lung rather than donor-derived *Cryptococcus* infection, traversing pulmonary capillaries into peripheral blood circulation for systemic dissemination. The *E. coli* infection brought about changes in the transplanted kidney's immune microenvironment that promoted *Cryptococcus* which is used to manifest transient or latent infection to spread through the blood, to colonize in the transplanted kidney and gradually expand its range. After that, *Cryptococcus* proceeded down the urinary tract to the bladder or even prostate duct. At the same time, *Cryptococcus* can breach the blood-brain barrier and enter the CSF. The possible routes of infection were shown in **Supplementary Figure 3**. However, since pathogenicity was related to *Cryptococcus* infection foci *in vivo*, the fungal load *in situ* and the body's immunity, sequences of *C. albidus* could be detected in CSF even in an absence of cryptococcal meningoencephalitis symptoms.

The inconsistent outcomes of the lesions in bladder and transplanted kidney may resulted from that the granuloma created barriers around the infection site to prevent drugs entering the renal lesions. In contrast, the bladder lesion was relatively superficial and had long-term exposure to running urine that contained antifungal metabolites. The fluconazole and flucytosine taken by this patient were metabolized by the kidney and excreted from the urine, so the therapeutic effect toward the bladder lesion was significantly superior to that of the transplanted kidney.

Currently, the detection technology of *Cryptococcus* antigens is based on *C. neoformans* and *C. gattii*. The sensitivity of *C. albidus* detection rate is 75% lower than that of *C. neoformans* and *C. gattii*, leading to the false-negative error in preliminary clinical screening (18). This may have contributed to the paradox between pathology-proven cryptococcoma and negative *Cryptococcus* latex antigen test of CSF and the culture of the blood, urine, CSF, sputum, feces and renal graft tissues in our study. Metagenome sequencing can act as an effective technical complement to pathogen detection in transplant recipients.

CONCLUSIONS

In conclusion, this study reported the first sequencing study of cryptococcoma and malacoplakia formed by *C. albidus* and *E. coli* in a transplanted kidney. This case suggests a possible synergistic relationship between *Cryptococcus* colonization and drug-resistant *E. coli* infection in the transplanted kidney. At the same time, we should be alert to the infection caused by rare *Cryptococcus* in clinical practice. In addition to traditional diagnostic methods such as culture and immunoassay, metagenome sequencing can be utilized as an auxiliary diagnostic tool.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA719067.

ETHICS STATEMENT

This study was approved by Nanfang Hospital Ethical Committee (NFEC-2020-044). Written informed consent was obtained from the patient for the publication of this case report.

AUTHOR CONTRIBUTIONS

YM, ZY, and WD participated in research design. ZY, YW, and YL participated in the writing of the paper. C-LW, JX,

and YM performed critical revision of the manuscript for important intellectual content. ZY, HS, and GC participated in the performance of the research. YM took charge for obtaining funding. XH and JG performed administrative, technical, or material support. RX and WZ performed statistical analysis. YM and C-LW supervised the study. All authors read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.721145/full#supplementary-material

Supplementary Figure 1 | Cystoscopy in Phase I. Pale round-shaped mucosal protrusions on the right side of the bladder, with 3 cm in diameter.

Supplementary Figure 2 | Cryptococcus counts after 8 h co-culture of Escherichia coli filtrate *in vitro*. No *E. coli* filtrate was added to the control group (group I), and 80 (group II), 160 (group III), 320 (group IV), 640 (group V) and 1,280 (group VI) μl *E. coli* filtrate was added to the solution of Cryptococcus respectively. $^{*}P < 0.05$.

Supplementary Figure 3 | Possible infection pathway of Cryptococcus (⟶) and *Escherichia coli* (⟶).

Supplementary Figure 4 | Dosages and whole blood trough levels of immunosuppressive regimens. MPA, mycophenolic acid.

Supplementary Figure 5 | White blood cell counts and lymphocyte counts in peripheral blood of the patient. WBC, white blood cell; LYM, lymphocyte.

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BRIEF RESEARCH REPORT
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Feasibility of Droplet Digital PCR Analysis of Plasma Cell-Free DNA From Kidney Transplant Patients

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Increasing research demonstrates the potential of donor-derived cell-free DNA (dd-cfDNA) as a biomarker for monitoring the health of various solid organ transplants. Several methods have been proposed for cfDNA analysis, including real-time PCR, digital PCR, and next generation sequencing-based approaches. We sought to revise the droplet digital PCR (ddPCR)-based approach to quantify relative dd-cfDNA in plasma from kidney transplant (KTx) patients using a novel pilot set of assays targeting single nucleotide polymorphisms that have a very high potential to distinguish cfDNA from two individuals. The assays are capable of accurate quantification of down to 0.1% minor allele content when analyzing 165 ng of human DNA. We found no significant differences in the yield of extracted cfDNA using the three different commercial kits tested. More cfDNA was extracted from the plasma of KTx patients than from healthy volunteers, especially early after transplantation. The median level of donor-derived minor alleles in KTx samples was 0.35%. We found that ddPCR using the evaluated assays within specific range is suitable for analysis of KTx patients' plasma but recommend prior genotyping of donor DNA and performing reliable preamplification of cfDNA.

Keywords: kidney transplantation, droplet digital PCR, plasma cell-free DNA, minor allele quantification, assay evaluation, graft health monitoring

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INTRODUCTION

Cell-free DNA (cfDNA) is gaining more and more attention and research toward its use as a diagnostic and prognostic biomarker for various medical conditions—prenatal diagnostics, oncology and solid organ transplantation [reviewed in (1–3)]. Fragmented DNA enters the bloodstream and other body fluids and provides genomic and epigenomic information of the source tissue through minimally invasive liquid biopsy.

While renal biopsy is still the gold standard for monitoring and confirming transplanted kidney (KTx) health, other non-invasive screening tests are being sought that would replace it or reduce its need. Unless the graft was donated by an identical twin, its genotype differs from that of the patient. Taking advantage of the different genotypes, donor-derived cfDNA (dd-cfDNA) secreted by the graft can be distinguished from the background of recipient-derived cfDNA secreted by other tissues. Analysis of dd-cfDNA has been shown to have the potential to detect cases of rejection episodes, with patients with rejection having higher plasma dd-cfDNA levels than patients with

stable grafts without rejection (4–7). A schedule of longitudinal dd-cfDNA analyses to monitor graft health was proposed, along with additional indicated analyses when graft damage is suspected and after changes in immunosuppression (8).

Donor-derived cfDNA can be quantified by several methods, including real-time PCR (qPCR) (9, 10), digital PCR (11–14) and next generation sequencing (4, 5, 7). Single nucleotide polymorphisms (SNPs) have been most commonly studied to distinguish recipient and donor DNA, but other polymorphisms—human leukocyte antigen mismatch (13), indels (9, 14) and copy number variations (15, 16)—have also been investigated. In this report, we used droplet digital PCR (ddPCR) as a method for quantifying dd-cfDNA in KTx based on SNP differences between donor and patient derived DNA with a critical proof-of-principle analysis of donated plasma samples using a novel set of SNP assays and also address the need for assay evaluation. In digital PCR, the reaction mixture is divided into individual PCR reactions (droplets in the case of ddPCR), which is expected to result in one or no template DNA molecules per reaction, and the Poisson distribution is adopted for absolute quantification of the template [reviewed in (17)]. Digital PCR has the advantage over qPCR of being more accurate at low target concentrations and not using standard curves, and over next generation sequencing of being less expensive, having a shorter turnaround time, and being easier to interpret without implementing bioinformatics algorithms.

MATERIALS AND METHODS

SNP Selection

One thousand Genomes Project VCF files for all chromosomes were downloaded[1] (18). Data were filtered stepwise for biallelic SNPs, European minor allele frequency > 0.40, global minor allele frequency > 0.45 and global fixation index < 0.02. Distances (CA01dist, CA01score) from SNPs to the nearest cfDNA fragment peaks were calculated using the CA01.bb file (18) and data were filtered for CA01dist < 19 and CA01score > 200. Using USCS Table Browser, all SNPs overlapping with repetitive elements were identified and removed. The surrounding sequences of the remaining 1,051 SNPs were screened for potentially useful primer pairs and probes using Beacon Designer 8.4 and SNPs for which no amplicons were found were removed. SNPs with overlap of potential amplicons with off-target SNPs in potential primer or probe sequences were identified using UCSC Table Browser and discarded. Screening for off-target potential of predicted primers was performed using Primer-BLAST. IDT OligoAnalyzer 3.1 was used to calculate ΔG of primer dimers, hairpin Tm and secondary structures. Based on these characteristics of the identified potential assays, SNPs were ranked and 43 with the best potential were selected.

Abbreviations: cfDNA, cell-free deoxyribonucleic acid; CNA, QIAamp Circulating Nucleic Acid Kit; CV, coefficient of variation; dd-cfDNA, donor-derived cell-free deoxyribonucleic acid; ddPCR, droplet digital polymerase chain reaction; FPS, false positive signal; gDNA, genomic DNA; KTx, kidney transplant; ME, QIAamp MinElute ccfDNA Kit; MM, MagMAX Cell-Free DNA Isolation Kit; qPCR, real-time polymerase chain reaction; SNP, single nucleotide polymorphism.

[1] ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/

gBlocks

One hundred sixty-one blood pressure dsDNA synthetic gBlock Gene Fragments (Integrated DNA Technologies) mimicking natural short DNA fragments were designed for each allele of the selected SNPs. Each gBlock had the same sequence as the corresponding genomic region and contained the SNP assay amplicon approximately in the middle with at least 29 bp of flanking sequence on each side. Prior to use, gBlocks were diluted in 2 mg/ml Sheared Salmon sperm DNA (Invitrogen).

Collection and Processing of Blood Samples

Clinical samples were collected and processed in accordance with the approval of Slovenian National Medical Ethics Committee (No. 0120-479/2016-2, KME 42/09/16). Approximately 10 ml of blood was collected in Cell-Free DNA BCT tubes (Streck) and stored at room temperature until further processing (maximum 72 h after blood draw). Plasma was separated from blood by two consecutive centrifugation steps (10 min at 1,600 g and 10 min at 16,000 g) at room temperature. The supernatant was immediately transferred to a fresh tube to reduce contamination of the plasma with cells and stored at −80°C until cfDNA extraction. The buffy coat layer was harvested and stored at −20°C until gDNA extraction.

DNA Extraction

Genomic DNA (gDNA) was isolated from a maximum of 200 μl of buffy coat using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. gDNA was eluted in 200 μl of the supplied AVE elution buffer. The isolated gDNA was stored at −20°C until further use.

In our kit comparison study, cfDNA was extracted from three 1 ml aliquots of the same plasma sample from apparently healthy donors ($N = 10$) according to the manufacturer's instructions using each kit: QIAamp Circulating Nucleic Acid Kit together with QIAvac 24 Plus Vacuum System (CNA; Qiagen), QIAamp MinElute ccfDNA Kit (ME; Qiagen) and MagMAX Cell-Free DNA Isolation Kit (MM; Thermo Fisher Scientific). Extractions with different kits were performed on three separate days, with one kit per day comprising all test samples. To ensure repeatability between laboratories, extractions were repeated in a different laboratory with a smaller subset of samples.

For isolation of cfDNA from 4 ml of plasma from KTx patients, the CNA kit was used according to the manufacturer's instructions. cfDNA was eluted in 45 μl of AVE buffer, aliquoted and stored at −20°C until further analysis.

The concentration of extracted DNA was determined fluorometrically using Qubit dsDNA HS Assay Kit and Qubit 3 Fluorometer (Thermo Fisher Scientific).

Capillary electrophoresis was performed using HT DNA High Sensitivity LabChip Kit and Caliper LabChip GX instrument and analyzed using LabChip GX Software Version 5.2.2009.0 (all from Perkin Elmer).

qPCR

The qPCR reactions were performed in a 384-well format on the ViiA 7 or QuantStudio 7 Flex Real-Time PCR

System using software-implemented experimental settings for genotyping. Ten microlitre qPCR reactions consisted of TaqMan Genotyping Master Mix (Themo Fisher Scientific), TaqMan Custom SNP Genotyping Assay (Themo Fisher Scientific; **Supplementary Table 1**), 5 ng of gDNA and nuclease-free water to final volume. The thermal profile included pre-read phase (60°C, 30 s), hold phase for denaturation (95°C, 10 min), 40 cycles of PCR phase (95°C, 15 s; 60°C, 1 min), followed by post-read phase (60°C, 30 s); the ramp rate was 1.6°C/s. Genotypes were called automatically based on the analysis algorithm embedded in the software using real-time dRn data and a quality score of 95.0.

ddPCR

The QX200 AutoDG ddPCR System (Bio Rad) was used for droplet generation and readout. Reactions in the final volume of 20 μl consisted of ddPCR Supermix for Probes (No dUTP, Bio-Rad), TaqMan Custom SNP Genotyping Assay (Themo Fisher Scientific, **Supplementary Table 1**), DNA template and nuclease-free water to the final volume. In the case of cfDNA analysis, 9 ul of cfDNA was used per reaction. Droplets were generated using the Automated Droplet Generator (Bio-Rad). The following cycling conditions were used on the C1000 or T100 Thermal Cycler (Bio-Rad): Initial denaturation at 95°C for 10 min was followed by 40 cycles of 94°C for 30 s, 60°C for 60 s and 1 cycle of enzyme deactivation at 98°C for 10 min. The ramp rate was 1.5°C/s. After cycling, plate was transferred to QX200 Droplet Reader (Bio-Rad).

Positive droplets were distinguished from negative droplets by applying a threshold for fluorescence amplitude in QuantaSoft software (Bio-Rad), version 1.7.4. The threshold value was set manually. The same threshold was used for each channel/assay throughout the reaction plate and similar thresholds were enforced for the same channel/assay in different reaction plates. Reactions with <10,000 detected droplets and saturated reactions with more than 100,000 target copies were rejected and excluded from the analysis. Data were exported and allele fractions along with coefficient of variation (CV) were calculated using Microsoft Excel software. A droplet volume of 0.739 nl was considered for the calculation of absolute measured copy number (19).

PCR Preamplification of cfDNA

Targeted multiplex PCR preamplification was prepared using Q5 Hot Start High-Fidelity Master Mix (New England Biolabs), 500 nm final concentration of each of the primers (Integrated DNA Technologies) used in assays targeting rs1707473, rs7687645, rs1420530 and rs6070149 (**Supplementary Table 1**), DNA template and nuclease-free water. The following reaction conditions were used in the C1000 or T100 Thermal cycler (Bio-Rad): an initial denaturation at 98°C for 30 s was followed by 8 cycles of 98°C for 10 s, 60°C for 30 s and 72°C for 20 s, and a final extension at 72°C for 2 min. The PCR products were then stored at −20°C until ddPCR analysis. Prior to ddPCR analysis, PCR products were diluted 10-fold with nuclease-free water.

Statistical Analysis

All tests were performed in RStudio, version 1.4.1106, using the packages ggpubr (version 0.4.0) and rstatix (version 0.7.0). The normality of the data was interrogated using the Shapiro–Wilk test. To compare the extraction yields of the three kits, the average yields across the three replicates of each sample were calculated and any influence of the kit on the extraction yield was investigated using the one-way repeated measures ANOVA test. Yields of KTx samples were compared to yields of samples from healthy donors using the Mann Whitney U test. For multiple comparisons, the Benjamini-Hochberg adjustment of *P*-values was used.

RESULTS

SNP Selection and Performance of SNP Detection Assays

Our approach to selecting biallelic SNP candidates that could more readily distinguish the DNA of one individual from another included a wide range of criteria. Considering high minor allele frequency, low global fixation index, proximity to cfDNA fragment peaks, no overlap with repetitive elements and comprehensive *in silico* amplicon analysis, 43 promising biallelic SNPs are suggested in **Table 1**. None of the SNPs have known clinical significance and they are all positioned in non-coding regions, either outside any gene regions or in introns or untranslated regions.

Of these, 7 SNP loci were randomly selected for assay design and evaluation of their performance. First, assays were tested for their performance characteristics in a genotyping qPCR. Allelic discrimination plots were examined as a checkpoint for reaction errors. Six of the assays tested showed good cluster separation with dense clusters at the expected positions in the allelic discrimination plots. No inhibition of qPCR reactions with extracted gDNA samples was detected. Cluster separation of the assay targeting rs7431017 was not as clear, but still sufficient.

Next, the assays were tested with ddPCR. Again, cluster separation was checked and the assay targeting rs7431017 was omitted due to cluster overlap. A representative scatterplot showing good separation of positive and negative droplets is shown in **Supplementary Figure 1A**. Interestingly, we noticed remarkable rain when analyzing gBlocks with all of our SNP assays, while there was significantly less rain present when analyzing gDNA or cfDNA.

To evaluate the performance of the ddPCR assay targeting rs1707473 in a scenario mimicking presence of donor-derived DNA in a background of recipient's DNA, 0.1–50% ratios of alleles G and T (0.1–33% minor allele fractions) were simulated with mixtures of synthetic gBlocks of the two alleles. The assay provides a linear signal over the whole wide tested range with R^2 of 0.999 for the allele T in a background of the allele G and 0.998 for the allele G in a background of the allele T (**Supplementary Figure 1B**).

Using gBlocks, CV values were also determined at 1% simulated dd-cfDNA with three different low copy number mixtures: 5/500, 10/1,000 and 20/2,000 copies of allele 1/allele

TABLE 1 | Proposed biallelic SNPs for distinguishing the cfDNA of two individuals.

SNP ID	Alleles	Chr: position (GRCh38.p12)	European MAF	Global MAF	Position in relation to genes
rs1935037	A, T	1:223323387	T = 0.4602	T = 0.4942	SUSD4 intron
rs10168994	T, C	2:193486802	C = 0.4026	C = 0.4754	/
rs745581	C, T	2:2729334	T = 0.4881	T = 0.4984	/
rs6546954	C, G	2:75182348	C = 0.4702	G = 0.4585	TACR1 and LOC105374811 intron
rs1707473*	T, G	3:146133737	T = 0.4950	G = 0.4930	PLOD2 intron
rs6775401	A, G	3:183462906	G = 0.4682	G = 0.4718	/
rs7431017*	A, C	3:76502252	A = 0.4811	C = 0.4559	ROBO2 intron
rs9289628*	C, T	3:98548445	T = 0.4901	C = 0.4896	/
rs7687645*	T, C	4:70350352	T = 0.4523	C = 0.4970	/
rs615954	G, C	5:123144242	C = 0.4761	C = 0.4884	PRDM6 intron
rs10078699	C, T	5:155125342	T = 0.4940	T = 0.4507	/
rs153749	A, G	5:171754251	G = 0.4205	G = 0.4764	/
rs2561182	G, C	5:69437818	C = 0.4911	C = 0.4784	MARVELD2 intron
rs7746234	C, T	6:138498373	C = 0.4066	T = 0.4930	NHSL1 intron
rs2517471	G, C	6:31084321	G = 0.4254	C = 0.4952	/
rs9656097	T, C	7:104526571	C = 0.4076	C = 0.4708	LHFPL3 intron
rs751966	G, A	7:135879129	A = 0.4871	A = 0.4679	/
rs2691527*	C, T	7:78173386	C = 0.4881	T = 0.4647	MAGI2 intron
rs6557883	T, C	8:26127967	C = 0.4811	C = 0.4992	LOC105379335 non-coding transcript
rs4733016	C, T	8:26991085	T = 0.4722	T = 0.4806	/
rs2974298	C, T	8:42518957	T = 0.5000	T = 0.4850	SLC20A2 intron
rs7011817	G, C	8:60212109	G = 0.4771	C = 0.4667	CA8 intron
rs2246293	C, G	9:104928557	G = 0.4871	G = 0.4892	LOC105376196 intron; ABCA1 upstream transcript
rs10901532	T, C	10:126136495	C = 0.4414	C = 0.4591	ADAM12 intron
rs7117897	T, C	11:129853970	C = 0.4632	C = 0.4561	TMEM45B intron
rs1166235	G, A	12:126874295	A = 0.4563	A = 0.4888	LINC02372 intron; LOC105370061 upstream transcript
rs10843022	C, G	12:27904982	G = 0.4414	G = 0.4503	/
rs7972248	C, T	12:3515931	C = 0.4980	T = 0.4579	PRMT8 intron
rs4770602	C, G	13:24136006	G = 0.4901	G = 0.4617	SPATA13 intron
rs7321093	T, C	13:28573704	C = 0.4831	C = 0.4872	LOC105370135 upstream transcript
rs2799341	T, C	13:53072591	C = 0.4036	C = 0.4501	/
rs3784165	G, C	14:31088123	C = 0.4135	C = 0.4696	AP4S1 intron
rs8014321	T, C	14:91501322	C = 0.4751	C = 0.4916	PPP4R3A intron
rs10148348	T, C	14:92866186	T = 0.4732	C = 0.4950	/
rs1420530*	C, T	16:52524817	T = 0.4821	T = 0.4892	TOX3 intron
rs1392494	G, A	16:59678252	A = 0.4781	A = 0.4894	/
rs1384366	G, A	17:78733702	A = 0.4553	A = 0.4665	CYTH1 intron
rs4789798	C, G	17:82573767	G = 0.4254	G = 0.4603	FOXK2 intron
rs12605230	G, C	18:38219647	C = 0.4871	G = 0.4996	/
rs6075029	C, T	20:16200100	T = 0.4980	T = 0.4573	/
rs6050798	C, T	20:2641352	T = 0.4891	T = 0.4681	TMC2 3' UTR; LOC105372505 intron
rs6070149*	C, T	20:57554682	T = 0.4791	C = 0.4732	/
rs243593	G, A	21:17783905	A = 0.4831	A = 0.4802	C21orf91-OT1 intron

SNPs marked with asterisk (*) were selected for assay design and evaluation of minor allele quantification performance. The European and global minor allele frequencies (MAF) of the SNPs were derived from the 1,000 Genomes Project (18).

2 per reaction. Each allele mixture was prepared and analyzed in 6 ddPCR reactions. CV was calculated for each of the alleles in the mixture and the results for assay targeting rs1707473 are summarized in **Supplementary Table 2**. As expected, the minor allele contributes the most to the overall CV value of the assay in duplex format, as the CV values are much lower for the major allele, which is present in higher copies per reaction (**Supplementary Table 2**). The limit of quantification of this assay, where CV falls below the set 20%, was found to be between 10 and 20 copies of the target per reaction. The limit of detection for the same assay was found at 5 copies per reaction (data not shown).

To assess possible bias due to synthetic gBlocks, the assay performance characteristics were also confirmed in a ddPCR reaction with cfDNA isolated from heterozygous healthy blood donors in the range of ∼3–60 copies per 20 µl reaction. In this case, the limit of quantification was determined to be in the range of 15–32 copies per reaction (data not shown).

Quantification of very low abundance alleles by SNP analysis (analogous to detection and quantification of the mutant in a high background of a wild-type allele) requires additional assessment of assay performance. A high background of the major allele vs. progressively lower abundance of the minor allele presents a potential hazard: a false positive signal (FPS) in the minor allele detection channel. The FPS should be measured and the detection cut-off for the minor allele defined with respect to the FPS. Therefore, we continued the characterization of the rest of the assays using artificial mixtures of gDNA and gBlocks with a constant background of ∼ 50,000 copies of major alleles (≈ 165 ng of human DNA) and a variable proportion of 0–0.5% copies of minor alleles. Analysis of such samples allowed us to be on the theoretically safe side for accurate quantification of 0.1% minor allele copies (50 copies per reaction) or even less. The samples to which no minor allele copies were added were used to estimate the FPS for each allele of all characterized assays.

The FPS is assay-dependent, reaching up to 0.14% in the case of assay targeting rs7687645, allele C (**Table 2**). To quantify the presence of minor alleles in the tested sample, we propose to subtract the FPS. In this way, the minor allele was successfully detected in all cases down to 0.1% minor allele copies without overlapping with the FPS. The data suggest reliable quantification of minor allele frequency down to 0.1–0.5%, depending on the assay/allele (CV $\leq$ 20%).

TABLE 2 | Characteristics of SNP ddPCR assays defined by measuring copies of alleles in mixtures of gDNA/gBlocks with a background of 50,000 copies of major alleles per ddPCR reaction.

Assay target	Target allele	FPS [%]	LOD [%]	LOQ [%]
rs1707473	G	0.00	<0.09	<0.09
	T	0.02	<0.13	0.13–0.23
rs2691527	C	0.03	0.05–0.08	0.03–0.08
	T	0.04	0.05–0.08	0.27–0.42
rs7687645	C	0.14	0.08–0.1	0.21–0.47
	T	0.03	0.08–0.1	0.16–0.38
rs1420530	C	0.03	0.05–0.08	0.08–0.26
	T	0.03	0.05–0.08	0.05–0.09
rs9289628	C	0.09	0–0.05	0.13–0.22
	T	0.07	0.05–0.08	0.13–0.24
rs6070149	C	0.03	0.05–0.1	0.08–0.16
	T	0.03	0.025–0.05	0.24–0.5

For each assay/allele, the false positive signal (FPS) of the background without target allele was measured. The limit of detection (LOD) was defined in a range of the minor allele proportion where the target signal significantly exceeds the false positive signal. The limit of quantification (LOQ) was defined in a range of minor allele proportion where the coefficient of variation of the ddPCR measurements did not exceed 20%. To narrow down or precisely define the ranges of LOD and LOQ, further measurements should be performed in the respective ranges of minor allele proportions.

Comparison of cfDNA Extraction From Plasma

We compared the yields of cfDNA isolation from plasma using three widely used commercial kits. Blood was collected from 10 apparently healthy donors, plasma was divided into 1 ml aliquots, and cfDNA was isolated from 3 aliquots using each kit. The results are shown in **Figures 1A,B**. The cfDNA yield was comparable as no significant differences were found between kits (one-way repeated measures ANOVA; $p = 0.152$), but appeared to be best with the CNA kit with a mean of 5.7 ng/ml plasma and a median of 6.1 ng/ml, compared to ME with a mean and median of 4.3 ng/ml plasma and MM with a mean of 4.6 and a median of 3.7 ng/ml plasma. Intersample variability appeared to be highest with the MM kit. The fold difference between the highest and lowest yields obtained was 5.3 with MM, 3.4 with CNA and 2.8 with ME. Intrasample variability (reproducibility between aliquots) was comparable for all three kits (mean coefficient of variance: CNA 18%, ME 13%, and MM 15%). It is possible that more reproducible results could be obtained with an automated protocol available for all three kits but not tested in our laboratory.

Fragment size analysis also revealed no significant differences between the kits tested (**Figure 1C**). Due to the lack of detectable high molecular weight fragments, no further assay-based quantitative analysis was performed to assess genomic contamination.

Given the lack of significant differences in DNA yields measured by Qubit fluorometer between the kits tested and in agreement with the comparable results obtained by capillary electrophoresis, accurate yields were determined by absolute copy number quantification by ddPCR only for the cfDNA extracted by the CNA kit, which was selected for further use in our work. Very good correlation was found between yields measured by Qubit and DNA copy number measured by ddPCR using different SNP detection assays (data not shown). No significant inhibition of ddPCR reactions was observed when analyzing cfDNA samples isolated by column-based cfDNA extraction using the CNA kit.

cfDNA From Plasma of Kidney Transplant Patients

Next, cfDNA was extracted with the CNA kit from 15 4 ml plasma samples donated by 8 KTx patients. Four patients

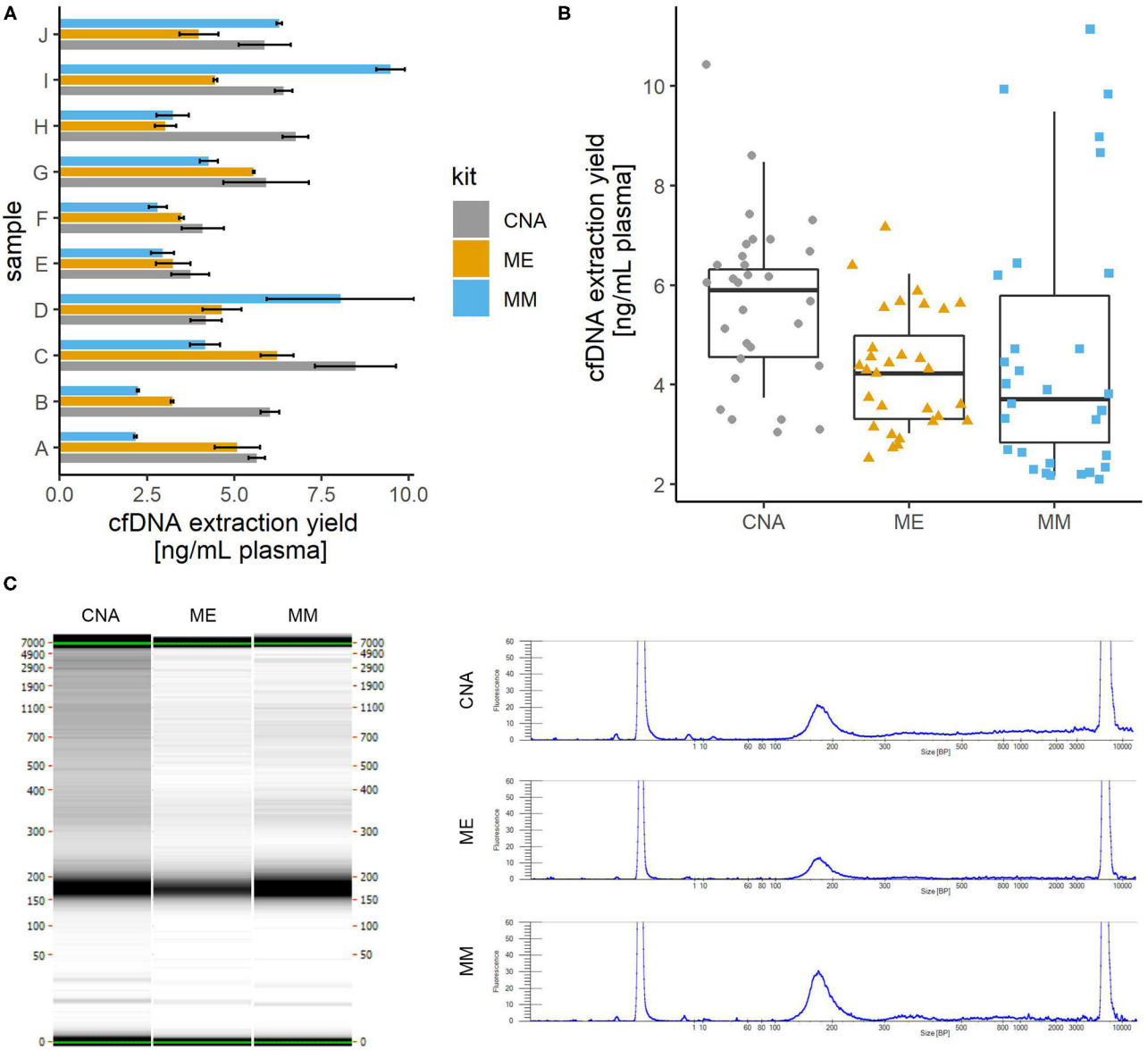

FIGURE 1 | Comparison of cfDNA isolation from plasma using three different commercially available kits. cfDNA was isolated from 1 ml of plasma using each kit in three replicates. **(A)** The average yield for each plasma sample. Error bars show the standard deviation of three replicates. **(B)** Distribution of yield of cfDNA isolation with three kits. Colored shapes show the individual yields of all samples/replicates and boxplots show the average yields across replicates. No significant differences were observed between the yields obtained with the three kits tested. **(C)** Comparison of fragment sizes. The left panel shows the capillary electrophoresis gel for one of the three replicates of the same sample extracted with three different commercial kits. On the right, the electropherogram of the same gel shows that the fragment size profile is very comparable between the kits, with a major peak around 170 bp representing mainly the mononucleosomal cfDNA fraction and a minor peak around 340 bp (probably dinucleosomal cfDNA). CNA, QIAamp Circulating Nucleic Acid Kit; ME, QIAamp MinElute ccfDNA Kit; MM, MagMAX Cell-Free DNA Isolation Kit.

donated blood on day 6 after transplantation, and 4 patients donated 3 or 2 samples at different time points more than 14 days and within 13 months after transplantation, with at least 10 days between consecutive donations. Extraction yields ranged from 15.3 to 124.2 ng with a median of 38.4 ng and a mean of 46.3 ng, corresponding to ∼ 4,600–37,600 haploid genome equivalents with a median of 11,600 and a mean of 14,000 haploid genome equivalents. On average, we extracted more cfDNA from plasma of KTx patients than from plasma of apparently healthy donors in the above test experiment ($p = 0.007$). The increase in cfDNA yield was particularly pronounced shortly after KTx ($p = 0.006$), whereas the mean extraction yield from samples collected more than 14 days after KTx was not statistically significantly different from the mean extraction yield from samples from healthy subjects ($p = 0.51$; **Figure 2A**).

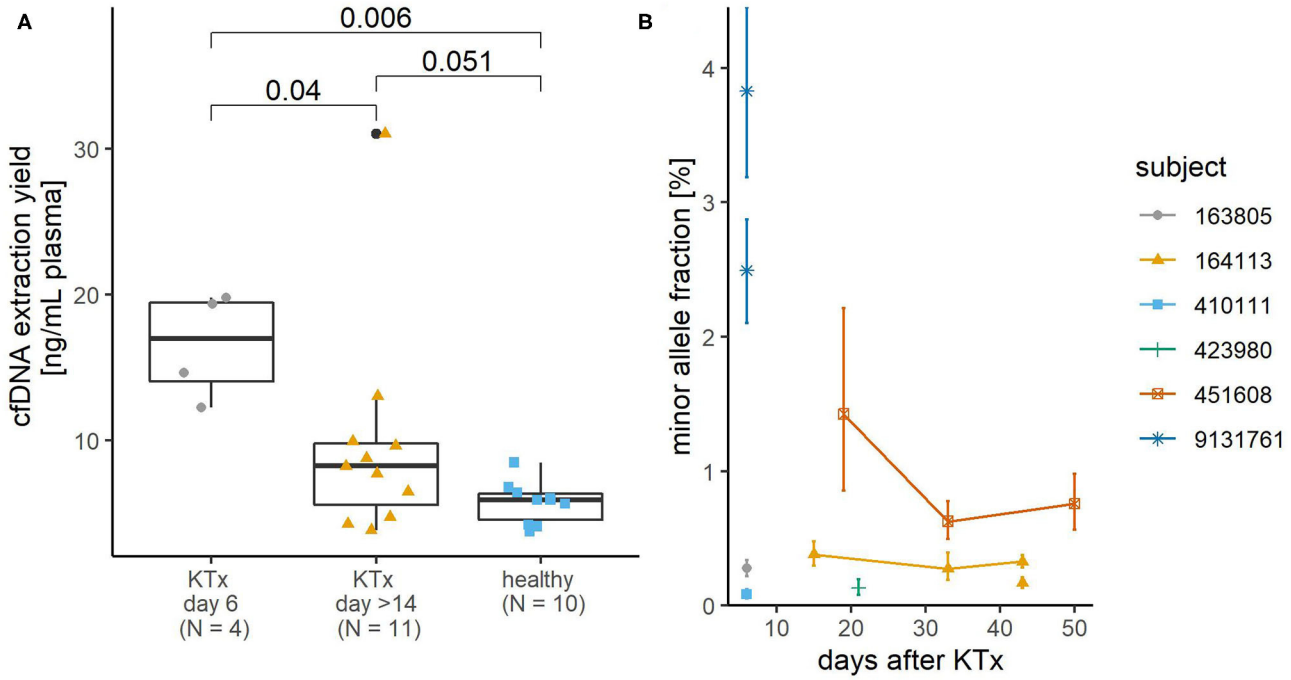

FIGURE 2 | Yield of plasma cfDNA from kidney transplant (KTx) patients and its donor-derived minor allele content. **(A)** The KTx cfDNA yield was higher than that of healthy volunteers. Colored shapes show the yields of each sample and boxplots summarize their distribution. *P*-values of pairwise comparisons using Mann Whitney *U*-test and Benjamini-Hochberg adjustment are shown. **(B)** Minor allele content in plasma cfDNA of KTx patients. The detected minor alleles are from the donor, but the zygosity status of the donor DNA is not known, so the exact proportion of donor-derived DNA would be 2-fold in the case of heterozygous donor DNA. For two samples (subject ID 9131761, 6 days after KTx and subject ID 164113, 43 days after KTx), the minor allele proportion was determined using two SNP assays, hence two values for the minor allele proportion of each sample. Error bars represent Poisson confidence intervals. In three cases where the confidence intervals are wider (subject ID 9131761, 6 days after KTx and subject ID 451608, 19 days after KTx), minor allele proportions were determined in non-preamplified cfDNA samples. In the other cases, ddPCR analysis was performed after targeted multiplex PCR preamplification of cfDNA.

Minor Allele Proportions in KTx Samples

At least the genotype of the patient and preferably that of the donor should be known to allow quantification of the dd-cfDNA. To identify the patient's genotype, the gDNA extracted from buffy coat from KTx patients was genotyped by qPCR at all six SNP sites. At least one homozygous SNP was detected for each patient. No donor tissue was available for genotyping. The corresponding plasma cfDNA was analyzed by ddPCR with assays targeting SNPs for which the patient's DNA was homozygous to search for and quantify the presence of foreign donor-derived alleles. Donor-derived alleles could only be quantified in three samples with relatively high minor allele proportions—1.43% and higher. Most other samples were subsequently PCR-preamplified and reanalyzed by ddPCR. The quantifiable minor allele proportions in these samples ranged from 0.08 to 0.76% (**Figure 2B**).

DISCUSSION

We have selected and proposed a new biallelic SNP set that has a very good potential to distinguish the DNA of two individuals. To our knowledge, this is the first reported SNP set matched to cfDNA fragment peaks, indicating increased protection of DNA from digestion (20) and enabling the design of PCR SNP detection assays in regions of reduced fragmentation. As a proof of principle, we evaluated the performance of six SNP detection assays that are now ready for use. In a background of 50,000 major allele DNA copies, they are able to quantify at least 0.5% and some even 0.1% minor allele copies. The most commonly used set of SNP assays for this purpose is reportedly capable of detecting minor allele content down to 2% with a CV of <15% (11), but has nevertheless been used to quantify 0.1%.

For use in clinical practice, an assay should be able to accurately detect dd-cfDNA fractions above the established threshold that marks possible rejection and half the threshold to allow analysis of informative heterozygous donor SNPs. Thus, considering the most commonly proposed threshold of 1% for KTx (4, 5, 7), an assay should reliably quantify down to 0.5% minor allele content. However, some studies suggest an even lower threshold (6, 21–23). It has been suggested that plasma dd-cfDNA fraction could be measured by ddPCR analysis of SNPs without prior knowledge of donor genotype (11), but low cfDNA levels and low dd-cfDNA fractions in KTx and also heart transplant patients argue against this.

Quantification of low-copy DNA is limited not only by assay performance characteristics but also, and probably more so, by stochastic effects associated with low copy numbers and

subsampling, which contribute significantly to measurement uncertainty at low limits of detection (24–27). Assuming 30 minor allele copies (100 pg of human DNA) as a theoretical lower limit for reliable quantification, a minor allele content of 0.1% requires ∼100 ng of cfDNA, which is not trivial to obtain from plasma of KTx patients. Considering the yield of cfDNA that we extracted from 4 ml of plasma from KTx patients, the theoretical limit of quantification for corresponding samples is in the range of 0.08–0.65%. Thus, based on our observation, sample size (i.e., volume of blood collected and cfDNA content in blood) is the most important limiting factor in cfDNA analysis.

The cfDNA extraction yield from plasma of our group of KTx patients was similar to that previously reported for KTx patients (21) but lower than that reported for liver transplanted patients (28). We observed a higher cfDNA extraction yield from plasma of KTx patients compared to plasma cfDNA from apparently healthy individuals, which is in agreement with Schütz et al. who also reported a higher total cfDNA in a group of KTx patients compared to a healthy control group or a group with other medical conditions (29).

Half of the KTx plasma cfDNA samples included in our study had a minor allele content of <0.35%, which is consistent with the reported median baseline values of 0.21–0.46% in KTx [reviewed in (8)]. The calculated inherent theoretical limit of minor allele quantification was higher than this in 4 of 15 (27%) cfDNA samples, implying that in some cfDNA samples the actual minor allele content is lower than its theoretically derived quantifiable limit. In such samples, reliable determination of the donor genotype would be hampered because it depends on accurate quantification of the minor allele content. Although the results of such samples would likely be adequately classified as sub-threshold (non-rejection), subsequent samples from the same patient would need to be repeatedly analyzed with many SNP assays until the donor's cfDNA content is high enough to allow reliable determination of his genotype. On the other hand, prior knowledge of the genotype would allow analysis with fewer assays and reduce overall costs. Additional one-time genotyping would also not significantly increase the turnaround time of the analysis. Therefore, in the case of KTx and possibly other solid organ transplants with low expected levels of donor DNA, such as heart transplants, other sources of donor DNA should preferably be genotyped in advance. Urine could be a suitable and readily available source of transplanted kidney DNA as suggested by Oellerich et al. (6).

Our results suggest that plasma cfDNA from KTx patients should first be preamplified in order to quantify minor allele content, which agrees well with a study in which donor-derived DNA was almost undetectable in non-preamplified plasma cfDNA from KTx patients (12). If only detection of a minor allele fraction above the 1% threshold was required to identify rejection cases, the yield of cfDNA extraction was high enough to allow analysis of ∼10 ng in a ddPCR reaction, and the donor genotype was known, preamplification could theoretically be omitted. Otherwise, an evaluated reliable preamplification should be performed that should not cause a significant shift in allele ratios.

All in all, our study confirms ddPCR as a feasible method for dd-cfDNA analysis in plasma from KTx patients, although we recommend prior genotyping of the donor and preamplification of cfDNA. In contrast to more complex and sophisticated next generation sequencing-based assays, it could be performed in any laboratory equipped with a ddPCR machine at a lower cost and results should be available within 2 days, with at most one additional day for one-time genotyping along with the first sample from each patient.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Slovenian National Medical Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS and MR conceived the study. SV designed and performed SNP selection. MA recruited KTx patients for blood sample collection. MS organized sample storage, processing, and distribution. BJ and MŠ designed and performed the experiments, data analysis, and data interpretation. DD and PK supervised the work and provided critical insights. MŠ drafted the manuscript with the assistance of BJ. All authors revised and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.748668/full#supplementary-material

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Proximal Tubule p53 in Cold Storage/Transplantation-Associated Kidney Injury and Renal Graft Dysfunction

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Kidney injury associated with cold storage/transplantation is a primary factor for delayed graft function and poor outcome of renal transplants. p53 contributes to both ischemic and nephrotoxic kidney injury, but its involvement in kidney cold storage/transplantation is unclear. Here, we report that p53 in kidney proximal tubules plays a critical role in cold storage/transplantation kidney injury and inhibition of p53 can effectively improve the histology and function of transplanted kidneys. In a mouse kidney cold storage/transplantation model, we detected p53 accumulation in proximal tubules in a cold storage time-dependent manner, which correlated with tubular injury and cell death. Pifithrin-α, a pharmacologic p53 inhibitor, could reduce acute tubular injury, apoptosis and inflammation at 24 h after cold storage/transplantation. Similar effects were shown by the ablation of p53 from proximal tubule cells. Notably, pifithrin-α also ameliorated kidney injury and improved the function of transplanted kidneys in 6 days when it became the sole life-supporting kidney in recipient mice. *in vitro*, cold storage followed by rewarming induced cell death in cultured proximal tubule cells, which was accompanied by p53 activation and suppressed by pifithrin-α and dominant-negative p53. Together, these results support a pathogenic role of p53 in cold storage/transplantation kidney injury and demonstrate the therapeutic potential of p53 inhibitors.

Keywords: cold storage, kidney injury, kidney transplantation, p53, proximal tubule

INTRODUCTION

Kidney transplantation is a preferred treatment for end stage renal disease (ESRD) for its advantage in better life quality and lower medical cost. However, the demand of optimal living donor kidneys has far exceeded the availability. As a result, deceased donors remain to be a major source for kidney transplants (1). From deceased donors to recipients, the kidneys are inevitably subjected to a series of ischemia and hypothermia; reperfusion and rewarming injury (2). Particularly, the preservation and transportation of these deceased donor kidneys are often associated with cold ischemia, which has been shown clearly to be an independent risk factor for delayed graft

function (DGF) and chronic nephropathy (3, 4). Strategies and approaches to alleviate cold storage/transplantation injury would improve the quality of the donor kidneys and set stage for greater acceptance of the grafts. In cold storage/transplantation, significant injury and cell death occurs in renal tubules, especially the proximal tubules, contributing to compromised graft function and survival (5). Basic research (6, 7) and clinical trial (8) revealed that inhibition of proximal tubular cell death with caspase inhibitors during cold storage improves graft function and histology. Moreover, we (9) and others (10–13) identified intrinsic mitochondrial pathway of apoptosis in cold storage/transplantation kidney injury. Despite these findings, the cellular and molecular mechanism of cold storage/transplantation kidney injury remains elusive and there is an urgent need for effective renoprotective approaches.

p53, a well-known tumor suppressor, is a stress response gene (14). In kidneys, p53 contributes to the pathogenesis of both acute and chronic kidney diseases (15, 16). In 2003, the Dagher lab demonstrated the protective effect of pifithrin-α in renal ischemia-reperfusion, suggesting the first evidence for a role of p53 in acute kidney injury (17). In 2004, we suggested the role of p53 in cisplatin-induced kidney injury (18). Later studies by us and the Padanilam lab demonstrated that knockout of p53 from proximal tubules alleviates ischemic kidney injury in mice, providing unequivocal evidence for the pathogenic role of p53 in AKI (19, 20). In view of these studies, the current study was designed to (1) delineate p53 activation in kidney cold storage/transplantation, (2) elucidate the role of p53 in cold storage/transplantation kidney injury, (3) identify the specific role of proximal tubule p53, and (4) test the therapeutic potential of p53 inhibitors in kidney cold storage/transplantation.

MATERIALS AND METHODS

Animals

Animals used in this study were housed in the animal facility of Charlie Norwood Veterans Affairs Medical Center (CNVAMC). Animal experiments were conducted with the approval of and in accordance with the guidelines established by the Institutional Animal Care and Use Committee of CNVAMC. Male mice on C57BL/6 background of 8–14 weeks of age were used. The kidney proximal tubule-specific p53 knockout mouse model was generated by crossing p53-floxed mice with PEPCK-Cre mice as described previously (19).

Abbreviations: AKI, acute kidney injury; BUN, blood urea nitrogen; CKD, chronic kidney disease; CNVAMC, Charlie Norwood Veterans Affairs Medical Center; Cyc B, cyclophilinB; DCD, donor of circulatory death; DGF, delayed graft failure; DMSO, dimethyl sulphoxide; ESRD, end stage renal disease; H&E, hematoxylin and eosin; IRI, Ischemia reperfusion injury; KIM-1, kidney injury molecular-1; KO, knock out; Nx, nephrectomy; PF, pifithrin alpha; PI, propidium iodide; p-p53 (s15), phosphor-p53 (Ser15); PUMA, p53 upregulated modulator of apoptosis; RPTCs, rat kidney proximal tubule cells; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UW solution, university of Wisconsin solution; WT, wild type.

Kidney Cold Storage/Transplantation

The kidneys from donor mice were transplanted into syngeneic recipients following previously described procedures with minor modifications (9, 21). The donor mice were anesthetized with pentobarbital (50 mg/kg intraperitoneally). The left kidney of the donor was excised and flushed with cold heparinized (Sigma H3393) University of Wisconsin (UW) solution (Belzer UW; Bridge to Life Ltd.) and stored in cold UW solution in ice-water bath for the indicated time. For recipient transplantation, the left kidney of the recipient was removed. The donor aortic cuff and renal vein were anastomosed to the recipient abdominal aorta and inferior vena cava, respectively, with 10-0 Ethicon sutures. Graft anastomosis time was standardized at 30 min. The ureteral implantation was accomplished by fixing to the exterior wall of the bladder. The end of the ureter was cut and the bladder wall was closed using a 10-0 Ethicon suture. During the recipient surgery, the animals were kept hydrated with normal sterilized saline instilled intraperitoneally. During surgery, the mice were kept on warm heating pads to maintain body temperature. After transplantation, recipient mice were euthanized at 24 h to collect transplanted kidneys for analysis of acute injury. To monitor the life-supporting function of transplanted kidneys, the native contralateral kidney was removed at day 5 post-transplantation to let the mice survive for another day to examine the function and histology of the transplanted kidney at day 6.

To determine the effect of pifithrin-α (Selleck, S2929) on acute kidney cold storage/transplantation injury, it was given three times: (1). Injected to donor mice overnight before donor kidney procurement; (2). Kept in UW solution at 50 μM during cold preservation according to cell experiment; (3). added to abdominal cavity immediately after transplantation (day 0) to recipient mice. To test its effect in the life supporting model, in addition to those three dosages, pifithrin-α was given two more dosages on day 2 and day 4 after transplantation. All the injection dosages were intraperitoneally administered at 2.2 mg/kg. The same volume of DMSO (the vehicle solution) was given as the control of every dosage. Purity of the pifithrin-α was shown to be 98.14% by the manufacturer (Selleck. TX) in HPLC assay.

Renal function was measured as serum creatinine and blood urine nitrogen (BUN) levels using commercial kits as previously described (22).

Cold Storage/Rewarming of Rat Kidney Proximal Tubular Cells

Rat kidney proximal tubule cells (RPTCs) were originally from U. Hopfer at Case Western Reserve University (Cleveland, OH) and cultured as described previously (18). For cold storage, cells were incubated in UW solution at 4°C for indicated time. For rewarming, UW solution was replaced with normal culture medium at 37°C for indicated time. Cell death was evaluated by phase-contrast and fluorescence microscopy after stained with Hoechst 33342. Cells showing cellular shrinkage, blebbing, and nuclear condensation and fragmentation were counted as apoptotic cells. The percentage of cell apoptosis was estimated in

four fields with ∼200 cells per field to estimate the percentage of apoptosis.

Stable Transfection of RPTCs With Dominant Negative p53

RPTCs stably transfected with dominant-negative p53 were generated in our previous work (18). Briefly, the dominant-negative mutant sequence of p53 with a point mutation (V143A) (a generous gift from Dr. Chi-Hui Tang, University of Texas M.D Anderson Cancer Center and Health Science Center, Houston, TX) was subcloned into pcDNA3.1 that contained a HA tag and hygromycin resistance cassette using *Hind*III and *Xba*I. After transfection using Lipofectamin 2000 (*Invitrogen*), the cells were maintained in culture medium containing hygromycin for selection and expanding. Positive clones verified by immunoblotting of HA were subjected to a second round of cloning to ensure the selection of pure colonies.

Determination of Caspase Activity

Caspase activity was measured by the enzymatic assay using the fluorogenic peptide substrate DEVD.AFC as described previously (23). The 1% Triton X-100 extracted cell lysate was added to the enzymatic reaction containing DEVD.AFC. The fluorescence at excitation 360 nm/emission 530 nm was monitored by a GENios plate-reader (Tecan US Inc., Research Triangle Park, NC, USA). A standard curve was constructed using free AFC in each measurement. Caspase activity was expressed as the nanomolar amount of liberated AFC by each mg protein of cell lysate.

Immunoblot Analysis

Immunoblotting was performed by standard method as previously described (24). The primary antibodies used in this study included p53 (#2524), p-p53 (s15) (#9284, #9286), PARP (#9532), cleaved caspase-3 (#9664), Bak (#12105), PUMA (#4976), HA tag (#5017), cyclophilin B (#43603) and GAPDH (#5174) from Cell signaling, and p21 (ahz0422) from *Invitrogen*. Each experiment was repeated for at least four times, and representative images are present.

Hematoxylin and Eosin Staining and Terminal Deoxynucleotidyl Transferase–Mediated Digoxigenin-Deoxyuridine Nick-End Labeling (TUNEL) Assay

For histology, kidney tissues were fixed for paraffin embedding and H&E staining. Tubular damage was scored by the percentage of renal tubules with cell lysis, loss of brush border, and cast formation (0, no damage; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%) (25). For TUNEL staining, kidney tissue sections were stained with *In Situ* Cell Death Detection Kit, TMR red (Roche Applied Science) and examined with fluorescent microscopy. The slides were examined with fluorescent microscopy, and the TUNEL-positive cells were counted from 10 randomly picked images for each specimen in the outer medulla and kidney cortex region.

Immunofluorescence of Tissue and RPTCs

Kidney samples were fixed, paraffined, rehydrated for immunofluorescence staining with procedure as previously described (24). The primary antibodies included anti-p53 (#2524) and anti-p-p53 (s15) (#9286) from Cell Signaling. Fluorescein-labeled Lotus Tetragonolobus Lectin (LTL, FL-1321; Vector Labs) or DAPI (Vector) was used to mark proximal tubules or nucleus, respectively. For quantification, positive cells were counted from ten randomly picked images for each specimen in the outer medulla and kidney cortex region. Proximal tubules were also stained with fluorescein-labeled LTL alone. For quantitative analysis, around 10 fields (X100 magnification) were randomly selected from each section and the percentage of normal LTL- positive tubule was measured with ImageJ.

RPTCs immunofluorescence was performed as described previously (26) using p53 antibody (#2524). Slides were mounted with anti-fade containing DAPI.

Immunohistochemistry

Immunohistochemistry staining for KIM-1 was performed as previously described (9). Tissue was exposed to goat KIM-1 antibody (Novus, AF1750) and ImmPRESS-AP (Alkaline Phosphatase) horse anti-goat IgG. The color was developed using vector red substate kit, Alkaline phosphatase (AP) (SK-5100). For quantification, around 10 fields (X100 magnification) were randomly selected from each section and the percentage of KIM-1 positive tubules was measured with ImageJ.

Immunohistochemistry staining for macrophage and neutrophil used an amplification protocol as formerly described (24). After primary antibody incubation with rat anti-macrophage (ab56297, Abcam) and rat anti-neutrophil (ab2557, Abcam), kidney tissue sections were blocked with avidin and biotin (vector laboratory kit, SP-2001) followed by biotinylated goat anti-rat secondary antibody (Millipore, AP183B). Following signal amplification with Tyramide Signal Amplification Biotin System (Perkin Elmer, NEL700A001KT), the sections were incubated with a VECTAS-TAIN® ABC kit (Vector Laboratories, PK-6100) and color was developed with a DAB kit (Vector Laboratories). For quantitative analysis, 10 to 20 fields (X200 magnification) were randomly selected from each section and the percentage of positive stained area was quantitated using ImageJ.

Statistical Analyses

The Student's t test was used to show the significant difference between two groups, and ANOVA was used for multigroup comparison. The Dunn's multiple comparisons and the Fisher least significant difference test were used for one-way ANOVA and two-way ANOVA, respectively. Linear regression analysis was performed using Prism. P values < 0.05 were considered significant. Data were expressed as mean ± SD. GraphPad Prism 8 was used for all calculations.

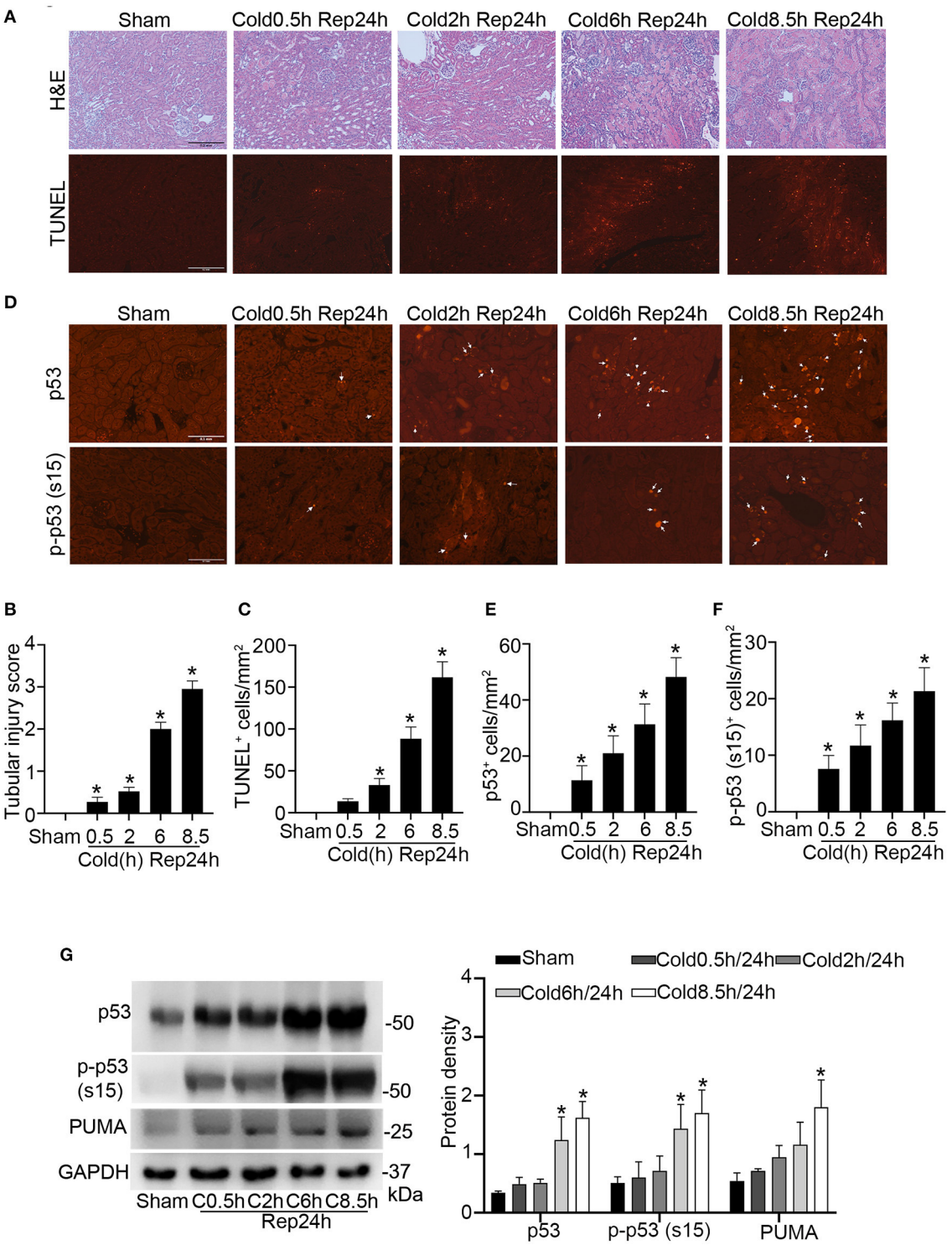

FIGURE 1 | Kidney injury and p53 activation during cold storage/transplantation. The left kidney was collected from B6 donor mice for 0.5–8.5 h of cold storage, followed by transplantation into syngeneic recipient mice for 24 h or sham operation as control. **(A)** Representative images of H&E staining and TUNEL staining. Scale bars = 0.2 mm. **(B)** Pathological score of tissue damage. **(C)** Quantification of TUNEL positive cells in kidney cortical and outer medulla tissues. **(D)** Representative images of p53 and p-p53 (s15) immunofluorescence. Scale bars = 0.1 mm. Arrow: positive staining. **(E,F)** Quantification of p53 and p-p53 (s15) positive cells in kidney cortical and outer medulla tissues. **(G)** Immunoblots analysis and quantification of p53, p-p53 (s15), and PUMA. GAPDH was used as loading control. Data are expressed as means ± SD; n ≥ 4. *$P < 0.05$, statistically significant difference from the sham control group.

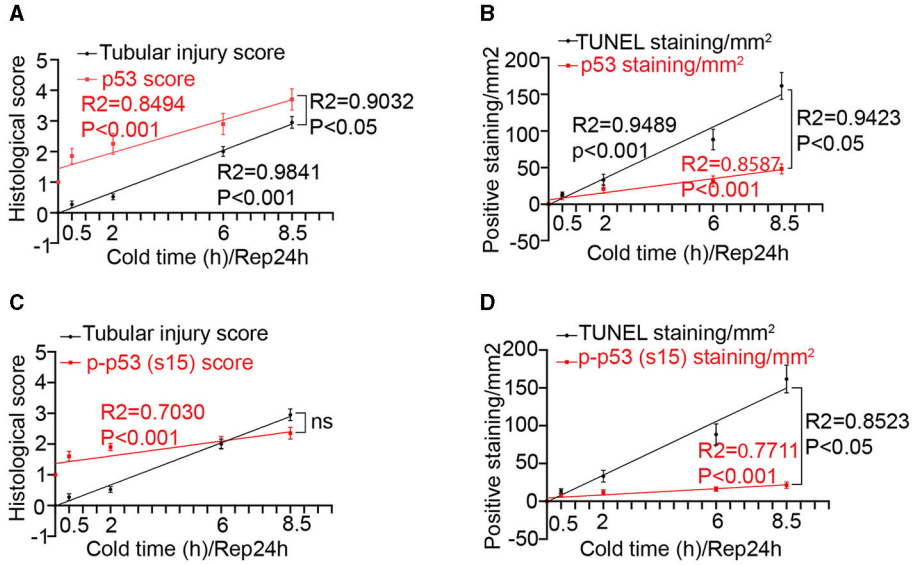

FIGURE 2 | Correlations between cold-storage time, tubular injury and p53 in transplanted kidneys. **(A)** Linear correlation and regression analysis of p53 staining score and tubular injury score with cold storage time. **(B)** Linear correlation and regression analysis of p53 staining and TUNEL staining with cold storage time. **(C)** Linear correlation and regression analysis of p-p53 (s15) staining score and tubular injury score with cold storage time. **(D)** Linear correlation and regression analysis of p-p53 (s15) staining and TUNEL staining with cold storage time. Data are expressed as means ± SD; n ≥ 4.

RESULTS

p53 Is Activated in kidney Transplantation After Cold Storage

To study kidney cold storage/transplantation, we used a mouse syngeneic kidney transplantation model, in which the left kidney was isolated from the donor B6 mouse for cold storage and then transplanted to the recipient B6 mouse. In histology (**Figure 1A**), kidney transplantation with short durations of cold storage of 0.5 and 2 h only showed injury in a few tubules. Six h of cold storage caused moderate (scored ∼2) while 8.5 h led to severe tubular damage (scored ∼3) (**Figure 1B**), which was characterized by tubular brush border shedding, cast formation, cellular necrosis and detachment from basement membranes. Corroborating with the histological observations, tubular cell death detected by TUNEL assay (**Figure 1A**) slightly increased with short cold storage followed by transplantation. However, TUNEL positive cells increased significantly after 6 h, and peaked at 8.5 h, of cold storage with transplantation (**Figure 1C**). In this model, we initially examined p53 by immunofluorescence. As shown in **Figure 1D**, kidney cold-storage/transplantation increased p53-positive cells. Consistently, kidney cold-storage/transplantation led to p53 phosphorylation at serine-15 [p-p53 (s15)], indicative of p53 activation. In quantification, 0.5 and 2 h of cold storage with transplantation moderately increased the number of p53-positive cells, while 6 and 8.5 h induced remarkable increases (**Figure 1E**). Along with total p53, the phosphorylated p53 (p-p53) increased albeit at a lower level in quantification (**Figures 1D,F**). By co-staining with LTL (marker for kidney proximal tubules) and DAPI, we detected p53 and p-p53 (s15) mainly in the nucleus of proximal tubule cells although occasionally observed p53 and p-p53 (s15) staining in distal tubules and interstitium (**Supplementary Figure 1**). Immunoblot analysis of kidney tissues was roughly consistent with the cold-storage time dependent increases of p53, p-p53 (s15) and its downstream gene like PUMA (**Figure 1G**).

p53 Activation Correlates With Cold-Storage Time and Tubular Injury in Transplanted Kidneys

We then conducted Pearson correlation and linear regression analysis to determine the relationships between p53 and tubular injury during kidney cold storage/transplantation. For the sake of analysis, we firstly determined the histological score of p53 and p-p53 (s15) staining on the basis of the average number of p53/p-p53 (s15)-positive cells per image in ×200 magnification according to our recent work (24): 1, no positive tubules; 2, 1 to 3; 3, 4 to 6; 4, 7 to 9; 5, 10 to 12; 6, 13 to 15; 7, 16 to 18; and 8, 19 to 21 positive tubules, respectively. As shown in **Figure 2A**, cold storage time correlated well with tubular injury ($R2 = 0.9841$ $P < 0.001$) and p53 activation ($R2 = 0.8494$ $p < 0.001$). Notably, tubular injury showed a good correlation with p53 activation with an R2 value of 0.9032. To reveal the association between p53 and cell death, the correlation between p53 staining and TUNEL staining was analyzed (**Figure 2B**). Cold storage time ($R2 = 0.9489$ $P < 0.001$) as well as p53 activation ($R2 = 0.9423$ $P<0.05$) showed significant positive correlations with TUNEL positive staining. Furthermore, p-p53 (s15) correlated well with cold storage time ($R2 = 0.7030$, $P < 0.001$ **Figure 2C**) and TUNEL

staining ($R2 = 0.8523$, $P < 0.05$, **Figure 2D**), but it did not show a significant correlation with renal tubular injury (**Figure 2C**).

Pifithrin-α Inhibits p53 and Protects Renal Tubular Cells During Cold Storage/Rewarming

For *in vitro* experiments, we took advantage of cold storage/rewarming model to mimic the ATP-depletion and replenishment *in vivo* (9, 11, 27, 28). Rat kidney proximal tubular cells (RPTCs) were cold (4°C) stored in UW solution followed by incubation in full culture medium at 37°C for rewarming. Immunoblot analysis showed that p53, p-p53 (s15) and downstream targets gene (e. g., Bak and p21) were upregulated during rewarming after 24 h of cold storage, while cold storage only without rewarming did not induce significant changes in these proteins (**Supplementary Figure 2**).

To determine the role of p53 in cold storage/rewarming-associated cell injury, we tested the effects of pifithrin-α, a pharmacological inhibitor of p53 (29). Cold storage only without

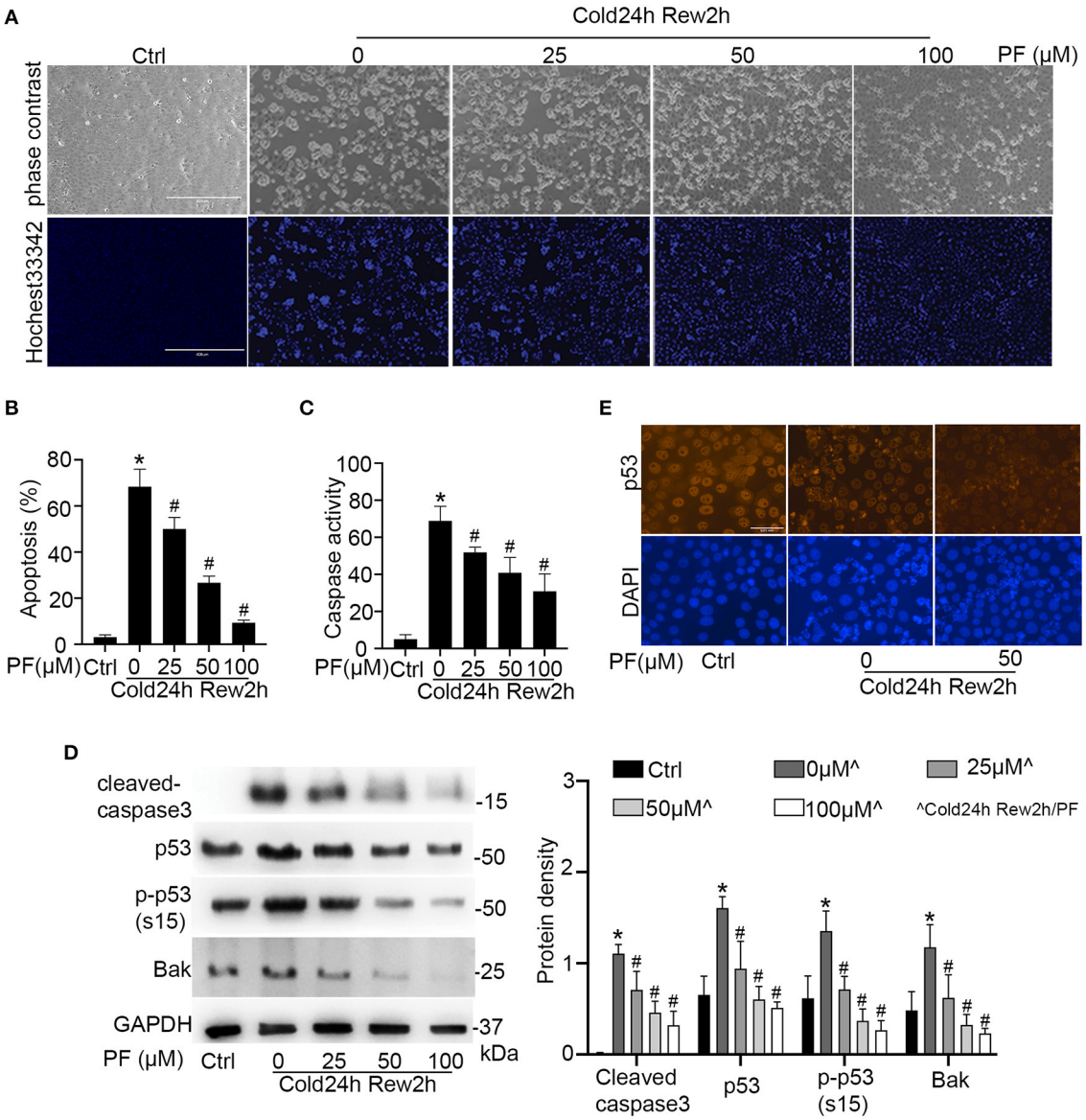

FIGURE 3 | Pifithrin-α inhibits p53 and protects renal tubular cells during cold storage/rewarming. RPTCs were incubated in UW solution in 4°C for 24 h followed by rewarming in full medium in 37°C for 2 h in the presence of 0, 25, 50, or 100 μM pifithrin-α (PF). **(A)** Representative images of cellular and nuclear morphology. Cell nucleus was stained with Hochest 33342. Scale bar: 0.4 mm **(B)** Percentage of cell death quantified by counting the cells with typical apoptotic morphology. **(C)** Caspase activity measured with DEVD.AFC as the enzymatic substrate. **(D)** Immunoblots analysis and quantification of cleaved-caspase3, p53, p-p53 (s15) and Bak with GAPDH as a loading control. **(E)** Immunofluorescence of p53. DAPI was used to mark nucleus. Scale bar, 0.05 mm. Data are expressed as mean ± SD (n≥ 5). *$P < 0.05$ vs. Control. $^{\#}P < 0.05$ vs. PF 0 μM (Cold 24 h Rew2h).

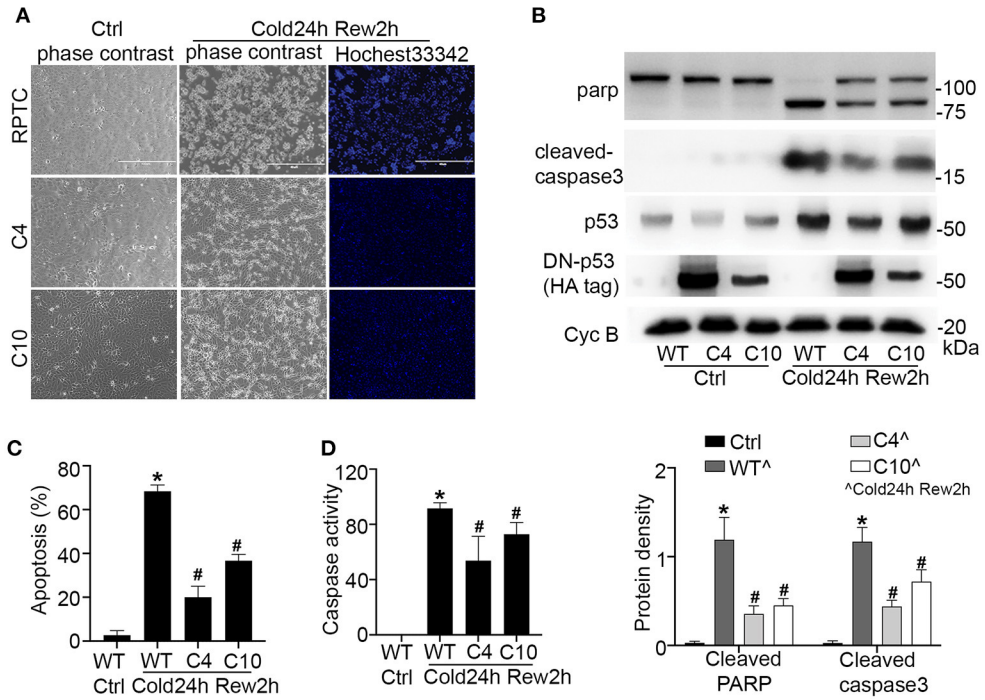

FIGURE 4 | Dominant negative p53 protects renal tubular cells during cold storage/rewarming. Stably DN-p53 transfected cell clones (C4, C10) and wild type RPTCs were incubated in UW solution in 4°C for 24 h followed by rewarming in full medium in 37°C for 2 h. **(A)** Representative images of cellular and nuclear morphology. **(B)** Immunoblots analysis and quantification of PARP, cleaved caspase 3, p53 and DN-P53 marked by HA-tag with cyclophilin B as loading control. **(C)** Quantification of cell death. **(D)** Caspase activity. Data are expressed as mean ± SD (n≥ 5). $^{*}P < 0.05$ vs. Control. $^{\#}P < 0.05$ vs. WT Cold24h Rew2h.

rewarming did not induce obvious apoptosis in RPTCs (data not shown). Two h rewarming after 24 h of cold storage induced 68% apoptosis that was characterized by nuclear shrink, fragmentation and cell detach (**Figure 3A**). Propidium iodide (PI) staining did not show significant necrosis during cold storage/rewarming (data not shown). Pifithrin-α suppressed apoptosis during cold storage/rewarming dose-dependently. While 25 μM of pifithrin-α reduced apoptosis from 68 to 50%, 50 and 100 μM of pifithrin-α suppressed it to 27 and 10% respectively (**Figures 3A,B**). The morphological analysis was verified by measurement of caspase activity (**Figure 3C**) and by immunoblotting of active/cleaved caspase 3 (**Figure 3D**). The inhibitory effect of pifithrin-α on p53 was confirmed by immunoblot analysis of p53, p-p53 (s15) and its target gene Bak (**Figure 3D**) and immunofluorescence of p53 (**Figure 3E**).

Dominant Negative p53 Protects Renal Tubular Cells During Cold Storage/Rewarming

To further verify the role of p53 in cold-storage rewarming injury, we tested RPTC cells that were stably transfected with dominant negative p53 (DN-P53) generated in our previous work (18). DN-P53 has a point mutation (V143A) that diminishes the DNA binding and gene transcriptional activity of p53. We compared regular/wild type (wt) RPTCs with two DN-p53 cell clones (C4, C10). The expression of HA-tagged DN-p53 in C4 and C10 cells (but not in wt cells) were confirmed by immunoblotting (**Figure 4B**). After cold-storage rewarming, C4 and C10 cells had 20 and 37% apoptosis, which were significantly lower than that of wt RPTCs (**Figures 4A,C**). C4 and C10 cells also had lower caspase activity (**Figure 4D**) and less cleaved PARP and caspase 3 (**Figure 4B**). Taken together, these results indicate that p53 plays an important role in cell injury and death in this cold storage/rewarming model.

Pifithrin-α Protects Kidneys During Cold Storage/Transplantation

We then determined the effect of pifithrin-α on kidney injury during cold storage/transplantation. To this end, the donor mice were pre-treated with pifithrin-α (drug administration detailed in materials and methods) to collect the left kidney for 8.5 h cold storage followed by transplantation for 24 h. The right kidney of donor mice without cold storage/transplantation was used as sham control. Pifithrin-α did not show effect on histology (data not shown) and protein expression in sham control. In histology analysis, cold storage/transplantation led to significant renal tubular damage (DMSO), which was reduced by pifithrin-α (PF). By semi-quantification, the tubular injury score was 3 in DMSO-treated group and 1.9 in pifithrin-α–treated mice

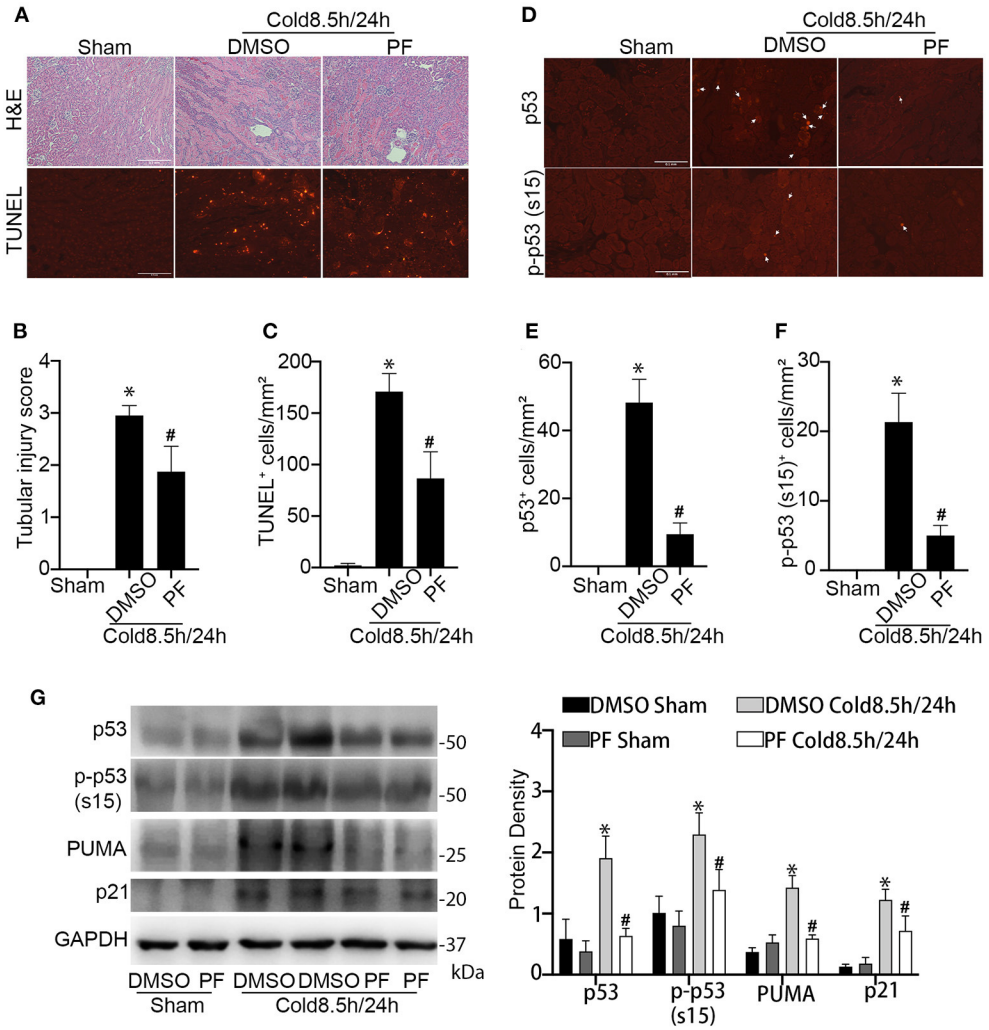

FIGURE 5 | Pifithrin-α protects kidneys during cold storage/transplantation. 2.2 mg/kg pifithrin-α or the vehicle solution DMSO was injected intraperitoneally to donor mice. After overnight treatment, donor kidneys were collected for 8.5 h of cold storage in UW solution in the presence of 50 μM pifithrin-α or DMSO. After kidney transplantation surgery, 2.2 mg/kg pifithrin-α or DMSO was added to the abdomen cavity of recipient mice. The transplanted kidneys were collected at 24 h after transplantation. The contralateral kidneys of donor mice were used as sham control. **(A)** Representative images of H&E staining of renal histology (Scale bar, 0.2 mm) and TUNEL assay (Scale bar, 0.1 mm). **(B)** Tubular damage score. **(C)** Quantification of TUNEL positive cells in outer medulla and cortical tissues. **(D)** Representative image of p53 and p-p53 (s15) immunofluorescence. Scale bars, 0.1 mm. Arrow: positive staining. **(E,F)** Quantification of p53 and p-p53 (s15) positive cells in outer medulla and cortical tissues. Quantitative data are expressed as mean ± SD ($n \geq 4$). $^{*}P < 0.05$ vs. sham control, $^{\#}P < 0.05$ vs. DMSO Cold 8.5 h/24 h. **(G)** Immunoblots analysis and quantification of p53, p-p53 (s15), p21 and PUMA with GAPDH as loading control.

(**Figures 5A,B**). Consistently, less tubular cell death was detected in pifithrin-α treated transplants, showing 171 TUNEL positive cells per mm^2 tissue in DMSO group and 87 in pifithrin-α group (**Figures 5A,C**).

The inhibition of p53 by pifithrin-α was verified by immunofluorescence and immunoblot analysis. As shown in **Figures 5D-F**, kidney transplantation after 8.5 h of cold storage induced p53 accumulation and phosphorylation in many kidney cells, which was markedly reduced by pifithrin-α. Immunoblotting of p53, p-p53 (s15), p21 and PUMA further supported p53 inhibition by pifithrin-α in cold storage/transplantation (**Figure 5G**).

Ablation of p53 From Proximal Tubules in Donor Kidneys Reduces Cold Storage/Transplantation Injury

To further establish the role of p53 in cold-storage transplantation injury, we took advantage of the conditional p53 knockout mouse model where p53 is specifically ablated from kidney proximal tubule cells. We isolated the kidneys from the p53 conditional knockout (KO) mice or their wild type littermates (WT) for 8.5 h of cold storage, and then transplanted into WT recipient mice for 24 h. As shown in **Figures 6A,B**, cold storage/transplantation induced significant tubular damage in the transplanted WT kidney mainly in outer stripe of outer

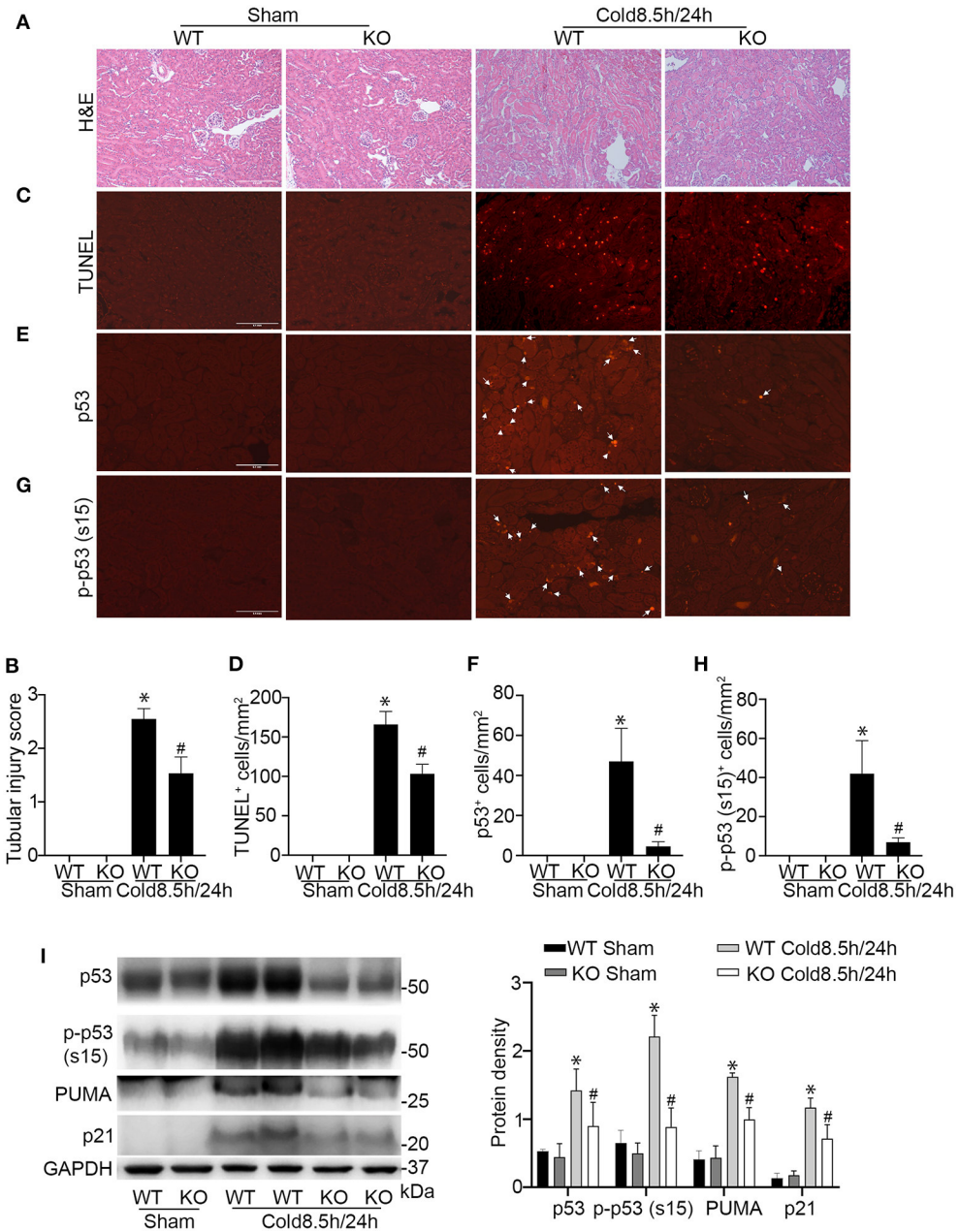

FIGURE 6 | Ablation of p53 from proximal tubules in donor kidneys reduces cold-storage transplantation injury. Donor kidneys were collected from p53 proximal tubules knockout mice (KO) and their wild-type littermates (WT), stored in cold UW solution for 8.5 h, and transplanted into WT recipient mice. The transplanted kidneys were collected 24 h after transplantation for analysis. The contralateral kidney of donor was used as sham control. **(A)** Representative images of H&E staining. Scale bar, 0.2 mm. **(B)** Pathologic score of tubular damage. **(C)** Representative images of TUNEL assay. Scale bar, 0.1 mm. **(D)** Quantification of TUNEL positive cells in outer medulla and cortical tissues. **(E)** Representative image of p53 immunofluorescence staining. Scale bars, 0.1 mm. Arrow: positive staining. **(F)** Quantification of p53 positive cells in outer medulla and cortical tissues. **(G)** Representative image of p-p53 (s15) immunofluorescence staining. Scale bars, 0.1 mm. Arrow: positive staining. **(H)** Quantification of p-p53 (s15) positive cells in outer medulla and cortical tissues. Quantitative data are expressed as mean ± SD (n ≥ 4). $^{*}P < 0.05$ vs. sham control, $^{\#}P < 0.05$ vs. WT Cold 8.5 h/24 h. **(I)** Immunoblots analysis and quantification of p53, p-p53 (s15), PUMA and p21 with GAPDH as loading control.

medulla and partially in cortex with an injury score of ∼2.5. In contrast, p53 KO graft showed a better preservation of the tubules at both sites, with overall injury score of ∼1.5. Consistently, kidney cell death detected by TUNEL assay was ameliorated in KO transplants. In quantification, WT grafts had about 166 TUNEL-positive cells/mm^2 tissue where 103 in KO kidney transplants (**Figures 6C,D**). Immunofluorescences and immunoblots of kidney cortex lysate showed massive p53 accumulation and phosphorylation in WT transplants along with target gene expression (**Figures 6E–I**), which was attenuated

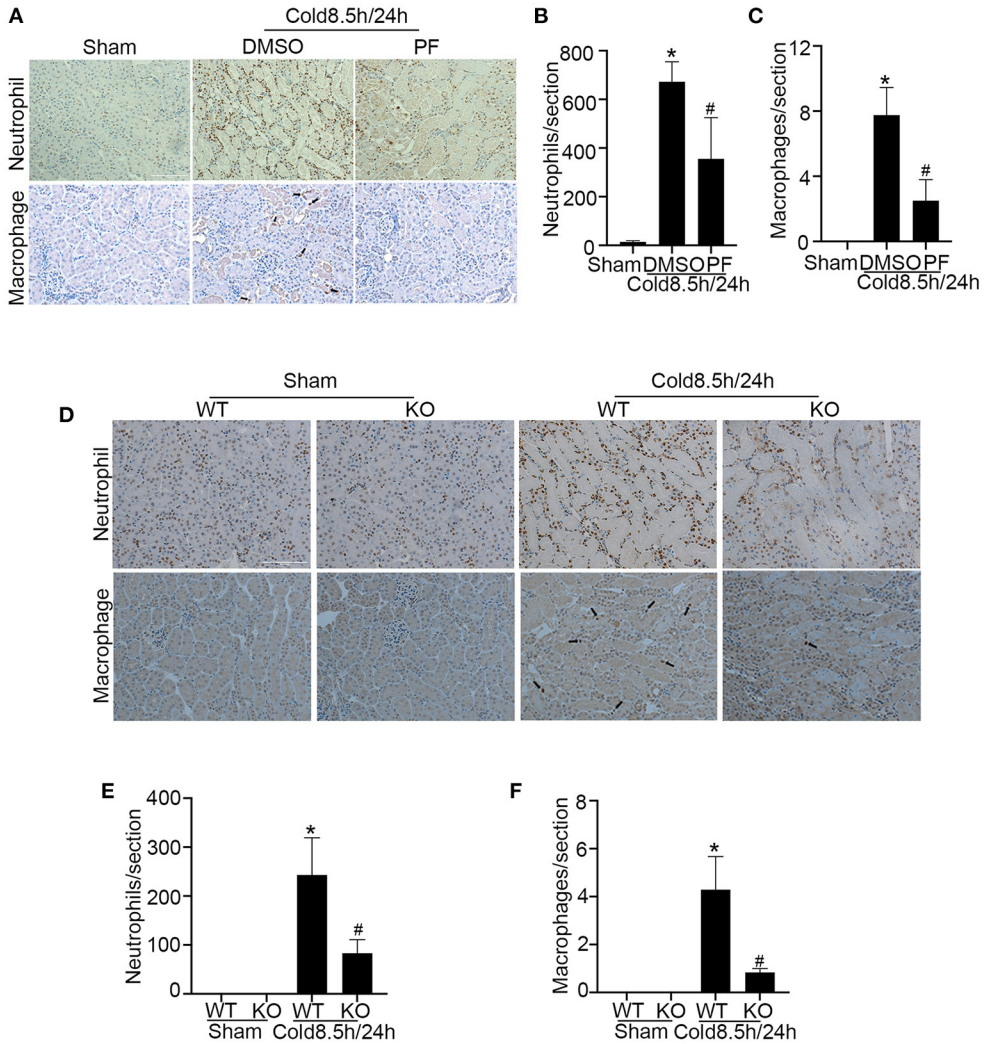

FIGURE 7 | p53 inhibition ameliorates inflammation in kidney cold storage/transplantation. **(A–C)** Pifithrin-α or the vehicle solution DMSO were given to donor B6 mice overnight, during 8.5 h of cold storage, and immediately after transplantation as detailed in **Figure 5**. The transplanted kidneys were collected at 24 h after transplantation for analysis. **(A)** Immunohistochemistry of neutrophils and macrophages. Scale bar, 0.1 mm. Arrow: positive staining of macrophages. **(B)** Quantification of positive neutrophils per cross-sectional area. **(C)** Quantification of positive macrophages per cross-sectional area. **(D–F)** Kidneys were collected from p53 KO and their WT littermates, stored in cold UW solution for 8.5 h, and transplanted into WT recipient mice. The transplanted kidneys were collected 24 h after transplantation for analysis. **(D)** Immunohistochemistry of neutrophils and macrophages. Scale bar, 0.1 mm. Arrow: positive staining of macrophages. **(E)** Quantification of positive neutrophils per cross-sectional area. **(F)** Quantification of positive macrophages per cross-sectional area. Data are expressed as mean ± SD ($n \geq 4$). $^{*}P < 0.05$ vs. sham control, $^{\#}P < 0.05$ vs. DMSO Cold 8.5 h/24 h or WT Cold 8.5 h/24 h.

in KO grafts to demonstrate decent knock out efficiency in proximal tubules.

p53 Inhibition Ameliorates Inflammation in Kidney Cold Storage/Transplantation

In addition to tubular damage, kidney cold storage/transplantation is often associated with inflammation which contributes to both acute and chronic graft dysfunction (30). We analyzed inflammatory cell infiltration in transplanted kidneys after cold storage. As shown in **Figure 7A**, cold storage/transplantation induced the infiltration of numerous neutrophils and a few macrophages in kidney interstitial tissues, which was significantly suppressed by pifithrin-α (**Figures 7B,C**). Consistently, p53 ablation also suppressed the infiltration of neutrophils and macrophages during kidney cold storage/transplantation (**Figures 7D–F**). These results indicate that p53 may contribute to the inflammatory response in cold storage/transplantation by inducing tubular injury.

Pifithrin-α Protects Against Kidney Injury and Dysfunction in Life-Supporting Grafts

To examine the therapeutic potential of p53 inhibition for transplanted kidneys, we established a life-supporting model, in which the recipient original kidney was removed on day 5

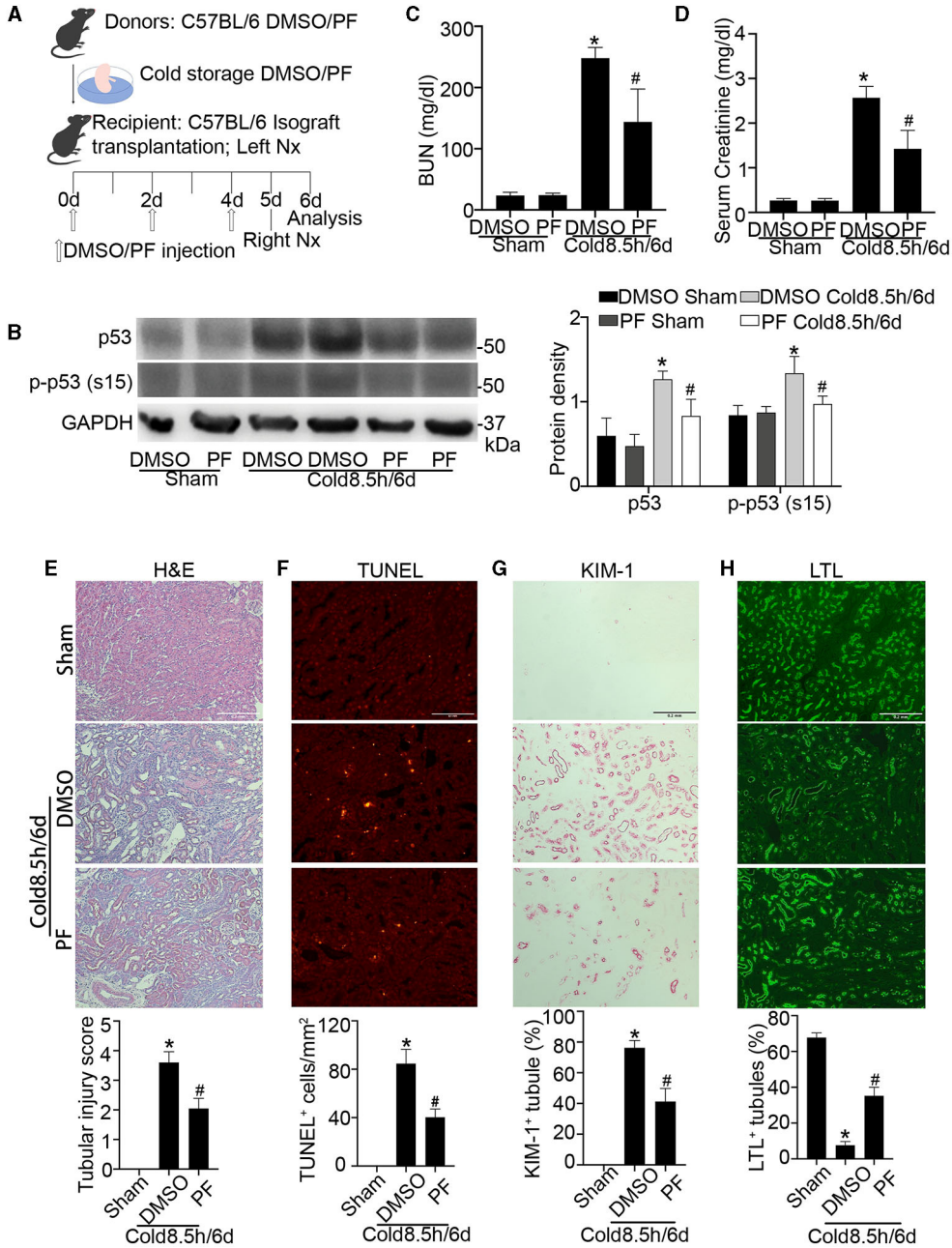

FIGURE 8 | Pifithrin-α protects against kidney injury and dysfunction in life-supporting grafts. 2.2 mg/kg pifithrin-α or the vehicle solution DMSO was injected intraperitoneally to donor mice. After overnight treatment, donor kidneys were collected for 8.5 h of cold storage in UW solution with 50 μM pifithrin-α or DMSO. After kidney transplantation, 2.2 mg/kg pifithrin-α or DMSO was given three times to the recipient mice: immediately after surgery, on day 2 and day 4. On day 5 post-transplantation, the native kidney of the recipient mice was removed so that the transplanted kidney became the life-supporting kidney. Samples were collected on day 6 for analysis. The contralateral kidneys of donor mice were used as sham control. **(A)** Diagram depicting the experimental procedure. **(B)** Immunoblots analysis and quantification of p53 and p-p53 (s15) with GAPDH as loading control. **(C)** BUN measurement. **(D)** Serum creatinine measurement. **(E)** Representative images of H&E staining and tubular injury score. Scale bar, 0.2 mm. **(F)** Representative images of TUNEL assay and quantification of TUNEL positive cells. Scale bar, 0.1 mm. **(G)** Representative images of KIM-1 immunohistochemistry and percentage of KIM-1 positive tubules. Scale bar, 0.2 mm **(H)** Representative images of LTL staining and percentage of LTL positive staining tubules. Scale bar, 0.2 mm. Quantitative data are expressed as mean ± SD (n ≥ 4). *$P < 0.05$ vs. sham control, #$P < 0.05$ vs. DMSO Cold 8.5 h/6 days.

after transplantation so that the transplanted kidney became the life-supporting kidney as described in our recent work (9). DMSO or pifithrin-α was given before donor harvest, included in UW solution during cold storage and every other day after surgery as illustrated in **Figure 8A** (drug administration detailed in materials and methods). p53 inhibition by pifithrin-α in this

experiment was verified by immunoblotting of p53 and p-p53 (s15) (**Figure 8B**). On day 6 after cold storage/transplantation, BUN and serum creatinine respectively increased to 248 mg/dl and 2.6 mg/dl in the mice receiving DMSO. Pifithrin-α significantly reduced BUN and serum creatinine to 144 mg/dl and 1.4 mg/dl (**Figures 8C,D**). In histological analysis, almost all tubules were injured with an injury score of 3.6 in DMSO group, whereas nearly half of renal tubules were preserved in mice receiving pifithrin-α with a tubular injury score of 2 (**Figure 8E**). Consistently, TUNEL assay showed that pifithrin-α reduced tubular cell death from 85 in DMSO group to 40 per mm^2 tissue (**Figure 8F**). We further examined KIM-1 and LTL, which are known markers for kidney tubular injury and normal proximal tubules respectively (31). As shown in **Figures 8G,H**, pifithrin-α reduced the number of KIM-1 positive tubules while increasing the percentage of normal LTL staining tubules. Together, these results demonstrate the beneficial effects of pifithrin-α on cold storage/transplantation in life-supporting grafts.

DISCUSSION

Cold storage is commonly used for transplantation of kidneys from deceased donors based on its superiority in lowering metabolism and preserving viability (32). However, prolonged cold storage inevitably causes kidney injury and graft dysfunction, which are associated with lower donor utilization rate and poor outcome of renal transplants (33, 34). There is an urgent need to develop new and effective strategies to improve organ preservation, including the addition of renoprotective agents to the preservation solution and machine perfusion (35, 36). To identify such strategies, it is critical to elucidate the molecular and cellular events triggered in cold storage/transplantation that leads to injury and dysfunction of renal grafts. In this study, we found that p53 was activated during kidney cold storage/transplantation in mice and this activation correlated well with kidney tubular injury. p53 was also activated during cold storage/rewarming in RPTC cells. Functionally, pharmacological and genetic inhibition of p53 afforded protective effects in these models. Especially, the ablation of p53 from kidney proximal tubules reduced cold storage/transplantation kidney injury in mice, pinpointing proximal tubules cells as the main cell type of p53 action. The pharmacological inhibitor of p53, pifithrin-α, not only attenuated acute kidney injury during cold storage/transplantation but also improved the graft function later on when it became the life supporting kidney, suggesting the therapeutic potential of p53 inhibitors.

p53 has been implicated in the pathogenesis of both acute and chronic kidney diseases (15). Particularly relevant to this study is the observation that p53 participates in tubular cell injury and death during renal ischemia-reperfusion injury (IRI) or ischemic AKI (19). In cold storage/transplantation, the donor kidney experiences warm ischemia during organ procurement and vascular anastomosis; and cold ischemia during organ preservation, followed by reperfusion after transplantation to the recipient (37, 38). Therefore, renal IRI is a primary cause of acute and chronic nephropathies in kidney cold storage/transplantation. In this regard, our current study has further proved the pathogenic role of p53 by using models of kidney transplantation, one of the most relevant clinical conditions of renal IRI. Consistent with this study, Imamura et al. reported that systemic administration of siRNA for p53 ameliorates structural and functional damage in rat models of rat kidney transplantation (39). In renal IRI models, p53 in kidney proximal tubules (and not in other kidney tubules) plays a critical role in tubular cell death and renal functional loss (19, 20). Consistently, in the current study, we provided specific evidence that proximal tubule p53 play critical role in cold storage/transplantation injury in mice, which is convincing for its great similarity to human genome.

In our study, p53 was mainly activated in renal tubular cells during kidney cold storage/transplantation (**Figures 1**, **6**). However, the mechanism of p53 activation under this condition remains unclear. A well-documented mechanism of p53 activation is DNA damage response, which includes a signaling cascade leading to the activation of upstream protein kinases to phosphorylate p53 (40, 41). Currently, it is unknown whether and to what extents DNA is damaged in kidney cold storage/transplantation, and whether the signaling pathway of DNA damage response is activated. Nonetheless, oxidative stress is associated with kidney cold storage/transplantation and, the beneficial effects of antioxidants, such as mitoQ (10, 28, 42), have been demonstrated for transplanted kidneys. Oxidative stress may lead to DNA oxidization, a known form of DNA damage, for the formation of oxidized nucleosides such as 8-oxo-2'-deoxyguanosine (8-oxo-dG). Therefore, oxidative stress is a likely cause of DNA damage response for p53 activation in kidney cold storage/transplantation.

The regulation of p53 is multifaceted and tissue/cell type specific (43). By using proximal tubule-specific p53 knockout model, we demonstrated the crucial role of p53 in proximal tubules during kidney transplantation with cold storage (**Figure 6**). Downstream of p53, both transcription-dependent and independent mechanisms may lead to nephrotoxicity (16, 18) and ischemic AKI (11, 19). In the current study, we examined down-stream genes of p53, such as p21 and PUMA. These genes were induced during kidney cold storage/transplantation, and the induction was attenuated by pifithrin-α and also by the ablation of p53 from proximal tubules (**Figures 5**, **6**). In addition, tubular cell death during cold storage/rewarming was suppressed by dominant negative p53 that has a loss of transcriptional function mutation (18, 44). These results suggest that p53 mediates cold storage/transplantation kidney injury mainly by inducing pro-death genes through its transcriptional mechanism.

Inflammation is another pathological feature of, and pathogenic factor in, renal IRI and subsequent renal interstitial fibrosis (45). In the present study, we showed that cold storage/transplantation induced massive neutrophil infiltration but only a few scattered macrophages in 24 h (**Figure 7**). It is acknowledged that inflammatory cells especially neutrophils

and associated cytokines play a pathogenic role in acute injury of kidney ischemia-reperfusion (46) However, the effect of pifithrin-α in inflammation and fibrosis is controversial from previous studies. It was reported that pifithrin-α exacerbated inflammation damage in rat (47) and mice (48) since inflammatory cell apoptosis may be suppressed. But knockout of p53 from kidney proximal tubules inhibited inflammation as the consequence of reduced tubular injury (19, 20). pharmacological and genetic blockade of p53 mitigated the infiltration of neutrophils and macrophages concomitantly with decreased tubular injury, indicating that reduced tubular damage led to the suppression of inflammation during kidney cold storage/transplantation. As for fibrosis, it was reported that pifithrin-α promoted renal fibrosis in rat model of ischemic AKI (47) although the mechanism is unclear. In contrast, it was also demonstrated that pifithrin-α attenuated fibrosis through facilitating G-M cell cycle progression after unilateral renal IRI in mice (49) and bilateral IRI in rats (50). Whether pifithrin-α can reduce renal fibrosis after kidney cold storage/transplantation warrants further investigation.

In conclusion, we have demonstrated an important role of p53 in kidney injury and dysfunction following cold storage/transplantation. In this regard, p53 in kidney proximal tubules is particularly important. Pharmacological inhibitors of p53, such as pifithrin-α, may have beneficial effects for improving the quality of donor kidneys from deceased donors and ultimately the outcome of renal transplants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Charlie Norwood Veterans Affairs Medical Center.

AUTHOR CONTRIBUTIONS

XX and ZD designed experiments and analyzed results. XX and JZ performed experiments. JZ and GZ instructed murine kidney transplantation surgery. PEPCK-Cre mice were kept by ZM. DN-p53 RPTCs were constructed by ML. XX, JZ, and ZD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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The Role of HLA Antigens and Steroid Dose on the Course of COVID-19 of Patients After Kidney Transplantation

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Background: Kidney transplant recipients appear to be at higher risk for critical COVID-19. Our analysis aimed to identify the possible risk factors for a severe course of the COVID-19 disease and to determine the influence of selected human leukocyte antigens (HLAs) on the course of the disease.

Methods: This is a retrospective, multicenter analysis that included patients that were confirmed to be severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) positive after kidney transplantation (KT). The group of patients was divided into two subgroups according to the course of the infection, as follows: non-hospitalized and hospitalized.

Results: A total of 186 patients (men, 69.4%) with confirmed SARS-CoV-2 positivity were included in the group. The following independent risk factors for the outcome of hospitalization were identified: the age at the time of infection [odds ratio (OR) = 1.19, $P < 0.0001$], a body mass index (BMI) >29.9 kg/m^2 (OR = 7.21, $P < 0.0001$), <7.5-mg prednisone dose/day (OR = 2.29, $P = 0.0008$), and HLA-DQ2 with a protective nature (OR = 0.05, $P = 0.0034$).

Conclusions: Higher doses of corticosteroids (>7.5 mg/kg) in standard immunosuppressive regimes and HLA-DQ2 appear to be protective factors in our analysis.

Keywords: kidney transplantation, COVID-19, HLA class I and class II typing, steroid dose, immunosuppression

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INTRODUCTION

The novel coronavirus, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was first identified toward the end of 2019 in Wuhan, in the Hubei province of China, and spread rapidly throughout the world. The clinical symptoms of the disease vary from a completely asymptomatic course through fever, cough, the development of bronchopneumonia, up to multiorgan failure (1).

Kidney transplant recipients appear to have a particularly high risk for critical COVID-19 illness due to chronic immunosuppression and coexisting conditions (2). In comparison with the general population, patients who underwent kidney transplantation (KT) from diabetes mellitus, arterial hypertension, or cardiovascular diseases suffer more often. Immunosuppressive treatment

is also a precondition for the worse course of the disease, most frequently with the combination of calcineurin inhibitors (CNI), mycophenolate mofetil (MMF), and corticosteroids (2–4).

Several analyses compared the course of COVID-19 infection in patients after KT to the non-transplanted population for their similarities, with large inter-individual differences. According to data from the European Renal Association COVID-19 Database register (ERACODA), the 28-day probability of death in 1,073 patients (28% after KT and 72% in a chronic dialysis program) was 21.3% in the patients who had kidney transplants and 25% in those undergoing dialysis (5). The available data from China show the high mortality of patients with end-stage kidney disease (33%) (6), and the data from Spain and Italy have shown the 30% mortality rate of dialyzed patients (7, 8). Known publications from New York reported a 16–30% mortality rate in the group of patients after KT (2, 9, 10).

The risk factors for hospitalization or death in this population remain to be similar to the general population (11). Death is more common among elderly patients and those with pre-existing pulmonary disease (11, 12).

The treatment of COVID-19 infection in patients who had kidney transplants does not significantly differ from the treatment of the non-transplanted population; however, the question remains whether and when it is necessary to discontinue immunosuppression. There are several protocols for the immunosuppressive management of transplanted patients with COVID-19, which reflect either the clinical progress of the disease, laboratory findings, or a combination of both (13–16). The data concerning the usage of hydroxychloroquine, remdesivir, or tocilizumab in this specific group of patients are more controversial than promising (17–19). The recently published data of using bamlanivimab for patients after solid organ transplantation are promising for the treatment of patients with mild-to-moderate COVID-19 who are at high risk of progression to hospitalization (20).

The human leukocyte antigen (HLA) is a critical component of the viral antigen presentation pathway in humans, and the HLA gene locus plays a fundamental role in human adaptive immunity. The genetic variability across the HLA alleles is known to be associated with outcomes in many different diseases and could be a key determinant in the susceptibility and severity of COVID-19. The HLA type influences the T cell-mediated response to viral infections and is therefore implicated in the morbidity and mortality of a SARS-CoV-2 infection (21, 22). Several smaller studies have indicated a possible association of the HLAs of group 1 (A1 and A2) with a more severe course of infection (23–25).

Our analysis aimed to identify the risk factors for a severe course (hospitalization) of COVID-19 disease in a group of patients after KT and the risk factors for COVID-19 fatalities, with a focus on the parameters before the infection (not on the parameters worsening infection during its course). Another aim was to determine the influence of the selected HLAs on the course of the disease.

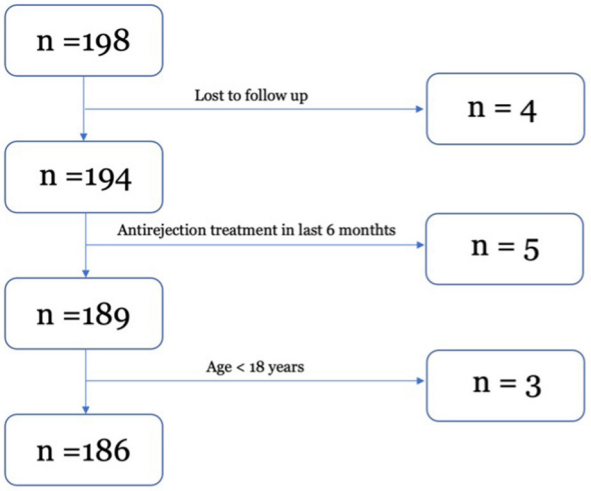

FIGURE 1 | Flowchart of the study.

MATERIALS AND METHODS

This was a retrospective, multicenter cohort analysis that included patients with kidney transplants who had positive test results for SARS-CoV-2 monitored in the Slovak Republic (transplantation centers Martin, Košice, and Bratislava) from March 2020 to December 2020. The positivity was confirmed by a real-time polymerase chain reaction (RT-PCR) test. We recorded the age at the time of test positivity, the time (months) from KT, a diabetes mellitus and arterial hypertension case history, and the body mass index (BMI) of all the patients. We evaluated the type and doses/levels of immunosuppression [tacrolimus, MMF, mycophenolic acid (MPA), and corticosteroids], HLA (A, B, DR, and DQ), and finally, the development of post-COVID syndromes. Post-COVID syndrome has been defined as the persistence of certain clinical difficulties at least 4 weeks after the infection has passed (i.e., fatigue, shortness of breath, insomnia, memory problems, "brain fog." heart palpitation, depression, and/or anxiety). The fatal outcome was defined as death in direct association with COVID-19, with respiratory failure confirmed by autopsy. In the patients who died, we recorded the time—the duration of illness (confirmed by positive test results) up to the time of death.

Inclusion criteria: Age >18 years, positive test results for SARS-CoV-2 by PCR
Exclusion criteria: Age <18 years, failed to follow-up, antirejection treatment in the last 6 months.

The flowchart of the study is shown in **Figure 1**.

Abbreviations: BMI, body mass index; CDC, Centers for Disease Control and Prevention; ERACODA, The European Renal Association COVID-19 Database; HLA, human leukocyte antigen; ICU, intensive care unit; KT, kidney transplantation; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PCR, polymerase chain reaction; TAC, tacrolimus; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

Immunosuppression

The maintenance immunosuppression consisted of tacrolimus and MPA (1,080-mg daily dose until the second week after transplantation, followed by a 720-mg daily dose, or adjusted individually according to the immunology risk of the patient). Furthermore, 500 mg of methylprednisolone was administered intravenously (IV) on day 0 and day 1, followed by the administration of 20 mg of prednisone until the 2nd week after transplantation, 15 mg of prednisone until the 4th week after transplantation, 10 mg of prednisone until the 12th week after transplantation, and 7.5 mg of prednisone until the 12th month after transplantation, with 5 mg of prednisone administered daily thereafter. The tacrolimus target levels were according to the protocols used in the Slovak Republic; 10–15 ng/ml 1–3 months after transplantation, followed by 5–10 ng/ml 3–6 months after transplantation, and 3–6 ng/ml for patients >6 months after transplantation.

The group of patients was divided into two subgroups according to the course of infection [based on the Centers For Disease Control (CDC) and Prevention Therapeutic Management of Adults With COVID-19 Last Updated: February 11, 2021], as follows:

- Subgroup 1: Patients not requiring hospitalization (asymptomatic, oligosymptomatic, or medium–severe course). Asymptomatic patients with maintained immunosuppression in the treatment regimen. Patients with a medium–severe course of COVID-19 had reduced immunosuppression [discontinuation of MPA or MMF, reduction of (CNI) by 50%].
- Subgroup 2: Severe course. Hospitalized patients with completely discontinued immunosuppression were administered 6 mg of dexamethasone IV for 24 h for a minimum of 10 days added to the treatment.

HLA Typing

The HLA typing of all patients was performed with PCR-based procedures using a sequence-specific primer (SSP). After the performance of the PCR, the amplified DNA fragments were size-separated using agarose gel electrophoresis and then visualized, documented, and interpreted.

Statistical Analysis

We used a certified statistical program, MedCalc version 13.1.2 (VAT registration no. BE 0809 344 640, Member of International Association of Statistical Computing, Ostend, Belgium), to perform statistical analyses. The continuous data were compared using Student's *t*-test, and a Wilcoxon rank-sum test was used for the analysis of the time after KT as it is non-parametric data. An χ^2-test and Fisher's exact test were used for categorical variables.

Univariable and multivariable logistic regressions were used to assess the monitored parameters to predict the risk of hospitalization and death. The statistically significant parameters assessed in the univariable analysis were entered into the multivariable model adjusted for the time after KT, sex, tacrolimus level, and MMF. The cut-off level for Prednison dose used in the logistic regression models was based on the average dose of Prednison in our group of patients.

A Kaplan–Meier survival analysis was used for the comparison of the survival rates of the hospitalized and non-hospitalized patients. We considered $P < 0.05$ to be statistically significant.

Ethical Approval

All procedures involving human participants have been approved according to the ethical standards of the institutional research committee, including the 1964 Helsinki Declaration and its later amendments of comparable ethical standards. The informed consent of the included participants was checked and approved by the ethical committees of the University hospital and the Jessenius Faculty of Medicine, and all signed informed consents are to be archived for at least 20 years after the research was completed.

The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

RESULTS

A total of 186 patients (men, 69.4%) with confirmed SARS-CoV-2 positivity were included in the group.

The group of patients was further divided according to the course of the disease into two subgroups. The criteria for the non-hospitalized patients were met by 147 patients. There were 39 patients who required hospitalization (subgroup 2), as displayed in **Table 1**.

At the time of the infection, all the monitored patients received prednisone as a part of their treatment; the patients in subgroup 2 received, on average, a lower dose of prednisone/day, and it was logically associated with the fact that they represented the group with the longer average duration of time after transplantation. The dosing of MPA and MMF was comparable in the monitored subgroups, as well as the average level of tacrolimus. As compared with subgroup 1, the patients in subgroup 2 had a higher age and a higher proportion of patients with diabetes mellitus. Three patients died in subgroup 1 and six patients died in subgroup 2. The post-COVID-19 difficulties of the group are shown in **Table 2**. Post COVID-19 syndrome was recorded more often in the hospitalized patients, with the most common symptom being fatigue.

We used a univariable analysis to determine the risk factors for the outcomes of hospitalization and death, as exhibited in **Table 3**.

We identified the following risk factors for hospitalization in the monitored group: age at the time of infection, a BMI >29.9 kg/m^2, diabetes mellitus, and a <7.5-mg dose of prednisone/day. On the other hand, HLA-B8 and -DQ2 appeared to be protective factors. The age at the time of infection and a <7.5-mg dose of prednisone/day were confirmed as risk factors for death. We identified the HLA-A2 and -DQ5 of the HLA antigens as risk antigens.

TABLE 1 | Basic group characteristics according to course of infection.

Characteristic	Non-hospitalized $n = 147$	Hospitalized $n = 39$	*P*-value
Gender—men (%)	67.3	76.9	0.2491
Age at the time of infection (in years)	51.7 ± 10.1	62 ± 11.2	**<0.0001**
Time after KT (months) (mean ± standard deviation)	78.7 ± 54.1 (median, 70)	104.9 ± 63.6 (median, 94)	**0.0104**
BMI (kg/m^2) (mean ± standard deviation)	28.8 ± 6	29.4 ±7.3	0.5971
Diabetes mellitus (%)	42.9	61.5	**0.0390**
Arterial hypertension (%)	91.8	100	0.0651
Tacrolimus in treatment (%)	91.8	92.3	0.9192
Average TAC level (ng/ml)	4.1 ± 1	4.1 ± 1	1.0000
MPA/MMF in treatment (%)	85.7	84.6	0.8628
Average MPA dose/day (mg)	668 ± 360	720 ± 311	0.4111
Average dose of corticosteroids/day (mg)	7.6 ± 2.9	5.9 ± 1.9	**0.0007**
Post COVID (%)	16.3	46.2	**0.0001**
HLA-A1 (%)	18.4	8	0.1183
HLA-A2 (%)	32	36	0.6372
HLA-A3 (%)	17	24	0.3179
HLA-B8 (%)	14.3	0	**0.0001**
HLA-B35 (%)	12.9	17.4	0.4706
HLA-B12 (%)	12.9	4.3	0.1295
HLA-DR1 (%)	9.5	13.3	0.4888
HLA-DR3 (%)	9.5	10	0.9251
HLA-DR11 (%)	10.9	10	0.8720
HLA-DR52 (%)	17	13.3	0.5784
HLA-DQ2 (%)	19	5.9	**0.0493**
HLA-DQ3 (%)	32	35.3	0.6969
HLA-DQ5 (%)	22.4	23.5	0.8843
HLA-DQ6 (%)	19	29.4	0.1587
Fatalities (%)	2 ($n = 3$)	15.4 ($n = 6$)	**0.0005**

KT, kidney transplantation; BMI, body mass index; TAC, tacrolimus; MPA, mycophenolic acid; MMF, mycophenolate mofetil; HLA, human leukocyte antigen.
The bold values mean statistical significant values.

TABLE 2 | Post-COVID difficulties.

$n = 42$	
Fatigue, $n = 21$ (%)	50
Shortness of breath, $n = 5$ (%)	11.9
Insomnia, $n = 3$ (%)	7.1
Memory problems, $n = 2$ (%)	4.8
Brain fog, $n = 2$ (%)	4.8
Heart palpitation, $n = 7$ (%)	16.7
Depression and/or anxiety, $n = 2$ (%)	4.8

The application of a multivariable analysis (logistic regression) adjusted for the time after KT, sex, tacrolimus level, and MMF dose confirmed the following as independent risk factors for hospitalization: age at the time of infection, a BMI > 29.9 kg/m^2, a <7.5-mg prednisone dose/day, and HLA-DQ2 with a protective nature (**Table 4**). The only independent risk factor for the fatal outcome was the age at the time of infection (**Supplementary Table 1**).

In the end, we compared the patients with a daily dose of steroids of <7.5 and >7.5 mg (**Table 5**). We confirmed that patients with a lower dose of steroids were significantly hospitalized more often. Seven patients died in this group in comparison with two patients in the group with a higher dose of steroids.

We confirmed the significantly worse survival in the group of hospitalized patients, as shown in **Figure 2**.

DISCUSSION

Our analysis aimed to identify the risk factors for hospitalization due to COVID-19 infection in patients after KT.

Our analysis confirmed that, similar to the general population, the risk group for a severe course of COVID-19 infection and for death is the age of the patient exceeding 59 years. Our data correspond with the conclusions of the ERACODA register, in which 28-day mortality was primarily associated with advanced age in kidney transplant patients (5). An analysis by French authors comparing the course of COVID-19 infection in patients after KT with a non-transplanted group also confirmed a significantly worse course of infection in the group of transplanted patients over 60 years of age. However, compared with the general population, the transplanted patients with a severe course of infection were younger (26).

It is not surprising that a higher BMI (>29.9 kg/m^2) was found to be a risk factor for hospitalization in patients with COVID-19. Obesity and diabetes mellitus were repeatedly confirmed as independent risk factors for a symptomatic course of COVID-19 in the group of patients after KT (27, 28).

The protective factor of the higher prednisone doses in the normal immunosuppression regime regarding the course of COVID-19 in the population of patients who underwent KT has not yet been described. Dexamethasone, the effect of which was confirmed in the RECOVERY study (29), is normally added to the treatment of all patients hospitalized with COVID-19. Glucocorticoids have been widely used in syndromes closely related to COVID-19 (30–34). However, the data that would support the assumption of the "preventative" effect of corticosteroids on the course of COVID-19 in patients after KT are missing. Patients after KT have generally been administered relatively low doses of prednisone in permanent immunosuppression regimes (from 20 mg in the early post-transplantation period to 2.5–5 mg/day in the late post-transplantation period).

In our group of patients, the dose of prednisone with <7.5 mg/day was a risk factor for hospitalization. The patients in subgroup 2 (those that required hospitalization) were administered with the lower average dose of prednisone (5.9 mg/day). The dosing of corticosteroids is gradually lower based on the time after transplantation; therefore, the patients with the lowest dose of corticosteroids were the patients with the longest duration of time after transplantation.

TABLE 3 | Univariable analysis (log regression).

Characteristic	Outcome of hospitalization OR (95% CI)	*P*-value	Outcome of death OR (95% CI)	*P*-value
Gender—men	1.61 (0.71–3.67)	0.2517	0.40 (0.07–2.20)	0.2961
Age at the time of infection (in years)	1.11 (1.06–1.16)	**< 0.0001**	1.36 (1.12–1.64)	**0.0013**
Time after KT <12 months	1.27 (0.32–4.96)	0.7233	0.64 (0.03–13.06)	0.7778
BMI >29.9 kg/m^2	5.62 (2.60–12.12)	**< 0.0001**	3.36 (0.76–14.82)	0.1089
Diabetes mellitus	2.13 (1.03–4.39)	**0.0400**	2.37 (0.54–10.38)	0.2523
Arterial hypertension	7.28 (0.42–125.84)	0.1718	1.54 (0.07–30.99)	0.7778
Tacrolimus in treatment	1.06 (0.28–3.98)	0.9235	1.27 (0.29–5.62)	0.7457
Average TAC-value >6 ng/ml	0.68 (0.22–2.12)	0.5139	1.35 (0.13–13.09)	0.7957
MPA in treatment	0.91 (0.34–2.45)	0.8625	3.57 (0.19–66.74)	0.3937
Average MPA dose >720 mg/day	1.07 (0.39–2.90)	0.8923	0.27 (0.01–5.22)	0.3937
Average prednisone dose ≤7.5 mg/day	4.66 (2.16–9.94)	**0.0001**	6.87 (1.23–38.31)	**0.0278**
HLA-A1	0.39 (0.08–1.83)	0.2334	0.44 (0.02–8.39)	0.5888
HLA-A2	1.20 (0.47–3.06)	0.6944	5.36 (3.02–9.52)	**0.0066**
HLA-A3	1.53 (0.52–4.49)	0.4324	2.2 (0.19–25.47)	0.5280
HLA-B8	0.03 (0.002–0.59)	**0.0207**	0.53 (0.02–10.10)	0.6791
HLA-B35	1.36 (0.39–4.71)	0.6194	1.22 (0.13–11.24)	0.8566
HLA-B12	0.61 (0.12–2.98)	0.5500	0.49 (0.02–9.28)	0.6390
HLA-DR1	1.46 (0.43–4.89)	0.5385	3.19 (0.58–17.33)	0.1791
HLA-DR3	1.05 (0.27–4.00)	0.9366	1.36 (0.15–11.93)	0.7771
HLA-DR11	0.51 (0.24–3.40)	0.8917	0.44 (0.02–7.99)	0.5805
HLA-DR52	0.76 (0.24–2.43)	0.6554	0.28 (0.01–5.09)	0.3934
HLA-DQ2	0.05 (0.002–0.95)	**0.0462**	0.57 (0.02–11.21)	0.7155
HLA-DQ3	1.24 (0.41–3.70)	0.6915	0.25 (0.03–2.14)	0.2098
HLA-DQ5	1.04 (0.30–3.56)	0.9425	7.5 (4.21–13.52)	**0.0033**
HLA-DQ6	1.74 (0.54–5.60)	0.3533	0.50 (0.05–2.48)	0.5322

OR, odds ratio; CI, confidence interval; KT, kidney transplantation; BMI, body mass index; TAC, tacrolimus; MPA, mycophenolic acid; HLA, human leukocyte antigen.
The bold values mean statistical significant values.

TABLE 4 | Multivariable analysis (log regression), outcome of hospitalization (adjusted for time after KT, sex, tacrolimus level, and MMF).

Characteristic	Outcome of hospitalization OR (95% CI)	*P*-value
Age at the time of infection (in years)	1.19(1.11–1.29)	**<0.0001**
BMI >29.9 kg/m^2	7.21 (1.33–8.94)	**<0.0001**
Diabetes mellitus	1.59 (0.50–5.05)	0.4305
Average prednisone dose ≤7.5 mg/day	2.29 (1.02–3.88)	**0.0008**
HLA-B8	2.08 (1.94–3.40)	0.9982
HLA-DQ2	0.05 (0.007–0.37)	**0.0034**

OR, odds ratio; CI, confidence interval; BMI, body mass index; HLA, human leukocyte antigen.
The bold values mean statistical significant values.

A great number of disease-protective and disease-susceptible HLA alleles have been well characterized in several viral infections (35). However, little data describe certain HLAs in relation to SARS-CoV-2. A group of Spanish authors assessed HLA class I in relation to the course of COVID-19 in the general population. In a group of 45 patients, they observed the higher values of SARS-CoV-2-binding peptides in the case of HLA-A2, but without any obvious correlation in the clinical picture (36). Another small-scale analysis performed on 62 patients described an association between HLA-A11 and higher mortality (37). In our group of patients in the univariable analysis, we recorded the occurrence of HLA-A2 as a risk factor for death; however, the multivariable analysis did not confirm this finding. On the contrary, a British analysis of 80 patients identified HLA-A2 as a possible protective factor for SARS-CoV-2 infection (38).

In our patient group, the presence of HLA-DQ2 appears to be a protective factor in the case of the need for hospitalization. The complex role of HLA-DQ2 on immune system reactivity is a matter of discussion. Certain studies support its potential in the promotion of interferon (IFN) production. However, other studies showed its negative impact on virus clearance (39). In a study conducted by Benlyamani et al. in critically ill patients, the results indicated the downregulation of HLA-DR molecules in the circulating monocytes (40). In the case of HLA-DQ, data regarding the incidence are available, but not regarding the development of the disease. Some data indicate that HLA-DQ1 can be associated with a higher incidence of COVID-19 (41). It is also important to note that, in a group of almost 2,000 patients from Italy and Spain, the correlation of HLA alleles with the incidence of COVID-19 was not proven (42).

TABLE 5 | Basic group characteristics according to steroid dose/day.

Characteristic	Subgroup 1 $n = 111$	Subgroup 2 $n = 75$	*P*-value
Gender—men (%)	70.3	68	0.7392
Age at the time of infection (in years)	51.8 ± 9.4	54.1 ± 15.6	0.2116
Time after KT (months) (mean ± standard deviation)	118.1 ± 67.8	64.3 ± 39.7	**<0.0001**
BMI (kg/m^2) (mean ± standard deviation)	28.8 ± 4.9	28.1 ± 7.6	0.4459
Diabetes mellitus (%)	43.2	52	0.2393
Arterial hypertension (%)	91.9	96	0.2653
Tacrolimus in treatment (%)	100	80	**<0.0001**
Average TAC level (ng/ml)	4.0 ± 1.1	4.1 ± 1	0.5291
MPA/MMF in treatment (%)	94.6	73.3	**<0.0001**
Average MPA dose/day (mg)	576 ± 262	864 ± 420	**<0.0001**
Post COVID (%)	24.3	20	0.4925
Hospitalization (%)	36	10.7	**0.0001**
Fatalities (%)	6.3	2.7	0.2634

KT, kidney transplantation; BMI, body mass index; TAC, tacrolimus; MPA, mycophenolic acid; MMF, mycophenolate mofetil. Subgroup 1: steroid dose <7.5 mg/day. Subgroup 2: steroid dose >7.5 mg/day.
The bold values mean statistical significant values.

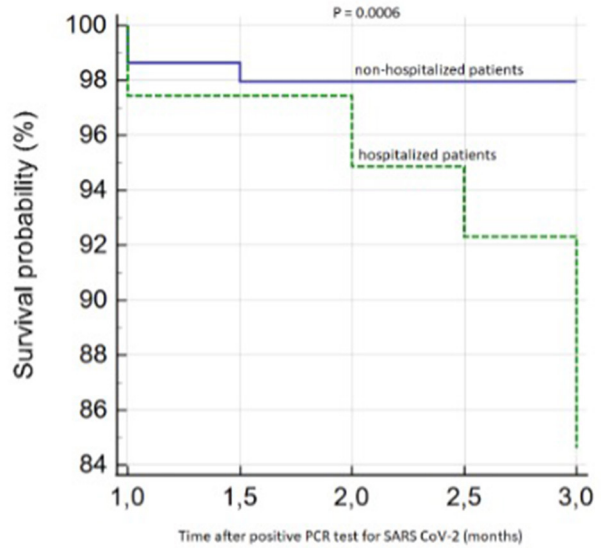

FIGURE 2 | Kaplan–Meier survival analysis (hospitalized vs. non-hospitalized patients).

According to the CDC, the most common post-COVID symptoms in the general population are fatigue, shortness of breath, or cognitive problems. The risk factors for post-COVID syndromes that have been identified include the need for hospitalization or being in the intensive care unit (ICU) (43–45). There were 42 patients with post-COVID-19 syndrome identified in our group of patients, and almost 50% of the patients from those who needed hospitalization developed post-COVID syndrome. The most common symptom was fatigue. At present, there is a lack of data about post-COVID syndromes in patients after KT and it needs further investigation.

The limitation of our analysis is the absence of data regarding the treatment and blood tests of patients during hospitalization because the patients were not hospitalized in one COVID center; therefore, the data about treatment would not be homogeneous. On the other hand, this analysis deals with the risk factors for hospitalization for COVID-19 before the onset of the infection. It is also the only analysis that identified certain protective/risk HLA antigens in patients after KT with COVID-19.

CONCLUSION

Patients after KT are high-risk patients for a severe course of COVID-19 infection. The obese and older patients (>59 years) can be considered as the group with the highest risks. The higher doses of corticosteroids in standard immunosuppressive regimes (>7.5 mg/kg) and HLA-DQ2 appear to be protective factors. However, these results should be supported with further data on larger samples of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Hospital's and Jessenius Faculty of Medicine's Ethical Committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ID participated in writing the paper, performance of the research, and data analysis. PS, KG, MV, TB, and ZŽ participated in data analysis. MJ participated in the research design and writing of the paper. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.730156/full#supplementary-material

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Increased Levels of sCD30 Have No Impact on the Incidence of Early ABMR and Long-Term Outcome in Intermediate-Risk Renal Transplant Patients With Preformed DSA

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Background: It is still incompletely understood why some patients with preformed donor-specific anti-HLA antibodies (DSA) have reduced kidney allograft survival secondary to antibody-mediated rejection (ABMR), whereas many DSA-positive patients have favorable long-term outcomes. Elevated levels of soluble CD30 (sCD30) have emerged as a promising biomarker indicating deleterious T-cell help in conjunction with DSA in immunologically high-risk patients. We hypothesized that this would also be true in intermediate-risk patients.

Methods: We retrospectively analyzed pre-transplant sera from 287 CDC-crossmatch negative patients treated with basiliximab induction and tacrolimus-based maintenance therapy for the presence of DSA and sCD30. The incidence of ABMR according to the Banff 2019 classification and death-censored allograft survival were determined.

Results: During a median follow-up of 7.4 years, allograft survival was significantly lower in DSA-positive as compared to DSA-negative patients ($p < 0.001$). In DSA-positive patients, most pronounced in those with strong DSA (MFI > 5,000), increased levels of sCD30 were associated with accelerated graft loss compared to patients with low sCD30 (3-year allograft survival 75 vs. 95%). Long-term survival, however, was comparable in DSA-positive patients irrespective of sCD30 status. Likewise, the incidence of early ABMR and lesion score characteristics were comparable between sCD30-positive and sCD30-negative patients with DSA. Finally, increased sCD30 levels were not predictive for early persistence of DSA.

Conclusion: Preformed DSA are associated with an increased risk for ABMR and long-term graft loss independent of sCD30 levels in intermediate-risk kidney transplant patients.

Keywords: kidney transplantation, donor-specific anti HLA antibodies, sCD30, risk stratification, ABMR, antibody-mediated rejection

INTRODUCTION

Antibody-mediated rejection (ABMR) caused by donor specific anti-HLA IgG antibodies (DSA) is responsible for the majority of graft losses after kidney transplantation and still remains one of the major challenges in transplant nephrology (1). Introduction of the single antigen bead (SAB) assays using Luminex technology has improved both sensitivity and specificity of detecting preformed DSA considerably but has left clinicians with the conundrum that many DSA-positive patients have favorable long-term outcomes.

Attempts have therefore been undertaken to improve the predictive value of the SAB assay. Analysis of immunoglobulin isotypes (2), subclasses (3, 4) or the capacity of the anti-HLA antibodies to bind and activate complement (5–7) have yielded mixed results.

CD30 is a 120 kD glycoprotein and part of the tumor necrosis factor (TNF) superfamily. Besides its constitutional expression on a variety of lymphoid neoplasms, most notably Hodgkin's lymphoma cells, it is expressed on activated T and B cells (8, 9). CD30 signaling via its receptor CD30 ligand (CD153) has been shown to play an important role in the generation of both memory CD8+ T cells and in regulating CD4+ T cell-mediated graft vs. host disease in animal studies (10). Cleavage of membrane-bound CD30 by metalloproteases generates the 85 kD protein soluble CD30 (sCD30). Although the exact biological function of sCD30 remains to be elucidated (11), elevated serum concentrations of sCD30 have been found to correlate with disease activity in patients with systemic lupus erythematosus, granulomatosis with polyangiitis and rheumatoid arthritis [reviewed in (8)]. In 2002, Pelzl et al. first reported increased pre-transplant sCD30 levels to be associated with reduced kidney allograft survival (12). Several following studies confirmed an association of elevated pre- and posttransplant levels sCD30 with rejection episodes or impaired allograft survival (13, 14), whereas other studies could not reproduce these findings (15, 16). Recently, Süsal et al. combined the T cell activation marker soluble CD30 (sCD30) and the SAB assay for risk stratification in two retrospective cohorts of sensitized kidney transplant patients. Remarkably, patients only exhibited an increased risk for graft loss in the presence of both elevated levels of sCD30 and DSA, whereas DSA-positive patients had comparable outcomes to DSA-negative patients in the absence of high sCD30 levels (11, 17, 18). These findings resulted in the hypothesis that DSA can only exert their detrimental effects in patients with a pre-activated cellular immunity as indicated by elevated pre-transplant levels of sCD30.

Abbreviations: AM, acceptable mismatch; ABMR, antibody-mediated rejection; CDC, complement-dependent cytotoxicity; CM, crossmatch; DGI, Deutsche Gesellschaft für Immungenetik; DSA, donor-specific antibodies; ET, Eurotransplant; ETKAS, Eurotransplant kidney allocation system; ESP, Eurotransplant senior program; FCM, flow cytometry crossmatch; GFR, glomerular filtration rate; HLA, human leukocyte antigen; IVIG, intravenous immunoglobulin; MFI, mean fluorescence intensity; PRA, panel-reactive antibodies; SAB, single antigen bead; sCD30, soluble CD30; TNF, tumor necrosis factor; UAM, unacceptable antigen mismatches; vPRA, virtual PRA.

Of note, the first cohort consisted of 80 highly-sensitized patients all with complement-dependent cytotoxicity panel-reactive antibodies (CDC-PRA) above 85%, 20% of whom were CDC-crossmatch (CDC-CM) positive prior to an intensive desensitization regimen including plasmapheresis and rituximab (17). The second cohort consisted of 385 at least moderately sensitized patients as indicated by either CDC-PRA positivity or ELISA-reactive anti-HLA antibodies. Induction treatment was variable with 11% receiving T-cell depletion and 53% receiving no induction regimen at all. Data on ABMR were not reported (11, 18).

Given the high immunological risk of the hitherto reported cohorts and their variable induction regimens, we asked whether a combination of preformed DSA and elevated sCD30 levels would also be predictive of early ABMR and accelerated graft loss in a homogenous group of intermediate-risk kidney transplant patients all treated with the same non-depleting induction regimen and tacrolimus-based maintenance immunosuppression. These patients had been transplanted prior to the clinical use of the SAB assay and pre-transplant risk stratification was solely based on a negative CDC-CM.

PATIENTS AND METHODS

Study Population

From all patients that received a living or deceased kidney transplant at our institution between January 2005 and December 2015 ($n = 686$), we retrospectively selected all those treated with an anti-IL2-receptor-based induction therapy (basiliximab, Simulect®, Roche, Basel, Switzerland) followed by a maintenance regimen consisting of a calcineurin-inhibitor, mycophenolate-mofetil and prednisolone ($n = 287$, **Supplementary Table 1**). Patients that simultaneously received multiple organs or had received an organ other than a kidney previously were excluded, as were ABO-incompatible living donor kidney transplantations. Kidney-only recipients treated without any induction therapy, depleting-antibody induction, i.e., anti-thymocyte globulins (ATG), or an mTOR-inhibitor-based maintenance regimen, were excluded as well as patients for whom no serum sample was available prior to transplantation ($n = 8$). During the study period, all recipients of a living donor transplant received basiliximab induction. For deceased donor transplantations, induction therapy was determined on an individual basis with no predefined criteria. All patients were transplanted with a negative CDC-CM using current sera. Donor and recipient characteristics as well as clinical data were obtained by careful chart review or were extracted from the Eurotransplant Network Information System (K_X_008). All retrospective analyses were performed with approval of the local Institutional Review Board.

Detection and Definition of DSA and Donor HLA Typing

Sera taken at the time of kidney transplantation were retrospectively screened for the presence of anti-HLA class I and class II IgG antibodies. Sera from patients with preformed DSA were additionally screened for the presence of DSA at day 14 post-transplantation. All sera were stored at −80°C and heat

inactivated at 52°C for 20 min prior to analysis. Screening was done using a commercial solid-phase microsphere-based assay (LSM12, One Lambda Inc., Los Angeles, CA, USA). Sera were analyzed on a LABScan 100 Luminex® (Luminex Corp., Austin, TX, USA) flow analyzer, applying a threshold ratio for positive results of 2.5. In positive sera, HLA specificity was determined by a single antigen assay for HLA class I and / or HLA class II antigens (LABScreen® Single Antigen, Class I or II, respectively, both One Lambda Inc.). The tests were performed according to the manufacturers' instructions, applying a baseline-adjusted MFI cut-off for positive reactions of 1,000. Donor-specificity of anti-HLA antibodies was defined based on the available donor HLA typing data. Donor HLA-typing was performed according to standard Eurotransplant protocols. Typing for HLA-A, B and DR was done for all donors. HLA Cw and DQ typing data were available for 95 (32.2%) and 275 (93.2%) donors, respectively. DP typing was not routinely performed and therefore, anti-DP HLA-antibodies were not evaluated for donor-specificity. If donor-specificity of anti-HLA antibodies could not be determined due to lack of high resolution typing of a donor, they were classified as non-DSA. This occurred in five recipients for HLA class I and in 14 patients for HLA class II antibodies, respectively. However, lack of high resolution typing in the corresponding donors resulted in no potential misclassification with respect to pre-transplant DSA status (yes/no). In case Luminex analysis revealed the presence of antibodies for all different splits of an HLA antigen, the bead with the highest MFI was used for MFI categorization. To categorize patients into DSA positive or negative, both a lower MFI threshold of 1,000 and 5,000 were applied as previously published (11, 19). In patients with more than one DSA, the one with the highest MFI (MFI^{max}) was used for categorization.

Measurement of SCD30

Pre-transplant sera were tested for sCD30 using the ELISA kit of eBioscience (San Diego, USA). Based on previous results, a value of 80 ng/ml was used as the most suitable cut-off for sCD30 testing (18).

Diagnosis and Treatment of Rejection

All rejection episodes were biopsy-proven. Biopsies were obtained either as protocol biopsies on days 14 and 90 post-transplantation or when clinically indicated. At the time of biopsy, specimens were evaluated according to the most recent Banff classification. For the current study, biopsies from DSA-positive patients were re-evaluated by an experienced nephropathologist (MB-H). Immunohistochemical staining for C4d was complemented when no C4d staining was performed at the time of biopsy and all biopsies were re-classified according to the BANFF 2019 classification (20). Subclinical borderline rejections were not treated. Both clinical and subclinical TCMR were treated with steroid pulses. In case of vascular or steroid-resistant TCMR, anti-thymocyte globulins were given. Any combination of steroids with plasmapherese, intravenous immunoglobulins and/or rituximab was considered adequate therapy for ABMR.

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). Survival analyses were performed by the Kaplan-Meier method and differences between groups compared using the log-rank test. Differences in baseline characteristics were analyzed by using the chi-square test (Fisher's exact when appropriate), Mann-Whitney U- or the Kruskall Wallis test. A $p < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics

64/287 patients (22.3%) had preformed DSA. DSA-positive patients were more likely to be female, more often underwent retransplantation and were more likely to receive a deceased-donor transplant compared to DSA-negative patients. The proportion of patients with elevated sCD30 levels was comparable between DSA-positive (39.1%) and DSA-negative (38.1%) patients (**Supplementary Table 1**). We next categorized patients according to pre-transplant DSA- and sCD30-status (DSA−/sCD30−, DSA−/sCD30+, DSA+/sCD30−, DSA+/sCD30+, **Table 1**). In DSA-positive patients, median MFI of the DSA with the highest MFI (MFI^{max}) was comparable between sCD30-positive (5,528, range 1,129–20,379) and sCD30-negative (5,168, 1,051–21,994) patients. Also, sCD30 concentrations were comparable in the two sCD30-positive groups. Median follow-up was 7.4 years (range 0–15.7) with no significant differences between the groups (**Table 1**).

Allograft Survival

Death-censored allograft survival was significantly lower in DSA-positive as compared to DSA-negative patients (10-year allograft survival 62.0 ± 7.3% vs. 85.9 ± 3.0%, $p < 0.001$; **Supplementary Figure 1**). When sCD30 was included into risk stratification, both sCD30-positive and sCD30-negative patients with preformed DSA had a significantly higher incidence of graft failure during follow-up as compared to DSA-negative patients (**Figure 1**). Of note, there was a trend toward accelerated graft failure in sCD30-positive as compared to sCD30-negative patients with preformed DSA (3-year allograft survival 83.3 ± 7.6 vs. 94.7 ± 3.6%, $p = 0.177$). Stratification of DSA-positivity by an MFI cutoff of 5,000 (DSA^{high}) revealed that sCD30-positive DSA^{high} patients had the worst 3-year allograft survival (75.0 ± 12.5%), whereas sCD30-negative DSA^{high} patients had a 3-year allograft survival comparable to patients without DSA (95.0 ± 4.9 vs. 96.7 ± 1.4 and 96.7 ± 1.9%, respectively, **Figure 2**). Irrespective of the MFI cutoff applied (1,000 vs. 5,000), however, graft loss was only delayed in DSA-positive sCD30-negative patients, resulting in a significantly reduced allograft survival compared to the DSA-negative patient groups during follow up (10-year allograft survival 64.0 ± 8.9 vs. 82.7 ± 5.5 and 87.7 ± 3.6%, respectively, **Figure 1**).

Incidence of Early ABMR

We hypothesized that the higher incidence of accelerated graft loss seen in sCD30-positive DSA-positive patients was due to a higher incidence of early rejection episodes, most notably

TABLE 1 | Baseline characteristics ($n = 287$).

	DSA−/sCD30− ($n = 138$)	DSA+/sCD30− ($n = 39$)	DSA−/sCD30+ ($n = 85$)	DSA+/sCD30+ ($n = 25$)	*p*-value
Donor					
Age, median (range)	57 (3–79)	56 (22–81)	56 (17–82)	52 (17–70)	0.580[#]
Female sex, *n* (%)	78 (56.5)	17 (43.6)	43 (50.6)	10 (40.0)	0.293[§]
Deceased donors, *n* (%)	78 (56.5)	29 (74.4)	42 (49.4)	19 (76.0)	0.016[§]
Recipient					
Age, median (range)	54 (19–74)	55,5 (31–73)	50 (17–78)	54 (18–73)	0.082[#]
Female sex, *n* (%)	34 (24.6)	17 (43.6)	33 (38.8)	11 (44.0)	0.029[§]
>1 KTX, *n* (%)	10 (7.2)	18 (46.2)	8 (9.4)	14 (56.0)	<0.001[§]
CDC-PRA <5, 5–84, >85 (%)					
Current	92.8/7.2/0	41/59/0	95.2/4.8/0	54.2/41.6/4.2	<0.001[§]
Highest	86.2/12.3/1.4	30.8/66.6/2.6	88.1/11.9/0	54.2/37.5/8.3	<0.001[§]
Number of HLA mismatches (A, B, DR), *n* (%)					0.177[§]
0	17 (12.3)	2 (5.1)	12 (14.1)	0 (0)	
1–2	35 (25.4)	8 (20.5)	22 (25.9)	10 (40.0)	
3–4	61 (44.2)	18 (46.2)	34 (40)	14 (56.0)	
5–6	25 (18.1)	11 (28.2)	17 (20)	1 (4.0)	
MFI^{max} median (range)	-	5,168 (1,051–21,994)	-	5,528 (1,129–20,379)	
sCD30 (U/ml), median (range)	-	-	99 (80–314)	100 (81–403)	
Follow-up (years) median (range)	7.6 (0–15.7)	8 (0–13.2)	6.8 (0–15.4)	6.85 (0.1–13.1)	0.298[#]

KTX, kidney transplantation; CDC-PRA, complement-dependent cytotoxicity panel reactive antibodies; MFI^{max}, mean fluorescence intensity value of the DSA with the highest MFI. P-values were obtained by [#]Kruskal-Wallis or [§]Chi-square testing. There were no statistically significant differences between the DSA+/sCD30− and the DSA+/sCD30+-group.

ABMR. At the time of biopsy, however, a higher incidence of both T cell-mediated rejection (TCMR) and ABMR was noted in sCD30-negative as compared to sCD30-positive patients with preformed DSA within the first year (**Table 2**). As our study cohort comprised patients biopsied between 2005 and 2015 with a much higher awareness of ABMR reflected in the more recent Banff classifications, all kidney biopsies from DSA-positive patients were reevaluated and graded according to the Banff 2019 classification (20). This resulted in a much higher and comparable incidence of early ABMR in both groups (41.0 vs. 41.7%, **Table 2**). There was no statistically significant difference in the incidence of C4d-positive ABMR (37.5 vs. 20%, $p = 0.41$) or moderate microvascular injury (g+ptc $\geq$ 2) between the groups (75 vs. 100%, $p = 1.00$). Finally, we observed a trend toward more mixed rejections (50 vs. 20%, $p = 0.218$) and ABMR with v-lesions in sCD30-negative as compared to sCD30-positive patients (43.8 vs. 20%, $p = 0.229$).

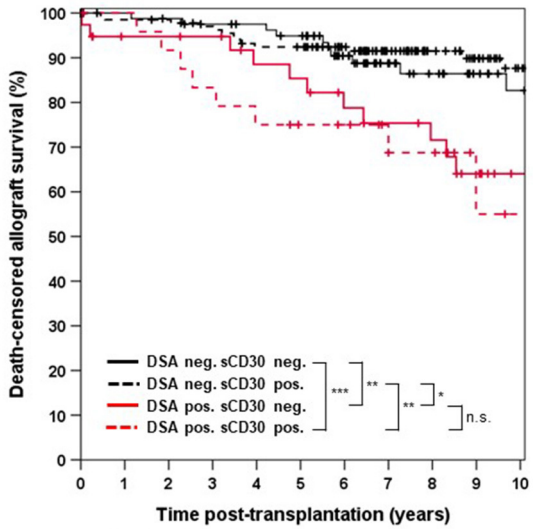
Early Loss of DSA and Outcome

We next asked whether elevated sCD30 levels as a surrogate marker of a preactivated immune system would be associated with a higher incidence of early DSA persistence. DSA-positive patients who lost their DSA as early as 14 days post-transplantation had very good outcomes, whereas persistence of DSA was associated with a significantly higher risk for impaired graft survival (10-year allograft survival 81.7 vs. 53.1%, $p = 0.049$, **Figure 3**). Of note, patients with persistent DSA had significantly higher MFI^{max} prior to transplantation compared to those patients in whom DSA were undetectable at day 14 (median 9,060 vs. 1,998, $p < 0.001$). However, the proportion of patients with persistent DSA 14 days post-transplantation was comparable between sCD30-positive (60%) and sCD30-negative (74.4%) patients (**Table 3**). Likewise, graft survival was not statistically different between these groups (**Figure 4**).

DISCUSSION

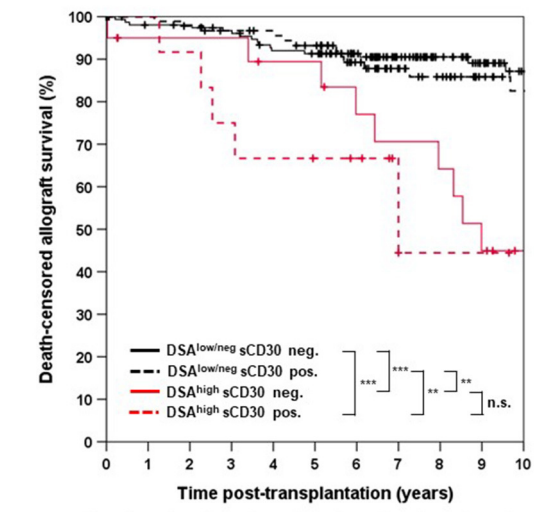
In this cohort of CDC-crossmatch negative patients with preformed DSA treated with a non-depleting induction regimen, both the incidence of early ABMR and long-term allograft survival were comparable in DSA-positive patients irrespective of the sCD30 status. Moreover, sCD30 status had no impact on the early dynamics of DSA post-transplantation.

In a previous study, Schaefer et al. described the incidence of ABMR and graft loss in a cohort of 80 highly sensitized patients with CDC-PRA > 85% that underwent desensitization prior to kidney transplantation (17). Detection of both DSA and high sCD30 levels prior to transplantation ($n = 18$) was associated with an increased risk for graft loss within 3 years following ABMR compared to the 43 DSA-positive but sCD30-negative patients (22 vs. 5%) (17). In our study, 2/16 (12.5%) sCD30-negative DSA-positive patients lost their graft within 3 years



DSA-/sCD30-	138	131	130	126	119	118	103	82	60	49	37
DSA-/sCD30+	85	81	79	76	74	71	56	41	34	27	22
DSA+/sCD30-	39	34	34	33	29	28	24	21	19	15	9
DSA+/sCD30+	25	24	22	20	18	16	15	12	10	4	3

FIGURE 1 | Death-censored allograft survival stratified by DSA and sCD30 status prior to transplantation. ***$p < 0.001$, **$p = 0.001$, *$p = 0.014$, and n.s. = non-significant.



DSA^{high}-/sCD30-	158	149	148	143	134	132	116	94	71	57	42
DSA^{high}-/sCD30+	96	91	88	85	82	78	63	48	41	30	25
DSA^{high}+/sCD30-	21	18	18	18	16	16	13	11	10	7	4
DSA^{high}+/sCD30+	12	12	11	9	8	7	6	3	1	1	0

FIGURE 2 | Death-censored allograft survival stratified by DSA^{high} and sCD30 status. DSA^{high} were defined as DSA with an MFI $\geq$ 5,000. DSA$^{low/neg}$ indicates either no DSA or DSA with MFI between 500 and 5,000. ***$p <$ 0.001, **$p < 0.01$, and n.s. = non-significant.

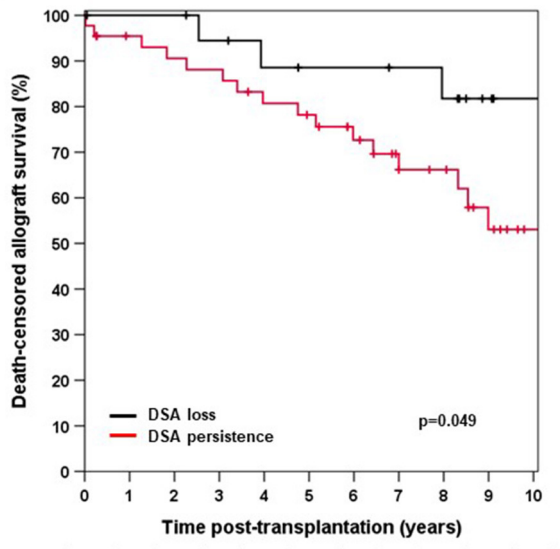
TABLE 2 | First year biopsy findings and treatment in DSA-positive patients.

	DSA+/sCD30− ($n = 39$)	DSA+/sCD30+ ($n = 24$)*
Indication biopsy, *n* (% of all biopsies)	21 (53.8)	15 (62.5)
Type of rejection at the time of biopsy		
No rejection, *n* (%)	19 (48.7)	19 (80.0)
Borderline, *n* (%)	3 (7.7)	0 (0.0)
TCMR, *n* (%)	8 (20.5)	3 (12.0)
ABMR, *n* (%)	9 (23.1)	2 (8.0)
Received ABMR therapy, *n* (%)	7 (77.8)	1 (50.0)
Reclassification according to Banff 2019		
No rejection, *n* (%)	14 (35.9)	11 (45.8)
Borderline, *n* (%)	6 (15.4)	1 (4.2)
TCMR, *n* (%)	3 (7.7)	2 (8.3)
ABMR, *n* (%)	16 (41.0)	10 (41.7)
C4d-positive ABMR, *n* (%)	6 (37.5)	2 (20.0)
Combined rejection, *n* (%)	8 (50.0)	2 (20.0)
ABMR with v $\geq$ 1, *n* (%)	7 (43.8)	2 (20.0)
Time until ABMR (days) median (range)	8 (1–208)	7 (3–27)
Indication biopsy, *n* (% of biopsies with ABMR)	13 (81.3)	8 (80.0)
Received ABMR therapy, *n* (%)	7 (43.8)	3 (30.0)
Received ABMR therapy but lost graft, *n*/all graft losses following early ABMR (%)	5/10 (50.0)	3/7 (42.9)

**1/25 patients did not receive a biopsy. There were no statistically significant differences between the groups.*

following ABMR as compared to 2/4 (50%) double-positive patients. Long-term follow-up of our study cohort, however, revealed a comparable incidence of graft loss following ABMR in both patient groups [7/10 (70%) in sCD30+DSA+ vs. 10/16 (62.5%) in sCD30-DSA+]. In our study, neither desensitization nor depleting induction treatment was performed. Consistent with other studies that re-evaluated histopathology specimens retrospectively (21), a considerable number of ABMR episodes had been missed at the time of biopsy (**Table 2**). Therefore, it is possible that in the absence of T cell help, reflected by low levels of sCD30, highly sensitized DSA-positive patients are particularly sensitive to both desensitization and/or rejection treatment.

In a second study on 385 sensitized (CDC-PRA- or anti-HLA ELISA-positive) patients that were transplanted between 1996 and 2011 without prior desensitization, Süsal et al. also found that pre-transplant DSA only carry an increased risk for graft loss within 5 years post-transplantation in the presence of high sCD30 levels. Data on the incidence of ABMR, however, were not reported (11, 18). 10-year follow-up revealed lower graft survival rates in all groups compared to our study even in the absence of both DSA and increased sCD30 levels (C. Süsal, data not shown), suggesting that the overall immunological risk was higher compared to our study population. Of note, all patients in the aforementioned studies received deceased-donor



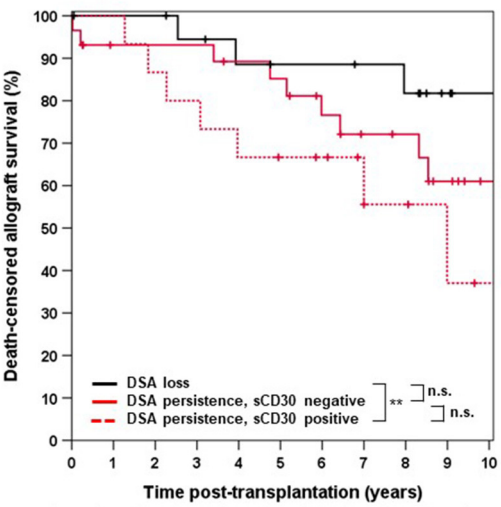
Loss	20	19	19	17	15	14	14	13	12	8	6
Persistence	44	39	37	36	32	30	25	20	17	11	6

FIGURE 3 | Death-censored allograft survival in DSA-positive patients in whom DSA were undetectable 14 days post-transplant (loss) or who had persistence of DSA (persistence).

TABLE 3 | DSA-status 14 days post-transplantation.

	DSA+/sCD30− (*n* = 39)	DSA+/sCD30+ (*n* = 25)	*p*-value
DSA persistence, *n* (%)	29 (74.4)	15 (60.0)	0.275[#]
DSA persistence in patients with ABMR, *n* (% of ABMR-pos. patients)	13 (81.3)	8 (100)	0.526[#]
Pre-transplant MFI^{max} of patients with DSA persistence, median (range)	7,478 (1,051–21,994)	10,178 (1,671–20,379)	0.240[§]
Pre-transplant MFI^{max} of patients with undetectable DSA at day 14, median (range)	1,664 (1,071–12,640)	2,037 (1,129–6,934)	0.395[§]

P-values were obtained by [#]chi-squared or [§]Mann-Whitney-U test.



DSA loss	20	19	19	17	15	14	14	13	12	8	6
DSA persistence, sCD30 negative	29	24	24	24	22	21	17	14	13	9	5
DSA persistence, sCD30 positive	15	15	13	12	10	9	8	6	4	2	1

FIGURE 4 | Death-censored allograft survival in DSA-positive patients in whom no DSA were detectable 14 days post-transplant irrespective of sCD30 status (loss) or who were either sCD30-positive (sCD30+) or sCD30-negative ($sCD30^{-}$) prior to transplantation and had persistence of DSA 14 days post-KTX. $^{**}p = 0.008$ and n.s. = non-significant.

transplants. When we excluded living donor transplantations, we still did not observe significant differences in ABMR or outcome in DSA-positive patients with vs. without elevated sCD30 levels (**Supplementary Figure 2**). Likewise, when we analyzed overall graft loss not censored for death as reported in the study by Süsal et al. the differences between the groups remained unchanged (**Supplementary Figure 3**). Therefore, besides an increased immunological risk, differences in immunosuppression and post-transplant management between the two cohorts might explain some of the differences observed. Of note, our cohort and the one studied by Süsal et al. were not tested for the occurrence of de novo DSA or sCD30 levels post-transplantation. It would be interesting to find out whether increased levels of sCD30 either prior to or early after transplantation are predictive for the development of *de novo* DSA as an additional risk factor for a reduced allograft survival (22).

Our study confirms our previous results that disappearance of DSA as early as 14 days post-transplant is associated with very good outcomes (23). Most studies on DSA persistence reported data between 3 months (24) and 1 year (25, 26) post-transplant. Therefore, our data are very important, as early adaptation of immunosuppression and close monitoring of patients with DSA persistence might improve outcome.

Our study has several limitations. Besides the small number of patients and the inherent limitations of a single-center design, donor HLA typing was incomplete (as it has been for long in the Eurotransplant kidney allocation system) and could not be completed as donor DNA was not available to us. However, as outlined in the methods section, binary assignment of donor-specificity was not affected by the incomplete HLA typing. In addition, there were no predefined criteria for the use of basiliximab induction during the study period and CDC-PRA-positivity might have been a selection criterion in some cases. However, as the key advantage of the SAB assay is the ability to detect the specific sensitization against non-self HLA as compared to the CDC-PRA (27), stratification of our cohort by

CDC-PRA and sCD30 status was not superior to a DSA-based approach (**Supplementary Figure 4**).

Our study has several strengths. First, both Luminex and ELISA analyses were performed retrospectively, so that risk stratification and treatment strategies were not influenced by DSA- and sCD30 status. Therefore, the prognostic value of these two biomarkers could be analyzed without the interference of desensitization or depleting induction therapy. Second, all analyses were performed from the same day of transplant serum and with the same reagents, reducing assay variability to a minimum. Third, follow-up was longer than in previous studies allowing for the detection of late allograft losses. Finally, meticulous re-analysis of histopathology according to the Banff 2019 classification revealed a comparable incidence of ABMR in DSA-positive patients irrespective of the sCD30 status, which further supports our outcome data.

In sum, in our cohort, preformed DSA were associated with an increased risk for ABMR and long-term graft loss independent of sCD30 levels in intermediate-risk kidney transplant patients. Therefore, determination of sCD30 in addition to SAB-determined DSA does not improve risk stratification prior to kidney transplantation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Regensburg University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TD analyzed data and wrote the manuscript. CB, ML, and BS performed Luminex analyses and data interpretation. KU and MB-H performed histological analyses of kidney biopsies. BB gave conceptual advice and contributed to the writing of the manuscript. DZ designed the study, analyzed data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.778864/full#supplementary-material

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Influence of Persistent Inflammation in Follow-Up Biopsies After Antibody-Mediated Rejection in Kidney Transplantation

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Background: Despite recent advances in immunosuppression treatment, antibody-mediated rejection (ABMR) remains the leading cause of kidney graft loss. Information about prognostic markers and the efficacy of treatment is scarce.

Methods: Retrospective study with kidney recipients diagnosed an active ABMR from January 1, 2004 to December 31, 2019 to explore the influence of persistent inflammation in follow-up biopsies on graft survival after ABMR treatment.

Results: About 116 patients were included. Active ABMR were treated with a combination of plasma exchange (PE), intravenous immunoglobulin (IVIg), rituximab, and steroids. At 6 months of treatment, 63 (54.3%) patients presented a stabilization or improvement in kidney-graft function. The effectiveness varied depending on the timepoint of the presentation between transplantation and rejection, which is lower for those with late ABMR (63 vs. 21% for early vs. late ABMR, respectively). Ninety patients (77%) underwent a control biopsy after ABMR treatment, from which 46 (51%) responded to the treatment. Microvascular inflammation (MVI) persisted in 64 (71%) biopsies, whereas tubulitis persisted in 17 (19%) biopsies. Death-censored graft survival at 1 year was significantly lower in patients with persistent MVI (86% vs. 95% without persistent MVI, $P = 0.002$), or with persistent tubulitis (44% vs. 66% without tubulitis, $P = 0.02$). In the Cox Regression analysis, the persistence of MVI [hazard ratio (HR), 4.50 (95%CI, 1.35–14.96), $P = 0.01$] and tubulitis [HR 2.88 95%CI (1.24–6.69), $P = 0.01$) in follow-up biopsies significantly increased the risk of graft failure.

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Conclusion: Persistent inflammation in follow-up biopsies after ABMR treatment was associated with an increased risk of graft loss, even without meeting Banff rejection criteria.

Study Registration: Agencia Española de Medicamentos y Productos Sanitarios (AEMPS): 14566/RG 24161. Study code: UTRINM-2017-01.

Keywords: kidney transplantation, antibody-mediated rejection, graft failure, follow-up biopsy, microvascular inflammation

INTRODUCTION

Along with the improvement of immunosuppression strategies, antibody-mediated rejection (ABMR), especially chronic active ABMR, has been increasing as the leading cause of late kidney graft failure (1, 2). Also, ABMR has been linked with worse patient survival (3–5). However, despite the clinical relevance of ABMR, there is no specific treatment for ABMR approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Plasma exchange (PE), intravenous immunoglobulin (IVIg), and corticosteroids constitute the most common strategy for ABMR treatment and are considered the standard of care for many kidney transplant societies. Also, rituximab is widely used as off-label to prevent and treat ABMR without any clear evidence of efficacy (6–9). Available information about its effectiveness and treatment complications is scarce; this makes it difficult to make decisions, especially when reassessing a kidney recipient after ABMR treatment. In this sense, the information derived from follow-up biopsies after ABMR treatment could be potentially useful when assessing ABMR prognosis.

Herein, we analyze the impact of PE, IVIg, steroids, and rituximab treatment after ABMR on kidney graft and we revise the impact of this treatment through follow-up biopsies in a cohort of patients after ABMR treatment to determine a prognostic marker of response, focusing on histological inflammation.

MATERIALS AND METHODS

Study Design and Patient Population

We performed a longitudinal single-center retrospective study, which included kidney recipients diagnosed with ABMR, according to the Banff 2017 classification. Concretely, we have identified kidney recipients who received a treatment for ABMR from January 1, 2004 to December 31, 2019 (including a combination of PE, IVIg, and rituximab) in the database of Renal Transplant Unit at Hospital Clinic de Barcelona; then biopsies at ABMR diagnosis were reanalyzed according to the criteria specified by Banff (2017). Recipients who received a multivisceral transplant, and those with transplant glomerulopathy (TG) in the initial biopsy, cg $\geq$ 1 in the Banff histopathological classification, were excluded (10).

Demographic, clinical, biochemical, histopathological, and immunological data were evaluated for both the donor and the recipient. Clinical characteristics, maintenance immunosuppression, and ABMR treatment were analyzed at the diagnosis and follow-up period. Charlson comorbidity index (CCI) was also assessed at ABMR diagnosis (11).

Infections that required hospitalization at least 48 h within the first year after ABMR diagnosis were recorded and described in relation to clinical variables.

The study was performed according to the Declaration of Helsinki principles and approved by the Hospital Research Ethics Committee. Study registration: Agencia Española de Medicamentos y Productos Sanitarios (AEMPS): 14566/RG 24161. Study code: UTRINM-2017-01.

Antibody-Mediated Rejection Diagnosis

The decision to perform a renal biopsy and patient treatment was based on the clinical judgment at ABMR diagnosis, including biopsies due to impaired renal function and protocol biopsies (at 3 or 12 months after kidney transplantation). Active ABMR was diagnosed and categorized according to the Banff criteria of 2017 (10). The day when the biopsy was diagnosed was considered as the date of active ABMR diagnosis. Immunologically, donor-specific antibodies (DSAs) were tested using Single Antigen Bead Test (LIFECODES® Single Antigen, Immucor, Georgia, US). In our Center, the Single Antigen Beads Test has been used since 2001. However, the criteria to consider an allele positive (the MFI over 1,500 and 4 times higher than the Lowest Reactive Antigen of the same locus) changed in 2017. Since 2017, the criterion is that the MFI is >750 and the cut-off for the ratio [MFI/LRA (Lowest Ranked Antigen)] is specific for each individual bead (12).

Definition of Outcomes

Our primary outcome was death-censored kidney graft survival at 1 year after ABMR diagnosis and at the follow-up period, between patients with and without persistent inflammation in the control biopsy, either microvascular (MVI, defined as g + ptc $\geq$ 1) or tubular ($t \geq 1$). Follow-up biopsies after ABMR treatment

Abbreviations: ABMR, antibody-mediated rejection; aABMR, acute antibody-mediated rejection; AEMPS, Agencia Española de Medicamentos y Productos Sanitarios; ATN, acute tubular necrosis; CCI, Charlson comorbidity index; CI, confidence interval; DSAs, donor-specific antibodies; eGFR, estimated glomerular filtration rate; EMA, European Medicines Agency; FDA, Food and Drug Administration; HLA, human leukocyte antigen; HR, hazard ratio; IFTA, interstitial fibrosis and tubular atrophy; IQR, interquartile range; IVIg, intravenous immunoglobulin; MVI, microvascular inflammation; OR, odds ratio; PE, plasma exchange; SCr, Serum Creatinine; TCMR, T-cell mediated rejection; TG, transplant glomerulopathy; TMA, Thrombotic microangiopathy,; UPCR, urine protein to creatinine ratio.

were performed according to the physician's criteria. Secondary outcomes were defined as kidney graft function at 6 months from the ABMR treatment and kidney graft function at the last follow-up. Kidney graft function was assessed by serum creatinine (SCr), estimated glomerular filtration rate (eGFR, according to the Chronic Kidney Disease Epidemiology Collaboration equation), and urine protein to creatinine ratio (UPCR) (13, 14). Early ABMR was defined as that which occurred within 6 months from the kidney transplant, while late ABMR was defined as that occurring after 6 months from the kidney transplant (15).

Patient survival was defined as the last day of follow-up or the date of death. Kidney graft failure was defined as one of the following: return to dialysis or re-transplantation. Response to ABMR treatment was defined as improvement or stabilization of eGFR at 6 months compared to eGFR at ABMR diagnosis.

PE was performed in Cobe Spectra or Spectra Optia separators (Terumo BCT, Lakewood, CO, USA) using 5% albumin (Albutein® 5%, Grífols, Spain) as a replacement solution. One plasma volume was exchanged in each session (16).

Statistical Analysis

Data are presented as mean (SD) for parametric variables, and median [interquartile range (IQR)] for the non-parametric ones. The corresponding tests used were the *t*-test, McNemar Test, Wilcoxon test, Chi-Square, and ANOVA as appropriate.

Kaplan–Meier was used to estimate graft survival and compared using the log-rank test. For the survival analysis throughout the follow-up, we used the Cox regression model, and the logistic regression model was used for the 1-year survival analysis using IBM SPSS Statistics 26.0 (SPSS, Inc; Chicago, Illinois) software for Windows. All the tests were two-tailed, and the significance level was defined as a P-value < 0.05.

RESULTS

Characteristics of Baseline Donor and Recipient

From January 2004 to December 2019, 116 kidney transplant recipients were diagnosed with an active ABMR (**Figure 1**). In 97 patients (83.6%), the biopsy was performed by indication due to impaired renal function, while the other 19 patients (16.4%) underwent a protocol biopsy at 3 or 12 months after transplantation. **Table 1** summarizes demographical and clinical data at ABMR diagnosis. Most of them were men (59.5%), with a mean age at the rejection of 50.8 ± 14 years, and a median follow-up from ABMR diagnosis of 33.5 [62.7] months. The median duration of dialysis was 4 [4] years. About 76% of the donors were deceased donors. Up to 48% of patients had received a previous kidney transplant, and 14% presented a positive cytometry crossmatch at transplantation.

Regarding the characteristics of rejection (**Table 1**), the median time from transplantation to active ABMR was 27.5 [287.7] days, with 70.7% (82) of the ABMR episodes diagnosed within 6 months after transplantation and which were considered as early ABMR [median of 16 (21.5) days from transplant]. Twenty-seven of the patients required dialysis after the diagnosis of ABMR.

Response to Treatment and Survival

All patients were treated for ABMR with a combination of corticosteroids, PE, IVIg, and rituximab. The active ABMR treatment protocol consists of a combination of five sessions of PE, IVIG 200 mg/kg every 2 PE, and two rituximab doses. However, in this cohort, 15 patients (12.9%) were not treated with rituximab by concerns of the treating physician because of a perception of increased risk of infections. Also, five patients (4.3%) did not receive PE treatment for problems related to vascular access. One plasma volume was exchanged in each session with a median of 5 [1] sessions.

The global response to ABMR treatment was 54.3%, with a significant increase in eGFR at 6 months after treatment and at the end of the follow-up period ($P = 0.003$). **Table 2** summarizes the changes in eGFR. Patients with an early ABMR [median 16 (21.5) days] have a better response than those with a late ABMR [median 25.9 (40) months], 67 vs. 23.5% for early and late ABMR, respectively, odds ratio (OR) 0.15 [95% CI 0.06–0.38], $P < 0.001$. Overall graft failure at 1 year and throughout the follow-up was 32.8 and 38.8%, respectively. Death-censored graft failure for the same time points was 25.9 and 31%, respectively. The presence of DSA at diagnosis was not associated with worse graft survival ($P = 0.15$).

The treating physicians decided follow-up biopsy after ABMR treatment in 90 patients. Demographic, clinical, biochemical and immunological characteristics from patients with and without follow-up biopsy have shown in **Supplementary Table 1**, whereas histological parameters in biopsy at ABMR diagnosis are presented in **Supplementary Table 2**. The prevalence of living donors, previous kidney transplants, and human leukocyte antigen (HLA) sensitization were higher in the follow-up of patients who underwent biopsy. There were no significant differences in death-censored graft survival, where $P = 0.31$ between patients with or without follow-up biopsy.

Histopathological findings at ABMR diagnosis and follow-up biopsies are summarized and compared in **Table 3**.

The presence of tubulitis at ABMR diagnosis was associated with an increased risk of graft loss at 1 year (OR 1.79 [95% CI 1.05–3.06], $P = 0.03$) and at follow up [HR 2.10 (95% CI 1.04–4.26), $P = 0.04$]. The combination of interstitial fibrosis and tubular atrophy (IFTA) and the coexistence of a T Cell-mediated rejection (TCMR) were associated with an increased risk of graft loss at follow-up [HR 1.62 (95% 1.09–2.40), $P = 0.02$ and HR 2.48 (95% CI 1.07–5.76), $P = 0.03$ for IFTA and TCMR, respectively; **Table 4A**].

Impact of Persistent Inflammation in Follow-Up Biopsies

A follow-up biopsy after ABMR treatment was performed in 90 patients (77.6%), with a median time from the treatment of 2 [4] months. In general, there was a significant decrease in inflammation and an increase in chronicity parameters between the biopsy at diagnosis and the follow-up biopsy (**Table 3**).

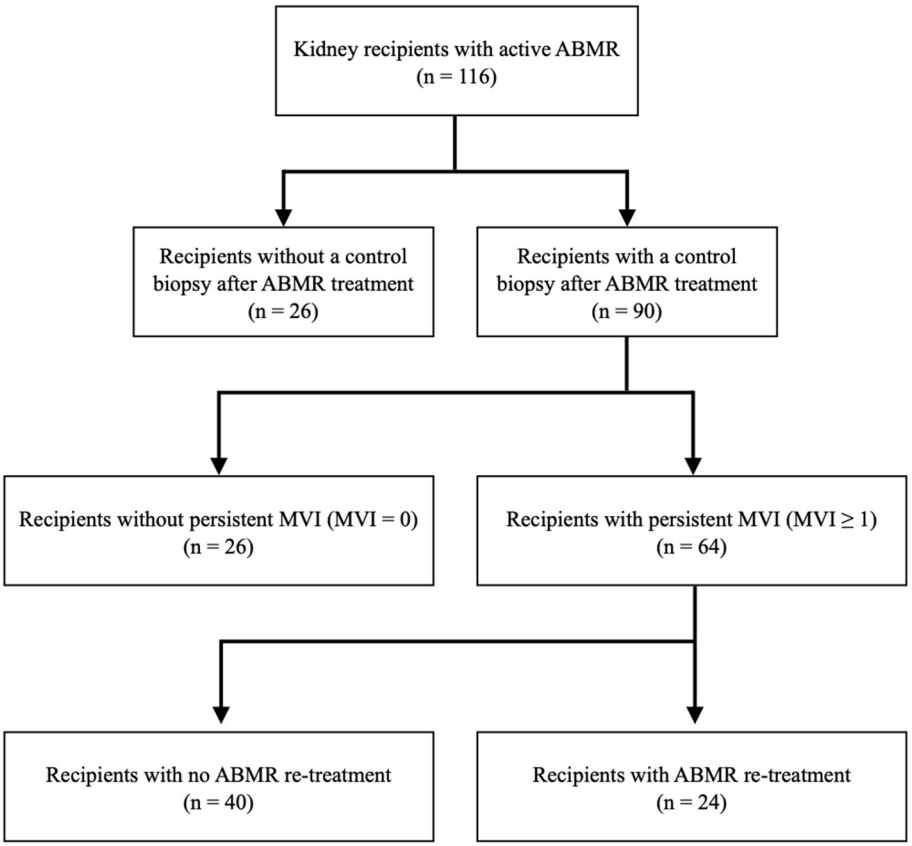

FIGURE 1 | Flowchart of the included patients. ABMR, antibody-mediated rejection; MVI, microvascular inflammation.

Although 46 out of 90 rebiopsied patients (51%) had responded clinically to ABMR treatment, about 64 (71%) follow-up biopsies presented a persistent MVI $\geq$ 1, and in 45 (50%), MVI $\geq$ 2 was detected. Twenty-six out of 46 patients with improvement in graft function had persistence of MVI $\geq$ 1. There were no statistically significant differences in baseline characteristics between patients with and without persistent MVI (**Supplementary Table 3**). There were also no significant histological differences in the biopsy at ABMR diagnosis between patients with and without the persistence of microvascular inflammation, except for a greater severity of MVI in patients with persistent MVI (**Supplementary Table 4**).

The time between kidney transplantation and graft biopsy was similar between patients with and without inflammation persistence in the follow-up biopsies ($P = 0.23$). The presence of DSA or the DSA title was similar between the two groups ($P = 0.09$). The presence of preformed DSA was associated with early rejection ($P = 0.007$; OR 5.1, CI95% 1.4–18.1). However, *de-novo* DSA was not associated with a higher risk of graft loss or persistence of MVI in the follow-up biopsies.

Death-censored kidney graft survival was significantly lower in those patients with persistent MVI (**Figure 2A**; Log-Rank, $P = 0.002$). Patients with glomerulitis (g) or peritubular capillaritis (ptc) had significantly lower death-censored kidney graft survival than patients without g or ptc at follow-up biopsy (**Figures 2B,C**).

In the Cox Regression analysis, the persistence of acute inflammatory lesions in the follow-up biopsies [g, ptc, MVI, tubulitis, acute tubular necrosis (ATN), and thrombotic microangiopathy (TMA)], significantly increased the risk of kidney graft loss throughout the follow-up (**Table 4B**). Also, the presence of TMA and ATN was associated with 1-year graft loss.

Here a multivariate analysis has many statistical limitations due to the number of events. Therefore, a bivariate analysis was performed instead of a multivariate analysis. After the adjusted analysis for the different histological characteristics, the persistence of MVI in the follow-up biopsies remains a risk factor for graft loss (**Supplementary Tables 5**, **6**).

There was no specific retreatment protocol, and therapy was the decision of the treating physicians. Twenty-four out of 64 (37.5%) patients with persistent MVI in follow-up biopsies were retreated with a combination of rituximab (5 patients) and PE (19 patients). Death-censored kidney graft survival was significantly higher for those patients with persistent MVI in control biopsy who were retreated (95 and 73% vs. 75 and 50% at 1 and 5 years after retreatment, respectively, $P = 0.04$; **Figure 3**). In the Cox regression analysis, ABMR retreatment for those patients with persistent MVI was associated

TABLE 1 | Demographic and clinical characteristics at diagnosis of antibody-mediated rejection (ABMR).

Donor	Patients ($n = 116$)
Age (years)	54.67 ± 14.29
Gender (male)	59 (50.9)
DDKT	83 (71.6)
DCD	12 (10.3)
Recipient age (years)	50.8 ± 14
Recipient gender (male)	69 (59.5%)
Dialysis vintage (years)	4 (4)
Dialysis modality	
Preemptive	19 (16.4)
Hemodialysis	88 (75.9)
Peritoneal dialysis	9 (7.7)
Hypertension (yes)	88 (75.9)
DM (yes)	20 (17.2)
Vasculopathy (yes)	19 (16.4)
HCV (yes)	29 (25)
ESKD etiology	
ADPKD	17 (14.7)
Urological	29 (25)
Glomerulonephritis	26 (22.4)
Diabetic nephropathy	12 (10.3)
Nephroangiosclerosis	7 (6)
Unknown	25 (21.6)
Previous KT (any)	56 (48.3)
HLA mismatches	4.1 ± 1.55
ABO incompatibility (yes)	16 (13.8)
Pre-transplant DSAs (any)	30 (25.8)
DSAs at ABMR diagnosis (any)	73 (62.9)
PRA > 50% (yes)	18 (15.5)
CF-CM at KT (positive)	17 (14.65)
Luminex at KT (positive)	
Class I	39 (33.6)
Class II	44 (37.9)
Induction Immunosuppression (yes)	112 (96.6)
Basiliximab	39 (33.6)
Rituximab	11 (9.5)
Thymoglobulin	68 (58.6)
Immunosuppression maintenance	
Tacrolimus	79 (68.1)
mTORi	38(32.8)
Mycophenolate	89 (76.7)
Steroids	97(83.6)
Characteristics of rejection at ABMR diagnosis	
Cellular rejection	16 (13.8)
Time from KT to ABMR (days)	27.5 [287.7]
ABMR < 6 months after KT (yes)	82 (70.7)
Need for dialysis at ABMR (yes)	32 (27.6)
ABMR treatment at diagnosis (yes)	116 (100)
Plasma exchange	111 (95.7)
IVIg	111 (95.7)
Rituximab	101 (87,1)
Corticosteroids	116 (100)

(Continued)

TABLE 1 | Continued

Data are mean ± SD, median [IQR] or n (%), unless otherwise specified. ABMR, Antibody-mediated rejection; KT, kidney transplantation; HCV, hepatitis C virus; DM, Diabetes Mellitus; ESKD, end-stage kidney disease; ADPKD, autosomal dominant polycystic kidney disease; HLA, human leucocyte antigen; DSAs, donor-specific antibodies; PRA, panel reactive antibodies; CF-CM, flow cytometry crossmatch; mTORi, mTOR inhibitors; DDKT, deceased donor kidney transplantation; DCD, donor after cardiac death; IVIg, intravenous immunoglobulin.

TABLE 2 | Creatinine, estimated glomerular filtrate, and proteinuria after ABMR diagnostic.

	At ABMR diagnosis (N =116)	At 6 months ($N = 98$)	At follow up ($N = 63$)	*P* value*
SCr (mg/dL)	3.67 ± 1.97	2.31 ± 1.49	2.3 ± 1.14	<0.001
eGFR (mL/min)	25.21 ± 16.3	37.63 ± 18.9	36.9 ± 20	0.003
Proteinuria (mg/g)	585 [980.5]	482 [1070]	437 [1194.5]	0.69

**Respect to baseline. ABMR, antibody-mediated rejection; SCr, serum creatinine; eGFR, estimated glomerular filtration rate.*

TABLE 3 | Banff histopathological findings at diagnostic and follow-up biopsies.

	At diagnosis ($n = 116$)	Follow-up biopsy ($n = 90$)	*P* value	Resolution/Presence at follow-up (n/n)
g	0.84 ± 0.95	0.88 ± 1.06	0.98	17 /45
ptc	1.43 ± 0.94	0.99 ± 1.01	0.0013	30/52
MVI (g+ptc)	2.27 ± 1.35	1.87 ± 1.78	0.02	17/64
t	0.45 ± 0.82	0.24 ± 0.57	0.03	18/16
i	0.68 ± 0.91	0.46 ± 0.77	0.01	17/24
ti	0.53 ± 0.8	0.53 ± 0.78	0.4	
v	0.2 ± 0.56	0.03 ± 0.23	0.002	
mm	0.09 ± 0.31	0.2 ± 0.56	0.075	
ah	0.31 ± 0.7	0.42 ± 0.72	0.21	
cg	0	0.19 ± 0.47	<0.001	-/14
ci	0.65 ± 0.76	1.07 ± 0.88	<0.001	5/65
ct	0.59 ± 0.73	1.06 ± 0.83	<0.001	6/79
cv	0.61 ± 0.75	0.81± 0.85	0.16	
C4d	95 (82)	65 (56)	<0.001	10/63
IFTA	0.54 ± 0.69	0.81 ± 0.98	0.009	

Results are shown as mean ± SD for quantitative and qualitative variables. Qualitative variables, as C4d, are shown in absolute number and the percentage in brackets (% positive). g, glomerulitis; ptc, peritubular capillaritis; MVI, microvascular inflammation; t, tubulitis; i, interstitial inflammation; ah, arterial hyalinosis; cg, transplant glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; IFTA, interstitial fibrosis + tubular atrophy; cv, vascular fibrous intimal thickening.

with a lower risk of graft loss [HR 0.40 (95% CI 0.16–0.99), $P = 0.048$].

Presence of tubulitis (t) was observed in 19% (17) of follow-up biopsies, from which 13 had t grade ≥ 1 without meeting the

TABLE 4 | Analysis for death-censored graft failure.

(A) Biopsy at ABMR diagnosis ($n = 116$)	At 1 year		At follow-up	
	OR (95% CI)	*P* value	HR (95% CI)	*P* value
Glomerulitis ≥ 1	0.73 (0.39–1.36)	0.31	0.92 (0.64–1.34)	0.68
Peritubular capillaritis ≥ 1	1.84 (1.01–3.35)	0.047	1.76 (0.68–4.56)	0.25
MVI ≥ 1	2.04 (0.54–7.66)	0.29	1.19 (0.93–1.51)	0.16
Tubulitis ≥ 1	1.79 (1.05–3.06)	0.03	2.10 (1.04–4.26)	0.04
Vascular inflammation ≥ 1	0.72 (0.22–2.32)	0.57	0.76 (0.23–2.50)	0.65
TMA ≥ 1	1.27 (0.14–11.6)	0.83	0.82 (0.11–6.02)	0.85
Total inflammation ≥ 1	1.32 (0.46–3.85)	0.6	1.07 (0.66–1.74)	0.78
ATN (yes)	0.49 (0.13- 1.84)	0.29	0.80 (0.38- 1.67)	0.55
Positive C4d (yes)	0.95 (0.25–3.69)	0.94	1.23 (0.42–3.57)	0.71
IFTA (yes)	3.10 (1.00–9.64)	0.05	1.62 (1.09–2.40)	0.02
Positive DSA	0.68 (9.20–2.30)	0.53	0.74 (0.36–1.5)	0.4
Cellular rejection (yes)	1.50 (0.39–6.20)	0.46	2.48 (1.07–5.76)	0.03
(B) Follow-up biopsy ($n = 90$)	**At 1 year**		**Throughout follow-up**	
	OR (95% CI)	*P* value	HR (95% CI)	*P* value
Glomerulitis ≥ 1	1.20 (0.37–3.89)	0.76	2,54 (1.17–5.49)	0.02
Peritubular capilaritis ≥ 1	4.83 (1.00–23.2)	0.049	2.99 (1.27–7.07)	0.01
MVI ≥ 1	1.73 (0.52–5.76)	0.37	4.50 (1.35–15.0)	0.01
Tubulitis ≥ 1	1.85 (0.79–4.31)	0.15	2.88 (1.24–6.69)	0.01
Vascular inflammation ≥ 1	1.09 (0.21–5,60)	0.99	0.85 (0.26–2.83)	0.79
TMA (yes)	11.41 (2.54–51.3)	0.001	3.70 (1.50–9.15)	0.005
Total inflammation ≥ 1	3.14 (0.93–10.6)	0.06	4.56 (1.53–13.6)	0.006
ATN (yes)	8.11 (1.72–38.2)	0.008	4.56 (1.53–13.6)	0.006
Positive C4d (yes)	2.34 (0.48–11.4)	0.29	1.77 (0.60–5.19)	0.29
TG (yes)	4.72 (1.27–17.6)	0.02	2.24 (1.33–3.77)	0.002
IFTA (yes)	1.43 (0.81–2.52)	0.21	1.62 (1.14–2.32)	0.008
Positive DSA (yes)	0.63 (0.16–2.47)	0.75	0.72 (0.32–1.62)	0.43
Cellular rejection (yes)	1.60 (0.22–3.00)	0.63	3.00 (1.06–8.80)	0.039

Univariate analysis. Results are shown as mean ± SD. MVI, microvascular inflammation; TMA, thrombotic microangiopathy; ATN, acute tubular necrosis; TG, transplant glomerulopathy; IFTA, interstitial fibrosis and tubular atrophy; DSA, donor-specific antibodies.

active or chronic TCMR diagnosis criteria. Patients with $t \geq 1$ in follow-up biopsies exhibited worse kidney graft survival rates at 1 year (66 and 44%, $P = 0.02$) (**Figure 4**). Moreover, the persistence of tubulitis in follow-up biopsies significantly increased the risk of kidney graft failure [HR 2.88 (95%CI 1.24–6.69), $P = 0.01$; **Table 4B** and **Supplementary Table 6**].

Chronic Lesions in Follow-Up Biopsies

The presence of TG [HR 2.24 (95% CI 1.33–3.77), $P = 0.002$], and IFTA [HR 1.62 (95% CI 1.14–2.32), $P = 0.008$) in follow-up biopsies were associated with an increased risk of kidney graft failure at follow-up. TG was also associated with an increased risk of 1-year kidney graft loss [OR 4.72 (95% CI 1.27–17.61), $P = 0.02$; **Table 4B**).

Given the different responses to treatment according to the time from transplantation to rejection, we analyzed the histological characteristics associated with graft loss adjusted for time to rejection (early/late) in the biopsy at ABMR diagnosis and in the follow-up biopsy. Results were shown in **Supplementary Table 7**. In the time-adjusted analysis, the presence of tubulitis or concomitant cellular rejection in the biopsy at ABMR diagnosis was associated with graft loss. In the follow-up biopsy, the persistence of MVI> 1, ATN, or TMA and the appearance of chronic lesions (cg, IFTA) were associated with graft loss.

Infectious Complications

During the year after ABMR treatment, 101 infections required hospital admission at least 48 h in 57 patients, which were supposed to be the infection rate of 0.87 infections/treated patient. The presence of the comorbidities of the recipients was associated with an increased risk of infectious complications with admission requirement, with CCI ≥ 4 associated with an increased risk of infections [OR 4.2 (95%CI 1.79–9.81), $P = 0.01$].

The potential association between the total immunosuppression received and infections was analyzed. The following items were not associated with the development of infections: time from transplantation to ABMR treatment ($P =$

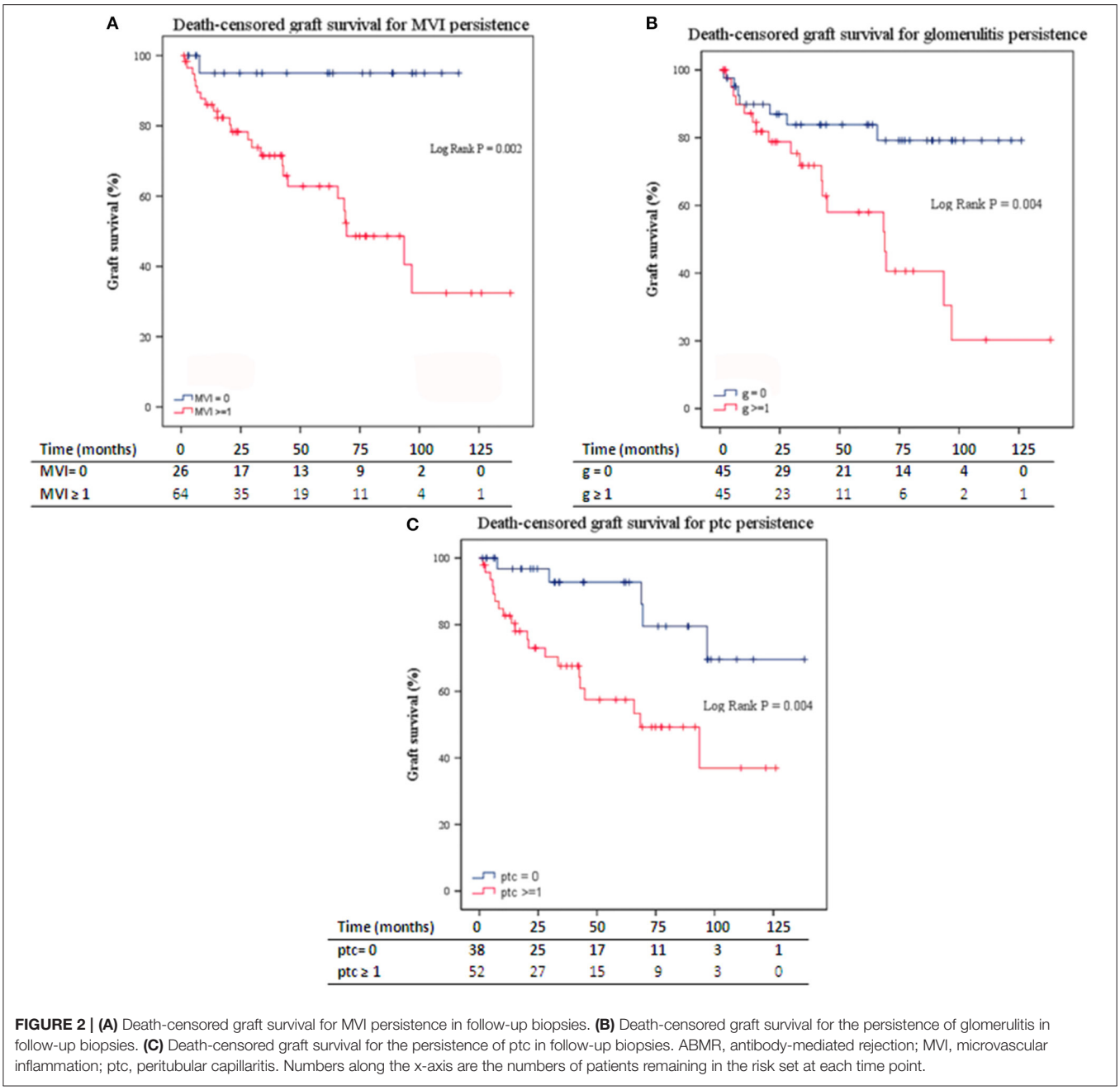

FIGURE 2 | (A) Death-censored graft survival for MVI persistence in follow-up biopsies. **(B)** Death-censored graft survival for the persistence of glomerulitis in follow-up biopsies. **(C)** Death-censored graft survival for the persistence of ptc in follow-up biopsies. ABMR, antibody-mediated rejection; MVI, microvascular inflammation; ptc, peritubular capillaritis. Numbers along the x-axis are the numbers of patients remaining in the risk set at each time point.

0.72), induction with thymoglobulin ($P = 0.31$), induction with rituximab ($P = 0.7$), any previous kidney transplantation ($P = 0.58$), and ABMR re-treatment ($P = 0.31$).

DISCUSSION

We analyzed short- and long-term kidney graft outcomes of recipients with persistent inflammation after active ABMR treatment, comparing them with those without inflammation on follow-up biopsies. In a cohort of 116 kidney recipients diagnosed with an active ABMR, 90 patients underwent a control biopsy after ABMR treatment. We observed persistent inflammation in the follow-up biopsies after ABMR treatment associated with a higher kidney graft failure rate. These findings suggest that persistent inflammation after an active ABMR had a prognostic value in kidney graft outcomes despite not strictly meeting any of the Banff categories. Moreover, they reinforce the importance of follow-up biopsies after ABMR treatment to guide the therapeutic decision-making of transplant physicians.

Active ABMR constitutes one of the most frequent complications in kidney transplantation (1, 4). Nevertheless, despite the therapeutic advances in immunosuppressive treatment during the last years, ABMR continues to be the

most common cause of kidney graft loss (1, 4, 5). At present, the most common strategy adopted by many transplant centers for active ABMR treatment comprises a combination of PE and IVIgs (5, 8, 17), which is considered by the transplant community as the standard of care despite the lack of a solid evidence which supports its usefulness. Also, rituximab is widely used, although there are no solid studies that have evaluated its efficacy (3, 5–7, 18). The degree of effectiveness of the treatment may vary according to the time of the treatment being considered. In this cohort, the current treatment for ABMR has relatively low efficacy, with only 54.3% of patients reaching a stabilization or improvement in kidney graft function of 6 months after treatment. Moreover, the effectiveness markedly varies depending on the timepoint presentation between transplantation and rejection; about 63% of patients with an early ABMR exhibited a significant response, while only 24% of those with a late ABMR responded to ABMR treatment. This observation is consistent with that reported in previous studies (19, 20) and suggests different immunological pathways between both types of acute antibody-mediated rejection (aABMR). Thus, it has been suggested that early rejection is associated with a donor presensitization, an observation that agrees with our results. Moreover, some studies have found an association within the DSA class (I or II) and the aABMR temporality, although these findings remain controversial (19, 20). These results contrast with ours, since we did not find any significant association with early/late aABMR and the DSA class. Nevertheless, it has to be noticed that we only included patients with an aABMR and those with a chronic ABMR were excluded.

Another important issue derived from the most used schemes for ABMR treatment is the severe complications which are derived from these immunosuppression regimens, especially infections and *de novo* neoplasms (1, 4, 5). In this study, we observed a high incidence of infections which required hospital admission for at least 48 h (0.87 infections/treated patient). Importantly, the risk of infection was associated with the comorbidities of the patients having a CCI $\geq$ 4, which is an independent risk factor for new onset of infections. It should be noted that most of the patients received treatment with rituximab, which has been associated with an increased risk of infections in postkidney transplantation, although there are no data from randomized studies which clearly demonstrate the association between rituximab and infections (21–23).

Therefore, since active ABMR treatment is linked to high morbidity and mortality in kidney transplant recipients, clinical, analytical, and histological prognostic markers are essential to identify those patients who will potentially benefit from those intensive immunosuppressive strategies to avoid futile interventions associated with a high rate of complications and poor benefit (3–5). In this sense, some studies have tried to validate scores based on a combination of clinical and histological variables both at the diagnosis and in the subsequent patient reassessment after rejection treatment (5).

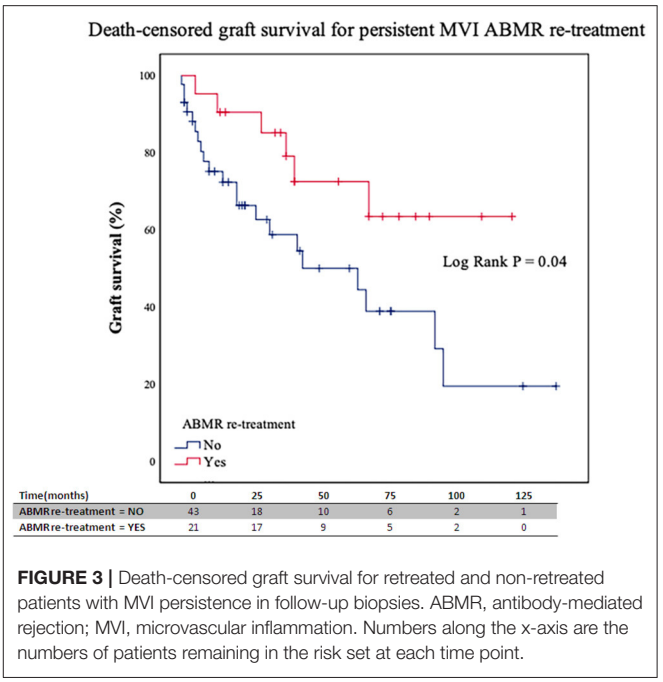

FIGURE 3 | Death-censored graft survival for retreated and non-retreated patients with MVI persistence in follow-up biopsies. ABMR, antibody-mediated rejection; MVI, microvascular inflammation. Numbers along the x-axis are the numbers of patients remaining in the risk set at each time point.

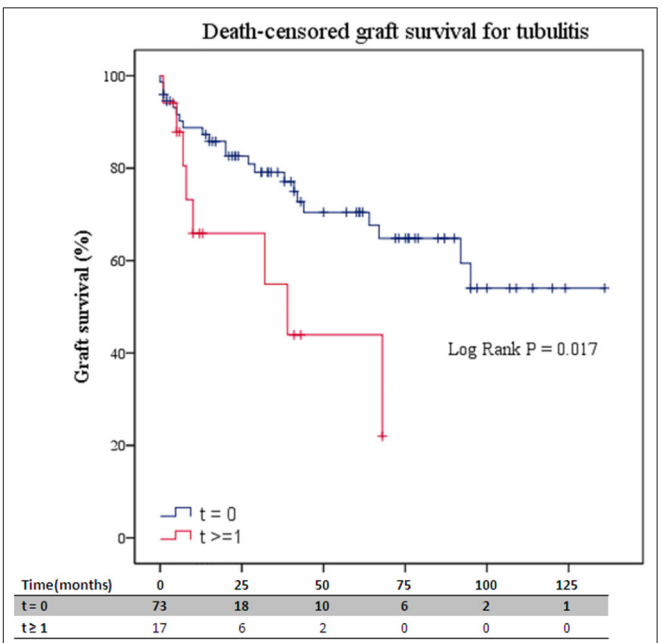

FIGURE 4 | Death-censored graft survival for tubulitis presence in follow-up biopsies. ABMR, antibody-mediated rejection; t, tubulitis. Numbers along the x-axis are the numbers of patients remaining in the risk set at each time point.

In the absence of conclusive data on the efficacy of retreatment, and considering the high rate of infections in patients with comorbidities, we would propose that such patients should not be retreated. Perhaps the development of new treatments with a better safety profile could be considered in these patients.

A controversial aspect that has not been solidly evaluated in previous studies is the usefulness of performing control graft biopsies after ABMR treatment, usually indicated in a heterogeneous manner and according to individual physician decision (6, 24, 25). In the present study, a control biopsy was performed in 78% of the included patients, of which 53% had presented a satisfactory response to ABMR treatment. Remarkably, we observed that 71% of the rebiopsied patients persisted with MVI $\geq$ 1. The negative impact of MVI $\geq$ 1 has been evidenced in previous studies as a potential predictor of the subsequent development of ABMR. However, only one of these studies has evaluated the specific influence of ptc in follow-up biopsies, where it was associated with a higher risk of graft loss (5, 24, 25). Other studies have demonstrated that inflammation in early protocol biopsies is associated with fibrosis progression and development *of de novo* DSAs (26).

More importantly, a key element from our results is that most of the rebiopsied patients had presented an improvement in graft function after the active ABMR treatment, and even in them, the persistence of MVI $\geq$ 1 was associated with worse kidney prognosis and the presence of any sign of inflammation (MVI, tubullitis, TMA, and ATN). An important point to cite is that 37.5% of the patients with MVI $\geq$ 1 were retreated, and this treatment was associated with a lower rate of graft loss than those with persistent MVI who did not undergo an ABMR retreatment. However, these results have to be taken with caution since the sample size is small, and a potential selection bias cannot be ruled out.

Another significant element of this work is the presence of tubulitis in follow-up biopsies. The coexistence of a TCMR with an ABMR is a previously studied condition that significantly worsens the prognosis of ABMR in kidney transplantation (27, 28). Only one previous study has evaluated the tubulitis presence in follow-up biopsies after treatment of an ABMR, in which it was not associated with an increased risk of graft loss at 6 years (5). In contrast, we have observed that $t \geq 1$, even without meeting criteria for TCMR diagnosis, was associated with substantially lower kidney graft survival.

Our findings suggest that persistent inflammation after aABMR treatment has a prognostic value, even when these inflammation signs did not fulfill any of the defined Banff categories and even when an initial improvement in kidney function is observed. This observation is consistent with the pathogenesis of chronic humoral damage, which is characterized by a sustained low-grade damage and glomerular basement membrane multilamination (transplant glomerulopathy, TG). This lesion has been associated with poor graft prognosis (29), as we have observed in the follow-up biopsies. This persistent glomerular inflammation may represent a sustained endothelial damage, over time, leading to TG.

This work has some limitations. Firstly, it is a retrospective study, and selection biases cannot be ruled out. The number of patients and events limit the statistical power, which prevents the performance of multivariate analyses. In Hospital Clinic de Barcelona, our guidelines include a follow-up biopsy after the ABMR treatment. However, the final decision for a kidney biopsy after ABMR treatment was based on clinical judgment. It should be noted that the rebiopsy periods were limited in time, and consistent to assess the response to treatment [mean time of 2 (4) months after treatment]. In addition, there were no differences in time to follow biopsy between the patients with and without persistent inflammation [2 (4.75) vs. 2 (4) months after treatment; $P = 0.98$, respectively].

Another important limitation that needs to be mentioned is the lack of a formal control group. Also, compliance is an important variable and could impact the analysis, although this is very difficult to address in a retrospective design. Moreover, the biological material to be examined with the molecular microscope (MMDx), which could provide additional information regarding the mechanisms of persistent inflammation (10), was not available.

Despite these limitations, we believe that the present study provides valuable information that reinforces the importance of follow-up biopsies after ABMR treatment, and revealed the persistence of MVI and tubulitis as markers of poor graft prognosis. As closing remarks, our study suggests the usefulness of systematically performing a follow-up biopsy after ABMR treatment, regardless of kidney graft function improvement after treatment, since the persistence of MVI or tubulitis seems to be associated with an increased risk of graft loss. Although it has not been established whether retreatment or other actions could modify this association, in this series, there was a better prognosis in the retreated patients with persistent inflammation than in the untreated patients ($p = 0.048$). More studies with a larger sample size are needed to confirm the findings of the present study, mainly focusing on evaluating the role of retreatment of patients with persistent inflammation in the control biopsy after treatment of ABMR.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee (CEIm) at Hospital Clinic de Barcelona. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GP, EM-M, and JRo collected, analyzed, interpreted the data, and prepared the manuscript. JRí performed the statistical analysis and interpreted the data. PV-A, DC, IR, ML, JC, FC, NE, FO, JMC, BB-G, and FD interpreted the data and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.761919/full#supplementary-material

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Spot Urine Protein Excretion in the First Year Following Kidney Transplantation Associates With Allograft Rejection Phenotype at 1-Year Surveillance Biopsies: An Observational National-Cohort Study

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Introduction: Urine protein excretion is routinely measured to assess kidney allograft injury, but the diagnostic value of this measurement for kidney transplant pathology remains unclear. Here we investigated whether spot urine protein excretion in the first year following transplantation associates with allograft rejection phenotype at 1-year surveillance biopsies and *de-novo* occurrence of donor-specific antibodies (DSA).

Patients and Methods: This prospective, observational national-cohort study included 139 non-sensitized patients who received a deceased donor kidney transplant between December 2014 and 2018. All patients received basiliximab induction and tacrolimus-based immunosuppression. Estimated protein excretion rate (ePER) was calculated monthly from spot urine protein-to-creatinine ratios. At 1-year, all recipients underwent surveillance graft biopsy and were screened for *de-novo* DSA. Screening-positive sera were subjected to single antigen bead (SAB) testing. The occurrence of *de-novo* DSA was determined based on SAB reactivity patterns using a mean fluorescence intensity threshold >1,000.

Results: Among the 139 study patients, 27 patients (19%) had histologic evidence of T cell-mediated rejection (TCMR), and 9 patients (7%) had histologic evidence of antibody-mediated rejection (AMR) at 1-year surveillance biopsy. One year after transplant, 19 patients (14%) developed *de-novo* DSA. Compared with patients without rejection and no *de-novo* DSA, mixed-effects linear regression analysis showed a significant difference in slope of ePER during the first year in patients with AMR and *de-novo* DSA at 1-year (46, 95% CI 25–68 mg/day/1.73 m^2 per month and 34, 95% CI 20–49 mg/day/1.73 m^2 per month, respectively). Patients with vascular TCMR also showed a significant difference in ePER slope over time compared with patients with non-rejection findings (31, 95% CI 9–52 mg/day/1.73 m^2 per month). The discriminatory power of ePER for intragraft rejection processes was better in patients with AMR (AUC 0.95, 95% CI 0.90–0.99; $P < 0.001$) than in those with TCMR (AUC 0.68, 95% CI 0.59–0.79; $P = 0.002$), with 89% sensitivity and 93% specificity for proteinuria >550 mg/day/1.73m^2.

Conclusions: An increase in ePER in the first year following kidney transplantation associates with AMR, vascular TCMR and *de-novo* DSA at 1-year and may be used as a non-invasive clinical marker of intragraft endothelial cell injury.

Keywords: kidney transplantation, antibody-mediated rejection, T-cell mediated rejection, donor-specific antibodies, urine protein excretion

INTRODUCTION

In recent years, antibody-mediated rejection (AMR) has been identified as the primary cause of allograft failure after kidney transplantation (1–3). This specific disease is diagnosed by means of needle biopsy. Although this invasive procedure has become safer and histologic interpretation more standardized, biopsy is usually indicated in deterioration of kidney function when allograft injury already occurs (4). Therefore, protocol biopsies have been proposed to detect changes before kidney dysfunction is apparent (5). Given that biopsy procedures are invasive, complications may occur; furthermore, sampling errors may jeopardize their diagnostic value. These shortcomings have stimulated research to identify non-invasive markers that are sufficiently diagnostic for specific transplant pathologies, and that can be used as an end point in clinical studies (6).

In patients with chronic kidney disease proteinuria is directly related to the underlying glomerular disease process and strongly associates with progression to end-stage kidney disease, with good specificity and sensitivity (7). Proteinuria is also routinely measured in kidney transplant recipients. Proteinuria, in the nephrotic range as well as lower grade, has been associated with inferior kidney transplant outcomes (8–10). Current clinical guidelines suggest that a kidney allograft biopsy should be performed when there is new onset of proteinuria or unexplained proteinuria $\geq$3.0 g/g creatinine or $\geq$3.0 g/24 h (11). However, these international guidelines are not evidence-based (evidence level 2C).

More recent data showed that proteinuria >1 g/24 h is a marker for allograft outcome with reasonable predictive accuracy, especially after the first 3 months post-transplantation (12). Although high-grade proteinuria has been related to transplant glomerulopathy and *de-novo* or recurrent glomerulonephritis (GN) (12–16), the association between low-grade proteinuria and the allograft pathology, in particular AMR, within the first year after transplantation has not been considered yet.

In this study, we aimed to assess the association and diagnostic performance of measuring proteinuria in spot urine samples during routine clinical follow-up in the first year following kidney transplantation with rejection phenotype at protocol-specified kidney biopsies and occurrence of *de-novo* donor-specific antibodies (DSA) at 1-year post-transplantation. In view of the great effect of specific diseases such as AMR on outcome after kidney transplantation, insight into the diagnostic value of proteinuria early after transplantation is particularly useful. Moreover, as many research teams are evaluating novel non-invasive biomarkers for kidney allograft injury, it is important to elucidate the diagnostic value of proteinuria measurement, a simple, inexpensive, and non-invasive marker that is already universally available.

Abbreviations: AMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific antibodies; eGFR, estimated glomerular filtration rate; GN, glomerulonephritis; MMF, mycophenolate mofetil; PCR, protein-to-creatinine ratio; ePER, estimated protein excretion rate; ROC, receiver operator characteristic; TAC, tacrolimus; TCMR, T cell-mediated rejection.

PATIENTS AND METHODS

Study Design

In this prospective, observational national-cohort study, we enrolled all consecutive adult recipients of a first deceased donor kidney transplant at the Department of Nephrology, University Medical Center Ljubljana between December 2014 and December 2018. All patients provided written informed consent. The National Medical Ethics Committee approved the study protocol.

Study Participants

Between December 2014 and December 2018, 211 adult patients received a deceased donor kidney transplant at our center. Sensitized recipients with preformed DSA and patients with prior transplants ($n = 51$), dual organ transplants ($n = 13$), and patients with early allograft loss within the first 90 days after transplantation ($n = 8$) were not candidates for the study. Finally, 139 patients were included in the study. All study participants were monitored regularly during the first year according to the protocol of the transplant unit of our department: twice a week in the first month, weekly in the 2nd and 3rd month, bi-weekly in the 4th and 5th month, and monthly thereafter.

The clinical data of the cohort were prospectively collected in electronic clinical patient charts, which were used for clinical patient management as well as being linked to the database used in this study.

All patients had standard immunologic risk and received basiliximab induction and tacrolimus-based immunosuppression. Patients with immediate graft function, diabetes mellitus, or previous cardiovascular events were candidates for rapid steroid withdrawal within the first week after transplantation.

Laboratory Assessment

Proteinuria was determined from second morning spot urine samples at month 1, and then monthly in the first year after transplantation in all study patients. Estimation of 24-h protein excretion rate (ePER, mg/day/1.73 m^2) was obtained by multiplying protein-to-creatinine ratio (PCR) and estimated creatinine excretion rate (17, 18). Urine creatinine was measured using non-isotope-dilution mass spectrometry standardized modified Jaffe reaction (calibration traceable to IDMS). Spot urine protein was measured by pyrogallol red-molybdate complex formation using a timed endpoint method. Measurements were performed on Dimension Xpand Plus Integrated Chemistry System using manufacturer's reagents (Siemens HealthCare GmbH, Erlangen Germany). Levels of ePER in the first year were then correlated with graft rejection status, rejection phenotype, and *de-novo* DSA formation at 1 year after transplantation.

At all-time points, data on serum creatinine were collected on the same day as PCR measurements. Glomerular filtration rate was estimated (eGFR) by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation (19).

Histologic Assessment of Biopsy Samples

In all patients, surveillance allograft biopsies were performed systematically at 1-year after transplantation. An indication kidney biopsy was considered if significant allograft dysfunction occurred before 1-year (e.g., associated with delayed graft function or an increase in serum creatinine of more than 20% from baseline without other obvious causes). Slides were stained with hematoxylin eosin, periodic acid–Schiff, and silver methenamine (Jones). An immunohistochemical C4d stain (monoclonal antibody, dilution 1:500; Quidel Corporation, Santa Clara, CA) was performed on frozen tissue. Two pathologists (NK and MF) independently reviewed all biopsies, blinded for the clinical data. The severity of histologic lesions was semiquantitatively scored according to the revised Banff 2013 criteria (20).

T cell-mediated rejection (TCMR) was reported as tubulo-interstitial (borderline and grade IA/B) or vascular (grade IIA/B). The phenotypes of AMR were classified as acute or chronic active. The diagnosis of acute AMR was based on morphologic evidence of acute tissue injury (i.e., peritubular capillaritis and/or glomerulitis) and positive C4d staining. The diagnosis of chronic AMR was based on the morphologic evidence of antibody-mediated chronic tissue injury, specifically glomerular double contours compatible with chronic glomerulopathy on light and/or electron microscopy.

At the time of protocol biopsies, systematic follow-up human leukocyte antigen (HLA) antibodies (ELISA HLA class I and class II Luminex Gen-Probe LifeCodes LSA screening) and their donor-specificity in case of positive screening (using Luminex Gen-Probe LifeCodes LSA Single Antigen Beads) were evaluated. The occurrence of *de-novo* DSA was determined based on single antigen bead reactivity patterns using a mean fluorescence intensity threshold >1,000.

Statistical Analysis

Dichotomous variables were compared with chi-squared test and continuous variables with the Student's *t*-test or the Wilcoxon-Mann-Whitney test as appropriate. For variance analysis of continuous variables in different groups, parametric one-way ANOVA and Kruskal-Wallis test were used.

The ePER trajectories were analyzed using linear mixed model regression, with ePER values from 1 to 12 months as dependent and time and the interaction of histologic phenotype/*de-novo* DSA occurrence and time as fixed effects. Furthermore, patient-specific random effect for intercept was specified. The covariance structure was specified as an autoregressive model of the first order.

Area under the receiver operator characteristic (ROC) curve analysis was performed to evaluate the diagnostic accuracy of ePER and to calculate the specificity and sensitivity for discriminating between biopsy specimens showing AMR, TCMR, and other non-rejection findings.

All tests were two-sided and $P < 0.05$ were considered to indicate statistical significance. All analyses were performed using the SPSS statistical software (IBM SPSS statistics, version 21.0, Armonk, NY, USA).

RESULTS

Study Population and Histologic Classification of Kidney Allograft Biopsy Specimens

The baseline patient, donor, and transplant-related characteristics of the study population according to histologic biopsy findings at 1-year after transplantation are provided in **Table 1**. Among the 139 patients, 36 (26%) had histologic evidence of allograft rejection at 1-year surveillance biopsies. Among them 27 patients (75%) were classified as having TCMR, and 9 patients (25%) were classified as having AMR. A total of 103 patients (74%) had no evidence of rejection and their biopsy findings are presented in **Table 1**. In ten patients (7%) early acute rejection occurred before 1-year. All patients underwent an indication biopsy due to allograft dysfunction in the first 3 months after transplantation. All rejection episodes were classified as TCMR (borderline or grade IA) and treated with pulse steroids (**Table 1**).

Patients with rejection phenotypes were transplanted from older donors and more frequently undergone rapid steroid withdrawal. In addition, 6 patients with allograft rejection at 1-year surveillance biopsy experienced early TCMR (**Table 1**); 4 patients had TCMR (grades IA or IIA), and 2 patients had histologic evidence of chronic active AMR. At 1-year after transplant, 19 patients (14%) developed *de-novo* DSA (4 patients' class I, and 15 patients' class II), and incidence of *de-novo* DSA occurrence was significantly higher in patents with histologic evidence of allograft rejection (**Table 1**).

Kidney Allograft Histology and Proteinuria

At 1-year after transplantation, spot urine protein excretion was in the low range with a mean ePER of 318 ± 308 mg/day/1.73 m^2.

TABLE 1 | Baseline characteristics of the study population according to histologic diagnosis in surveillance kidney allograft biopsies performed at 1 year after transplantation*.

Variables	All patients (*N* = 139)	Rejection (*n* = 36)	Other findings (*n* = 103)	*P*-value
Recipients				
Age (years)	49 ± 14	50 ± 16	49 ± 13	0.86
Males (%)	101 (73)	27 (75)	74 (72)	0.72
Original kidney disease				0.45
Diabetes (%)	11 (8)	2 (6)	9 (9)	
Hypertension (%)	16 (12)	7 (19)	9 (9)	
GN (%)	41 (29)	8 (23)	33 (32)	
Polycystic (%)	16 (12)	3 (8)	13 (13)	
Pyelonephritis/reflux (%)	8 (6)	3 (8)	5 (5)	
Other/undefined (%)	27/20 (19/14)	6/7 (17/19)	21/13 (20/12)	
Time on dialysis (years)	1.9 (0.8–3.2)	2.2 (0.9–3.8)	1.8 (0.8–3.0)	0.14
Last PRA (%)	0 (0–4)	0 (0–7)	0 (0–4)	0.49
Donors				
Age (years)	48 ± 13	52 ± 13	46 ± 12	0.022
Expanded criteria donor (%)	39 (28)	13 (36)	26 (25)	0.21
Transplant-related				
HLA mismatches	2.9 ± 1.1	3.0 ± 1.1	2.8 ± 1.1	0.19
Delayed graft function (%)	28 (20)	9 (25)	19 (19)	0.40
Treatment with TAC/MMF/St	82 (59)	15 (42)	67 (65)	0.024
Treatment with TAC/MMF	57 (41)	21 (58)	36 (35)	0.014
Previous rejection#	10 (7)	6 (17)	4 (4)	0.029
Biopsy diagnosis at 1-year				
TCMR (%)		27 (75)	–	–
Borderline		8 (22)	–	–
Grade IA/B		10 (27)	–	–
Grade IIA/B		9 (25)	–	–
AMR (%)		9 (25)	–	–
Acute		7 (19)	–	–
Chronic active		2 (6)	–	–
Recurrent GN (%)##		–	6 (6)	–
CNI nephrotoxicity (%)		–	14 (14)	–
BKVAN (%)		–	5 (5)	–
No major abnormalities (%)		–	78 (75)	–
De-novo DSA at 1-year	19 (14)	11 (31)	8 (8)	0.001

GN, glomerulonephritis; PRA, panel reactive antibodies; HLA, human leukocyte antigen; TAC, tacrolimus; MMF, mycophenolate mofetil; St, steroids; TCMR, T cell-mediated rejection; AMR, antibody-mediated rejection; CNI, calcineurin inhibitor; BKVAN, BK virus associated nephropathy.

**Data are presented as means ± SD, medians (interquartile ranges), or as total numbers (percentages).*

#The diagnosis was made based on indication biopsies performed before 1-year after transplantation. All rejection episodes were diagnosed in the first 3 months and classified as TCMR (borderline or grade IA).

##All recurrent GN include recurrent IgA nephropathy.

TABLE 2 | Graft function, Banff histologic scores, and incidences of rejection phenotypes and occurrence of *de-novo* DSA according to levels of ePER (in tertiles) at 1-year after transplantation*.

Parameter at 1-year	Tertiles of ePER at 1-year (mg/day/1.73 m^2)			*P*-value
	<180 (*n* = 46)	180–300 (*n* = 45)	>300 (*n* = 48)	
ePER (mg/day/ 1.73 m^2)	125 ± 32	222 ± 32	594 ± 308	<0.001
eGFR (ml/min/ 1.73 m^2)	67 ± 17	69 ± 20	63 ± 21	0.139
Banff scores (mean ± SD)				
t score	0.15 ± 0.60	0.29 ± 0.59	0.83 ± 1.08	<0.001
i score	0.09 ± 0.35	0.20 ± 0.46	0.69 ± 0.93	<0.001
ti score	0.48 ± 0.75	0.69 ± 0.85	1.27 ± 0.92	<0.001
ptc score	0	0.02 ± 0.15	0.21 ± 0.54	0.004
g score	0.07 ± 0.32	0.04 ± 0.25	0.20 ± 0.45	0.038
v score	0.04 ± 0.29	0.02 ± 0.15	0.08 ± 0.28	0.491
ci score	0.74 ± 0.68	0.98 ± 0.72	1.17 ± 0.83	0.024
ct score	1.07 ± 0.39	1.11 ± 0.49	1.23 ± 0.56	0.239
ah score	1.35 ± 0.57	1.44 ± 0.58	1.38 ± 0.61	0.699
cg score	0.06 ± 0.32	0.02 ± 0.15	0.19 ± 0.45	0.048
c4d score	0.07 ± 0.25	0.09 ± 0.29	0.33 ± 0.83	0.030
Rejection phenotype				
AMR	0	1	8	<0.001
TCMR	3	10	14	0.018
Borderline	1	4	3	0.382
Grade IA/B	2	3	5	0.516
Grade IIA/B	0	3	6	0.048
De-novo DSA	1	4	14	<0.001

ePER, estimated protein excretion rate, eGFR, estimated glomerular filtration rate; t, tubulitis; i, interstitial inflammation; ti, total inflammation; ptc, peritubular capillaritis; g, glomerulitis; v, intimal arteritis; ci, interstitial fibrosis; ct, tubular atrophy; ah, arteriolar hyalinosis; cg, glomerular basement membrane double contours; c4d, staining for C4d on endothelial cells of peritubular capillaries by immunofluorescence; TCMR, T cell-mediated rejection; AMR, antibody-mediated rejection; DSA, donor-specific antibodies.

**Data are presented as means ± SD or as total numbers.*

The patients were stratified into three groups according to tertiles of ePER (**Table 2**). eGFR did not differ significantly among patients with different levels of ePER. Greater levels of ePER were significantly associated with higher Banff histologic scores related to tubulointerstitial inflammation and microvascular injury, and patients in the highest tertile had higher incidence rates of AMR and vascular TCMR. In addition, greater levels of ePER were associated with higher c4d histologic scores, chronic glomerulopathy (cg score), and higher incidences of *de-novo* DSA occurrence at 1-year (**Table 2**).

Among patients with no evidence of rejection, levels of ePER at 1-year were slightly higher in those with recurrent GN than in those with other non-rejection findings, but the difference was not statistically significant (268 ± 109 vs. 218 ± 22 mg/day/1.73 m^2; $P = 0.169$).

The course of ePER with respect to allograft histology and occurrence of *de-novo* DSA is illustrated in **Figure 1**. During the 12-month period, patients with AMR, TCMR, and non-rejection findings had ePER slopes of 38 (95% confidence interval

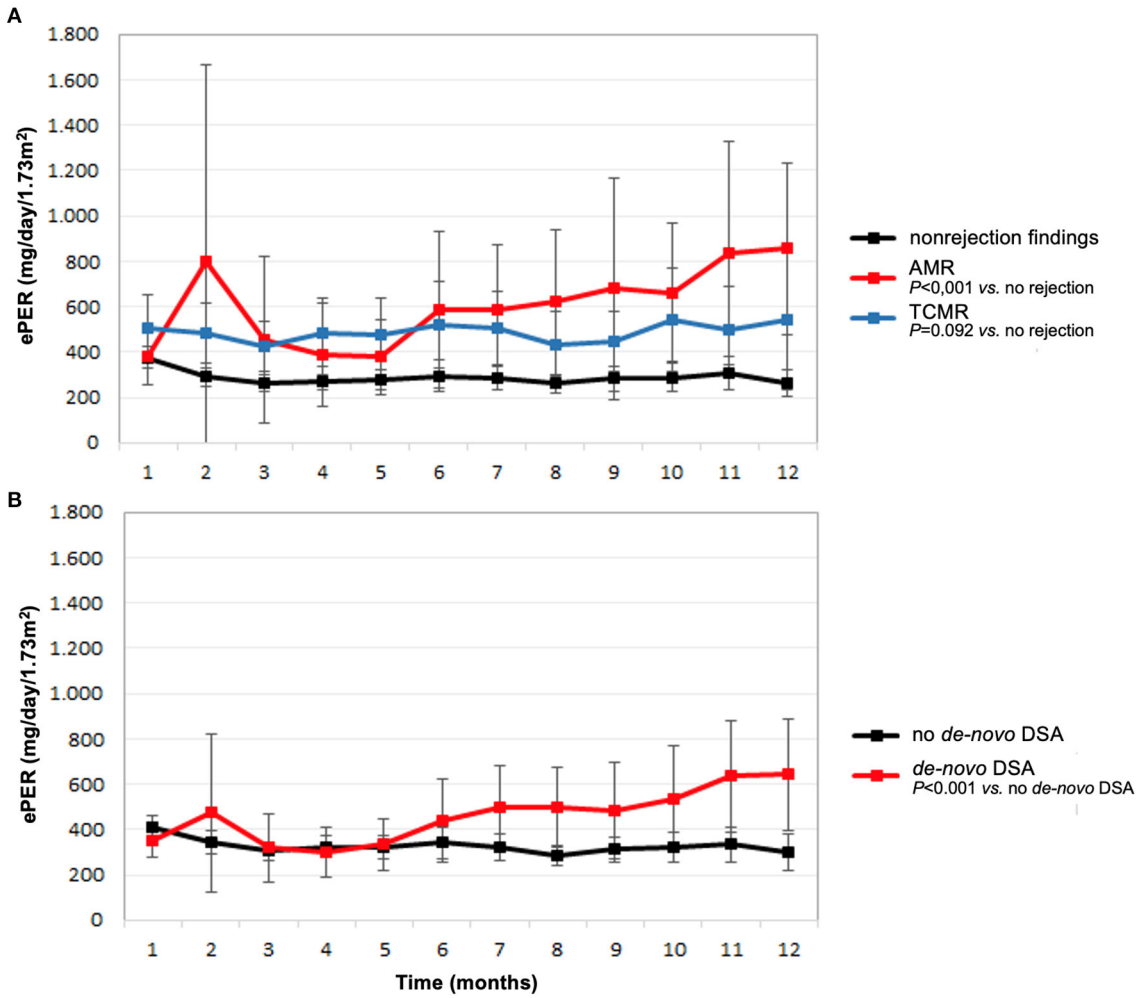

FIGURE 1 | Estimated protein excretion rate (ePER, mean and 95% CI) in the first year following kidney transplantation according to main histologic findings at 1-year surveillance biopsies **(A)** and *de-novo* occurrence of donor-specific antibodies (DSA) **(B)**. AMR, antibody-mediated rejection; TCMR, T cell-mediated rejection.

[CI] 1 to 76), 5 (95% CI −11 to 22), and −6 (95% CI −11 to −1) mg/day/1.73 m^2 per month, respectively (**Figure 1A**). The difference between patients with AMR and non-rejection findings of 46 (95% CI 25–68) mg/day/1.73 m^2 per month was statistically significant ($P<0.001$). The difference between patients with TCMR and no rejection of 11 (95% CI −2–25) mg/day/1.73 m^2 per month was not statistically significant ($P = 0.092$).

Patients with *de-novo* DSA had a significant increase in ePER during the first year compared with patients without *de-novo* DSA 1-year after transplantation (**Figure 1B**). During the 12-month period, patients with *de-novo* DSA and no *de-novo* DSA had ePER slopes of 28 (95% CI 10–46) and of −6 (95% CI −11–1) mg/day/1.73 m^2 per month, respectively. The difference of 34 (95% CI 20–49) mg/day/1.73 m^2 per month was statistically significant ($P < 0.001$).

Compared with patients without evidence of rejection, ePER slopes increased progressively in patients with higher histological grade of TCMR, and the increase was highest in patients with vascular TCMR (**Figure 2**). During the 12-month period, patients with borderline, tubulointerstitial, and vascular TCMR had ePER slopes of −21 (95% CI, −41 to −2), 12 (95% CI, −26–51), and 24 (95% CI, −1–50) mg/day/1.73 m^2 per month, respectively. A statistically significant difference in ePER slope was noted between patients with vascular TCMR (grades II/A,B) and no rejection (31, 95% CI 9 to 52 mg/day/1.73 m^2 per month; $P = 0.005$). The difference between patients with borderline TCMR and no rejection (15, 95% CI −7–37 mg/day/1.73 m^2 per month) and between patients with TCMR grades I/A,B and no rejection (15, 95% CI −5–35 mg/day/1.73 m^2 per month) was not statistically significant ($P = 0.184$ and 0.138, respectively).

Proteinuria as a Biomarker for Rejection-Associated Allograft Injury

Next, we examined the diagnostic performance of ePER for rejection injury phenotypes in surveillance allograft biopsies at 1-year after transplantation. ePER at 1-year post-transplant was significantly associated with the presence of AMR. The ROC

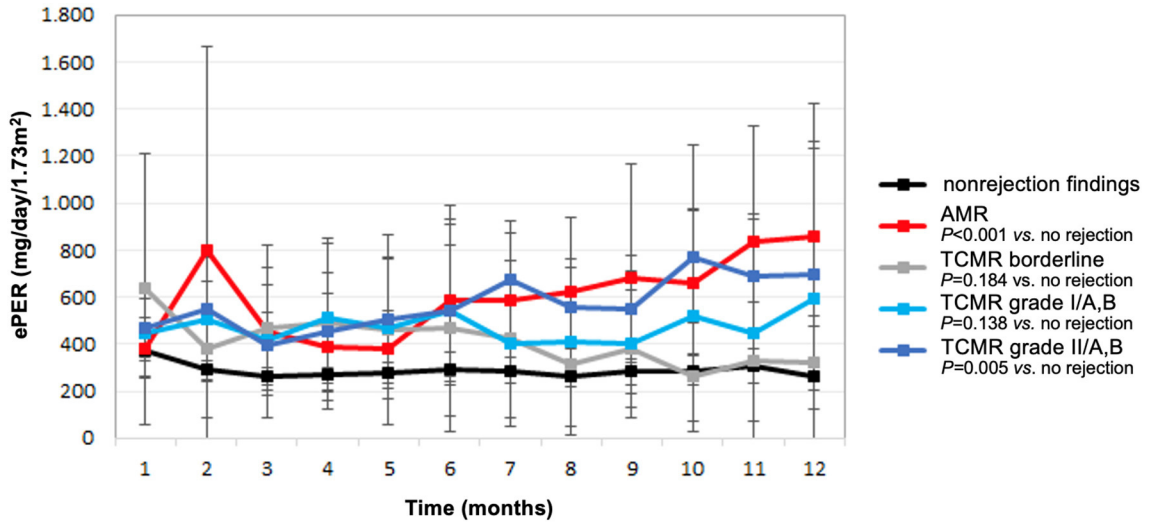

FIGURE 2 | Estimated protein excretion rate (ePER, mean and 95% CI) in the first year following kidney transplantation according to allograft rejection phenotypes at 1-year surveillance biopsies. AMR, antibody-mediated rejection; TCMR, T cell-mediated rejection.

AUC was 0.95 (95% CI 0.90–0.99; $P < 0.001$). The threshold of ePER that gave the maximal sensitivity and specificity for AMR was 550 mg/day/1.73 m^2; at this threshold, the AMR can be predicted with a sensitivity of 81%, and a specificity of 80%. The diagnostic accuracy for TCMR was lower with an AUC of 0.68 (95% CI 0.59–0.79; $P = 0.002$). However, the diagnostic accuracy was better for vascular TCMR with an AUC of 0.77 (95% CI 0.66–0.89; $P = 0.007$).

DISCUSSION

The goal of this study was to quantify the changes in spot urine protein excretion that occur during the first year after kidney transplantation in a low-risk cohort of non-sensitized patients with stable kidney function and to investigate whether post-transplant proteinuria is associated with significant allograft pathology at 1-year. The results indicate that kidney allograft rejection and rejection phenotype at 1-year surveillance biopsies are associated with levels of ePER in the first year following transplantation. This simple diagnostic tool measured in spot urine specimens obtained longitudinally in the first-year post-transplant from patients with biopsy-confirmed tubulointerstitial TCMR and other non-rejection findings was relatively flat and distinct from the progressive increase observed in patients with AMR. In addition, *de-novo* DSA occurrence at 1-year was also associated with an increase in ePER in the first year. Moreover, ePER at 1-year was highly specific for endothelial response to injury associated with *de-novo* DSA formation, AMR and high-grade vascular TCMR. These findings are important given that spot urine protein excretion can be easily measured and followed after transplantation.

An increase in serum creatinine is often the first clinical indicator of rejection. However, it lacks sensitivity and specificity. The limitations associated with monitoring rejection by measurements of serum creatinine have been recognized previously by the observation that 30% of graft biopsies performed in patients with stable kidney function reveal histological features of rejection (21). More recently, subclinical AMR has been reported in patients with preformed anti-HLA antibodies (22). In subclinical AMR, the serum creatinine level was stable, but protocol biopsy specimens showed glomerulitis, peritubular capillary infiltration by leukocytes, and positive staining of peritubular capillaries with an anti-C4d antibody. Since AMR is associated with endothelial response-to-injury (23), one would expect an increase in urine protein excretion. Our study demonstrated that levels of proteinuria increased in the first year following transplantation among patients with histologic signs of endothelial cell injury, specifically in patients with AMR and *de-novo* occurrence of DSA. In addition, preceding TCMR in the first months after transplantation was associated with rejection phenotypes at 1-year, including chronic active AMR, as described previously (24). Therefore, persistent or increasing proteinuria may indicate ongoing rejection, even in the absence of allograft dysfunction and despite augmented immunosuppression.

Protein excretion from native kidneys falls rapidly after transplantation and *de-novo*, persistent or worsening proteinuria is usually indicative of graft pathology (25). In the largest study to date, 58% of transplanted patients with proteinuria ≥150 mg/day had transplant-specific lesions (acute rejection, transplant glomerulopathy, interstitial fibrosis/tubular atrophy) on biopsy compared with only 11% with glomerulonephritis (10). However, detailed information on the natural history of proteinuria early after transplantation and allograft injury phenotypes has not been available. In our study, we observed that in the first year following transplantation, when patients

still had preserved allograft function, spot urine protein excretion was greater in patients with rejection phenotypes at 1-year surveillance biopsies. Our main observation was that proteinuria significantly increased in the patients in whom a 1-year surveillance biopsy showed AMR and in the patients who developed *de-novo* DSA. Additionally, the slope of proteinuria could discriminate between AMR and non-rejection findings. These findings fit well with recent observations of Fotheringham et al. who demonstrated that spot urine protein excretion is associated with DSA detection (26).

Next, ePER >550 mg/day/1.73 m^2 was a specific non-invasive marker for highly relevant intragraft injury processes such as AMR and vascular TCMR in our study. The high specificity of proteinuria for these treatable diagnoses in surveillance biopsies provides the evidence of current clinical guidelines that advocate the routine measurement of proteinuria (11). In addition, clinical guidelines suggest that a kidney biopsy should be performed when there is new onset or unexplained proteinuria ≥3.0 g/g creatinine or ≥3.0 g/day. Our data illustrate that this threshold is very conservative and that early detection of proteinuria >500 mg/day could be a more sensitive threshold. However, the association between proteinuria and allograft histology was weak in the first 6 months following transplantation, likely reflecting the contribution of residual kidney function of the native kidneys in the first months (27, 28).

High specificity and sensitivity of proteinuria for AMR, vascular TCMR, and *de-novo* DSA formation demonstrate acceptable diagnostic performance of low-grade spot urine protein excretion for intragraft microcirculation inflammation and glomerular injury. Similarly, previous study from Naesens et al. (12) demonstrated that many patients with significant histologic injury had low-grade proteinuria <1 g/day, illustrating that surveillance biopsies could thus be warranted in the absence of significant proteinuria or allograft dysfunction for the timely detection of subclinical injury. In this light, our study confirmed that allograft rejection processes, specifically AMR, and *de-novo* DSA occurrence may associate with low-grade proteinuria and an increase in proteinuria already in the first year following transplantation.

The results of our study are subject to several limitations. Our strategy of including only patients with a functioning kidney beyond 90 days after transplantation may have excluded from analysis some allografts that failed early post-transplant because of rejection. This may have biased our findings toward later events. However, proteinuria in the first months after transplantation is difficult to interpret as it may originate from native kidneys or can result from injury in the grafted kidney (e.g., ischemia-reperfusion injury) (28, 29). Whether our results also apply to immunologically high-risk transplants with preformed DSA, and whether the association between the histology of AMR and proteinuria would be more pronounced in this specific high-risk patient cohort, could not be inferred from our data. Furthermore, we could not investigate the association between proteinuria in the first year and recurrent glomerulonephritis because the number of patients with recurrent disease was small and the fact that recurrence of most common glomerular diseases (e.g., IgA nephropathy) usually occurs later after transplantation. In addition, a review from Akbari et al. (30) showed that in kidney transplant population the ability of spot urine protein measurements to predict 24-h protein excretion is modest and 24-h urine collection should be considered before making further decisions. However, recent observational study showed that spot and 24-h measurements of protein excretion are similar predictors of doubling of serum creatinine, graft loss, and patient death and that spot urine samples are a suitable alternative to 24-h urine collection (31). Unfortunately, we do not have outcome data to determine whether an increase in low-grade proteinuria in the first year following transplantation associates with inferior transplant outcomes.

In conclusion, this study found that in kidney transplant recipients an increase in low-grade spot urine protein excretion in the first year following transplantation associates with AMR and *de-novo* DSA formation at 1-year post-transplant. The analysis of the diagnostic performance of low-grade proteinuria for treatable subclinical disease processes (specifically AMR, vascular TCMR, and *de-novo* DSA occurrence) in surveillance biopsies provides the scientific underpinning of the current clinical guidelines to routinely measure proteinuria early after transplantation, and to pursue a histologic diagnosis even when proteinuria >500 mg/day is detected. Further studies in an independent cohort are needed to prospectively validate these findings.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Medical Ethics Committee of the Republic of Slovenia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MO, GM, and MA collected data. NK and MF performed histological analysis. JB-P interpreted the data and critically revised the manuscript. MA participated in the statistical analysis. All authors participated in research design, performance of the research, preparation of the manuscript, and have approved the final version of the manuscript.

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Intimal Arteritis and Microvascular Inflammation Are Associated With Inferior Kidney Graft Outcome, Regardless of Donor-Specific Antibodies

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Background: The prognostic role of intimal arteritis of kidney allografts in donor-specific antibody negative (DSA–) antibody-mediated rejection (ABMR) remains unclear.

Methods: Seventy-two out of 881 patients who had undergone kidney transplantation from 2014 to 2017 exhibited intimal arteritis in biopsies performed during the first 12 months. In 26 DSA negative cases, the intimal arteritis was accompanied by tubulointerstitial inflammation as part of T cell-mediated vascular rejection (TCMRV, $N = 26$); intimal arteritis along with microvascular inflammation occurred in 29 DSA negative (ABMRV/DSA–) and 19 DSA positive cases (ABMRV, DSA+, $N = 17$). In 60 (83%) patients with intimal arteritis, the surveillance biopsies after antirejection therapy were performed. Hundred and two patients with non-vascular ABMR with DSA (ABMR/DSA+, $N = 55$) and without DSA (ABMR/DSA–, $N = 47$) served as controls. Time to transplant glomerulopathy (TG) and graft failure were the study endpoints.

Results: Transplant glomerulopathy -free survival at 36 months was 100% in TCMRV, 85% in ABMR/DSA–, 65% in ABMRV/DSA-, 54% in ABMR/DSA+ and 31% in ABMRV/DSA+ (log rank $p < 0.001$). Death-censored graft survival at 36 months was 98% in ABMR/DSA-, 96% in TCMRV, 86% in ABMRV/DSA–, 79% in ABMR/DSA+, and 64% in ABMRV/DSA+ group (log rank $p = 0.001$). In surveillance biopsies, the resolution of rejection was found in 19 (90%) TCMRV, 14 (58%) ABMRV/DSA–, and only 4 (27%) ABMRV/DSA+ patients ($p = 0.006$). In the multivariable model, intimal arteritis as part of ABMR represented a significant risk for TG development (HR 2.1, 95% CI 1.2–3.8; $p = 0.012$) regardless of DSA status but not for graft failure at 36 months.

Conclusions: Intimal arteritis as part of ABMR represented a risk for early development of TG regardless of the presence or absence of DSA. Intimal arteritis in DSA positive ABMR represented the high-risk phenotype.

Keywords: antibody-mediated rejection, intimal arteritis, kidney transplantation, vascular rejection, rejection diagnostics

INTRODUCTION

Antibody-mediated rejection (ABMR) represents a major obstacle in achieving long-term graft function and survival (1). Its diagnosis is based on histology and detection of donor-specific antibodies (DSA) (2). Overlapping phenotypes and cases with incomplete manifestation represent a considerable part of diagnoses in clinical practice (3).

Intimal arteritis, i.e., v-lesion defined by Banff histological criteria, is a morphologic feature of vascular rejection. It represents a diagnostic and therapeutic challenge as it is involved in histologic criteria of both T cell-mediated rejection (TCMR) and ABMR (2). Although, previously intimal arteritis was recognized as the rejection phenotype often resistant to steroid treatment (4), more recently, the association of intimal arteritis with DSA was described (5). Lefaucheur et al. revealed a detrimental impact of intimal arteritis associated with DSA on graft prognosis exceeding other rejection phenotypes, and thus any grade of intimal arteritis has been included in the Banff classification as a histologic feature of ABMR (6).

Almost half of the patients with histological features of ABMR in DSA negative patients do not fit the current Banff classification (7). Intimal arteritis is frequently present in both TCMR and ABMR occurring early after kidney transplantation (8). Although a negative prognostic role of DSA in ABMRV has been well-documented (5), the outcome of ABMRV in DSA negative patients remains poorly understood. Therefore, in this study, we compared the outcome of intimal arteritis as part of both T cell-mediated vascular rejection (TCMRV) and antibody-mediated vascular rejection (ABMRV) with ABMR without intimal arteritis in patients with and without DSA in terms of premature graft loss and transplant glomerulopathy (TG) development.

MATERIALS AND METHODS

Study Design and Population

In this single center retrospective observational cohort study, we evaluated the outcome and clinical relevance of intimal arteritis in the early posttransplant biopsies performed within 12 months from January 2014 to December 2017. For comparison, we retrospectively reviewed medical records of 881 patients who had undergone kidney transplantation at the same time to identify those with histologic features of ABMR in early biopsies and enrolled them for further investigation. Data collection was finalized in January 2021 when all the study subjects reached a 3-year follow-up. Demographics of the study cohort are given in **Table 2** and the enrollment is described in **Figure 1**. All patients signed the informed consent with case or surveillance biopsies and medical data assessment, and the local Ethical Board approved the study under No.: 15-265191A.

Histopathology and the Definition of Rejection Phenotype Categories

Kidney allograft biopsies were performed using a percutaneous ultrasound-guided 16G biopsy needle. Diagnoses of acute rejection were established at a median time 70 days after transplantation by for-cause biopsies in 120 (69%) of patients. Fifty-two (31%) rejections were found in protocol biopsies at the third month after transplantation.

Biopsy samples were assessed for Banff scored lesions (2) as glomerulitis (g), peritubular capillaritis (ptc), transplant glomerulopathy (cg), intimal arteritis (v), interstitial inflammation (i), tubulitis (t), mesangial matrix increase (mm), vascular intimal fibrosis (cv), arteriolar hyaline thickening (ah), interstitial fibrosis (ci), or tubular atrophy (ct). The microvascular inflammation (MVI) score was defined as the sum of glomerular (g) and peritubular capillary inflammation (ptc). Immunofluorescence detection of C4d was performed in all cases.

Patients with glomerulitis, peritubular capillaritis, intimal arteritis, and/or TG were divided into five groups (TCMRV $N = 26$, ABMRV/DSA– $N = 29$, ABMRV/DSA+ $N = 17$, ABMR/DSA– $N = 47$, ABMR/DSA+ $N = 55$) according to the histopathological finding and presence or absence of DSA (**Table 1**).

T cell-mediated vascular rejection was characterized by intimal arteritis and tubulointerstitial inflammation (TI) in the absence of glomerulitis of rejection origin, C4d, and DSA. ABMRV included features of MVI and C4d (positive or negative) in addition to v-lesion. Patients were further divided according to their DSA status as ABMRV/DSA– when DSA were negative and ABMRV/DSA+ when DSA were positive. ABMR, defined by the presence of MVI and C4d (positive or negative), were further divided according to DSA status as described above (ABMR/DSA–, ABMR/DSA+). Four phenotypes fulfilling criterion 1 (histologic evidence of acute tissue injury) and 2 (evidence of recent antibody interaction with endothelium) of Banff ABMR definition were called histologic ABMR groups.

Anti-HLA Antibody Testing

Identification of circulating donor-specific anti-HLA antibodies (DSA) was performed by Luminex bead-based assay (One Lambda Inc., Canoga Park, CA, USA). Class I A, B, and class II DR antibodies were evaluated for specificity in all recipients before transplantation. Class II DP and DQ antibodies were evaluated for specificity after transplantation because donors' HLA DP or DQ typing was not available at the time of transplantation. DSA positivity at the time of transplantation was revealed in 14 (82%) of the patients with ABMRV/DSA+ and 47 (86%) of them with ABMR/DSA+. In the rest of the patients from these phenotype categories, DSA was detected at the time of diagnostic biopsy.

Immunosuppressive Treatment

Patients received maintenance immunosuppression based on tacrolimus, mycophenolate mofetil, and corticosteroids. All patients received induction treatment. Patients at low risk ($N = 73$) received basiliximab whereas those at high risks, such as retransplants and patients with anti-HLA antibodies or DSA, received rabbit anti-thymocyte globulin (rATG) ($N = 49$). Patients with DSA positivity (<5,000

TABLE 1 | Definition of the rejection phenotype groups.

	g	ptc	v	I	t	C4d	DSA
TCMRV	0	≥0*	≥1	0–3	0–3	0	Neg
ABMRV/DSA–	0-3	≥0*	≥1	0–3	0–3	0–3	Neg
ABMRV/DSA+	0–3	≥0*	≥1	0–3	0–3	0–3	Pos
ABMR/DSA–	0–3	≥0*	0	0–3	0–3	0–3	Neg
ABMR/DSA+	0–3	≥0*	0	0–3	0–3	0–3	Pos

**In presence of tubulointerstitial inflammation ptc alone was not evaluated as ABMR criterion.*

TABLE 2 | Overview of the baseline characteristics of all rejection phenotype groups.

	Total *N* = 174	TCMRV *N* = 26	ABMRV/DSA– *N* = 29	ABMRV/DSA+ *N* = 17	ABMR/DSA- *N* = 47	ABMR/DSA+ *N* = 55	*P*-value
Age, yr	53 (21–81)	54 (23–70)	56 (23–81)	49 (25–78)	56 (21–71)	48 (23–78)	0.121
Gender (female)	62 (36)	8 (31)	11 (38)	5 (29)	15 (32)	23 (42)	0.768
Dialysis vintage, m	26 (0–263)	20 (0–263)	25 (0–118)	32 (0–131)	21 (0–128)	38 (0–139)	0.046
PRA max, %	7 (0–100)	2 (0–69)	2 (0–22)	34 (0–100)	3 (0–98)	32 (0–100)	**<0.001**
HLA mismatch	3 (0–6)	4 (1–6)	3 (0–6)	4 (2–6)	3 (0–6)	3 (1–6)	0.14
1st transplantation	123 (71)	24 (92)	27 (93)	8 (47)	45 (96)	19 (35)	**<0.001**
DSA at Tx, *n* (%)	61 (35)	0	0	14 (82)	0	47 (86)	0.756*
DSA class I, *n* (%)	23 (13)	0	0	3 (18)	0	20 (36)	0.148*
DSA class II, *n* (%)	28 (16)	0	0	8 (46)	0	20 (36)	0.720*
DSA both classes, *n* (%)	21 (12)	0	0	6 (36)	0	15 (28)	0.525*
FACS T at biopsy	104/19 (18)	14/2 (14)	26/3 (12)	14/3 (21)	18/0	32/10 (31)	**<0.001**
FACS B at biopsy	104/31 (29)	14/1 (7)	26/0	14/8 (57)	18/1 (5)	32/21 (65)	**<0.001**
Cold ischemia, h	14 (0.3–24)	13 (0.3–21)	13 (0.3–20)	16 (0.5–23)	14 (0.5–23)	15 (0.6–24)	0.26
Deceased donor, *n* (%)	146 (84)	17 (65)	23 (79)	15 (88)	39 (83)	52 (95)	0.018
CKD diagnosis							0.039
Diabetes	23 (13)	3 (12)	6 (21)	2 (12)	11 (23)	1 (2)	
Vascular + TIN	41 (24)	10 (39)	8 (28)	5 (29)	6 (13)	12 (22)	
Glomerulonephritis	50 (29)	4 (15)	5 (17)	5 (29)	14 (30)	22 (40)	
Hereditary nephropathy	35 (20)	6 (23)	5 (17)	1 (6)	8 (17)	15 (27)	
Other	25 (14)	3 (12)	5 (17)	4 (24)	8 (17)	5 (9)	
Induction therapy							**<0.001**
Basiliximab	73 (42)	17 (65)	22 (76)	2 (12)	28 (60)	4 (7)	
rATG	49 (28)	8 (31)	7 (24)	7 (41)	15 (32)	12 (22)	
rATG, PE, IVIG	52 (30)	1 (4)	0	8 (47)	4 (8)	39 (71)	
Maintenance IS							
Tac/MMf/steroids	163 (94)	25 (96)	25 (86)	15 (88)	44 (94)	54 (98)	0.058
Time of diagnosis, d	70 (2–357)	74 (3–204)	30 (5–357)	24 (7–351)	80 (2–301)	61 (5–323)	0.976
For–cause biopsy	120 (69)	17 (65)	25 (86)	16 (94)	26 (55)	36 (66)	**0.009**
Treatment of rejection							**<0.001**
Methylprednisolone	81	18 (69)	8 (28)	1 (6)	37 (79)	17 (31)	
rATG	27	7 (27)	15 (52)	3 (18)	2 (4)	0	
PE, IVIG	58	1 (4)	6 (21)	13 (76)	5 (11)	33 (60)	
None	8	0	0	0	3 (6)	5 (9)	

**Comparing ABMR/DSA+ and ABMRV/DSA+ rejection phenotypes.*

MFI) at transplant had undergone plasma exchange (PE) prior to transplantation and intravenous immunoglobulin (IVIG) as additional treatment ($N = 52$). The later described multivariable Cox regression model was adjusted based on induction immunosuppression to eliminate confounding variables.

Patients with rejection were treated by steroid pulses [18 (69%) TCMVR; 8 (28%) ABMRV/DSA–; 1 (6%) ABMRV/DSA+;

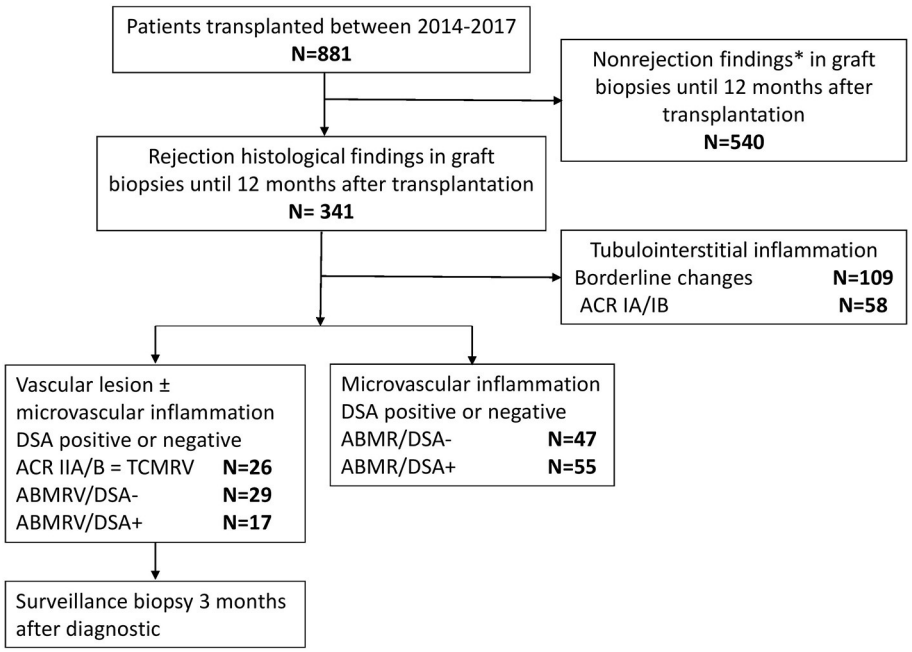

FIGURE 1 | Study flowchart. * Including parainfectious changes, tubulointerstitial inflammation in the presence of BKV, and suspected recurrence of glomerulonephritis.

37 (79%) ABMR/DSA-; 17 (31%) ABMR/DSA+] or rATG [7 (27%) TCMRV; 15 (52%) ABMRV/DSA–; 3 (18%) ABMRV/DSA+; 2 (4%) ABMR/DSA–, none of ABMR/DSA+] and/or plasmapheresis/IVIG [1 (4%) TCMRV; 6 (21%) ABMRV/DSA-; 13 (77%) ABMRV/DSA+; 5 (11%) ABMR/DSA–; 33 (60%) ABMR/DSA+]. Altogether, eight patients were not treated mostly due to concomitant infectious complications.

Surveillance and Subsequent Biopsies

Altogether, 124 patients from the whole cohort underwent at least one biopsy after the diagnostic examination. All patients who experienced intimal arteritis in early biopsies were eligible for surveillance biopsy at 3 months after the first biopsy. After obtaining written informed consent, 60 out of the 72 patients (83%) who experienced intimal arteritis underwent the surveillance biopsy. Patients from ABMR/DSA– and ABMR/DSA+ groups underwent subsequent biopsies either at the time of center protocol biopsy at the third month or when clinically indicated due to graft function worsening or proteinuria.

Statistical Analyses

Continuous variables were expressed as median and range. Categorical variables were expressed as *n* and percentage of the total. Categorical Banff histologic scores were expressed as count per category. The Chi-square, ANOVA, Kruskal–Wallis, and Wilcoxon tests were used for hypothesis testing when appropriate. $p < 0.05$ were considered statistically significant. Survival analyses were performed with the Kaplan–Meier method using the log-rank test. To identify factors associated with death-censored graft failure and the development of TG, univariable Cox regression was created. For the multivariable model, all variables with $p < 0.1$ were selected, and only variables without missing data were included. Data analyses were performed using IBM SPSS 22 (SPSS, Inc. Chicago, IL), R Studio 4.0.3. (2020-10-10), and GraphPad Prism5 (Graph Pad, San Diego, CA).

RESULTS

Demographics and Clinical Characteristics

In 174 patients, five different rejection phenotypes were identified: 26 TCMRV, 29 ABMRV/DSA–, 17 ABMRV/DSA+, 47 ABMR/DSA–, and 55 ABMR/DSA+ (**Table 1**). Diabetes, ischemic nephropathy, and glomerulonephritis were the most common original diseases. DSA– phenotypes were more common after the first transplantation. DSA positive phenotypes were more frequent in retransplantation. Peak PRA was significantly higher in DSA positive groups. Cohort demographics are given in **Table 2**.

Surveillance Biopsies

Surveillance biopsies at 3 months after vascular rejection diagnosis were performed in 21 out of 26 patients (81%) with TCMRV, 24 out of 29 (83%) patients with DSA negative ABMRV, 15 out of 17 (88%) patients with DSA positive ABMRV. At case biopsy, DSA negative patients with ABMRV did not differ from DSA positive ABMRV patients in any of the histologic scores, indicating acute inflammation (g, ptc, i, t, v). The only significant difference was the intensity of C4d ($p = 0.003$). Surveillance

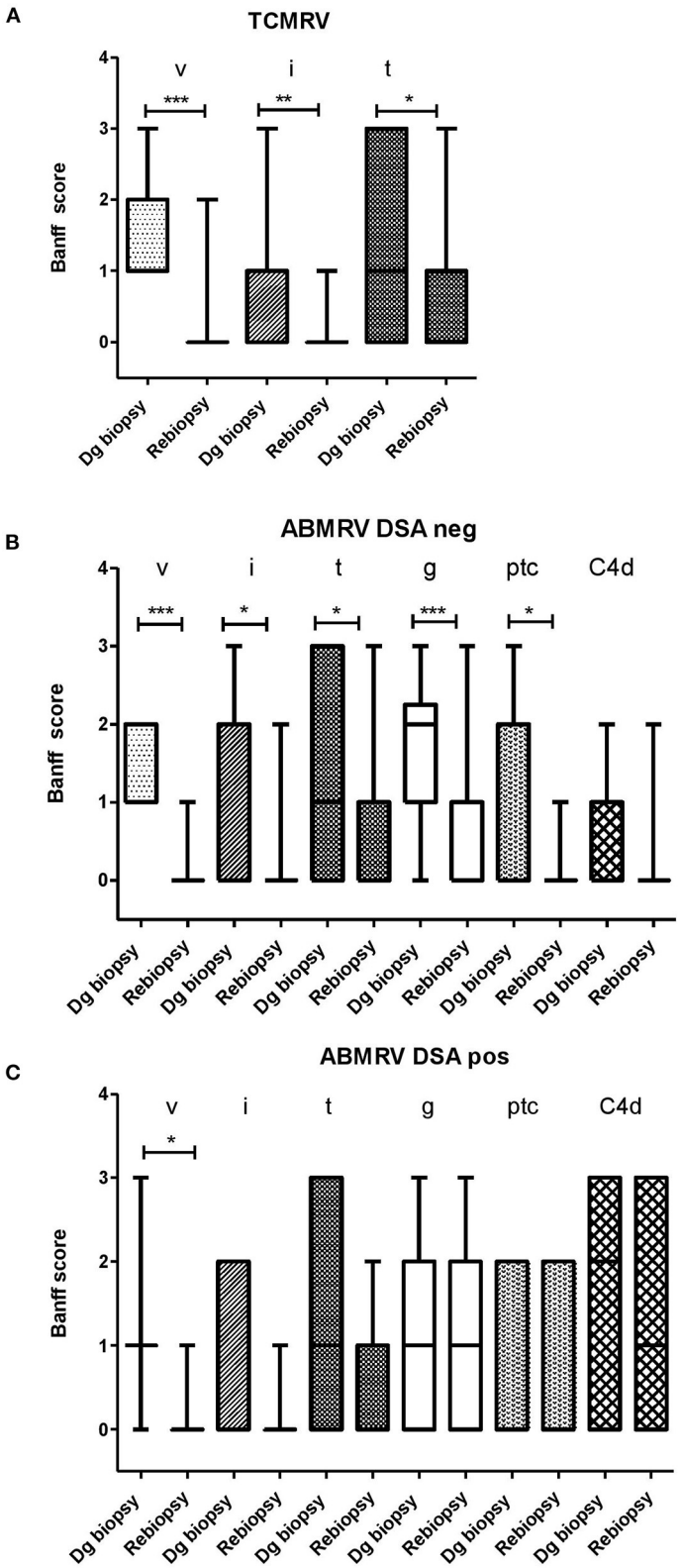

FIGURE 2 | The development of Banff score between diagnostic (dg biopsy) and rebiopsies in a subgroup of patients with consented rebiopsies. **(A)** TCMRV ($n = 21$) **(B)** ABMRV DSA– ($n = 22$) and **(C)** ABMRV DSA+ ($n = 15$). Differences were calculated by Wilcoxon matched-paired signed rank test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

biopsies revealed the resolution of rejection in 19 (90%) TCMRV cases, 14 (58%) ABMRV/DSA- cases, and only four (27%) ABMRV/DSA+ cases ($p = 0.006$). When comparing case and surveillance biopsies, patients with TCMRV had significantly improved i, t, and v scores, and DSA– patients with ABMRV had significantly improved in g, ptc, i, t, and v scores; however, in DSA+ ABMRV cases, only the v-score had improved (**Figure 2**, **Table 3**).

Development of TG

Significant differences between ABMR rejection phenotypes were found when we studied the development of the TG. For cause and surveillance, biopsies at the 3-year follow-up were included in the analysis. Estimate of the cumulative proportion of patients without TG (TG-free survival) at 36 months was 100% in TCMRV, 85% in ABMR/DSA-, 65% in ABMRV/DSA–, 54% in ABMR/DSA+, and 31% ABMRV/DSA+ (log rank $p < 0.001$). Pairwise comparisons showed a significantly longer time to TG in the TCMRV group than in other groups (log rank $p < 0.05$). Interestingly, DSA– ABMRV patients had a significantly shorter time to TG development than DSA– ABMR patients (mean for survival time without TG was 26 and 32 months, respectively) (log rank $p = 0.035$) (**Figure 3**).

Cox regression models were performed to identify risk factors for the development of TG in patients with histologic ABMR. Risk factors identified in the univariable analysis were included in the multivariable model (retransplantation status, DSA positivity, rATG therapy, rATG + PE/IVIG therapy, intimal arteritis at case biopsy, and C4d positivity). Multivariable model adjusted for the variables mentioned above found intimal arteritis to be associated with TG development (HR 2.1, $p = 0.01$) (**Table 4**).

Kidney Graft Survival

The graft survival was significantly impaired in DSA+ patients with intimal arteritis as a part of ABMR compared with others (**Figure 4**). Death-censored kidney graft survival at 36 months was 96% in TCMRV, 98% in ABMR/DSA–, 86% in ABMRV/DSA–, 79% in ABMR/DSA+, and 64% in the ABMRV/DSA+ group (log rank $p = 0.001$). Interestingly, DSA– patients with intimal arteritis as part of ABMR experienced similar graft survival as DSA+ patients with ABMR ($p = 0.507$).

Cox regression assessing risk factors for graft failure in ABMR revealed PRA > 20% (HR 4.4, $p = 0.001$), DSA positivity (HR 3.9, $p = 0.007$), retransplantation (HR 4.0, $p = 0.002$), induction with ATG and ATG with additional PE, IVIG (HR 4.1, $p = 0.021$), and C4d positivity (HR 2.5, $p = 0.041$) in the univariable analysis (**Table 5**). None of these variables were found to be significant in the multivariable model.

DISCUSSION

Intimal arteritis, a diagnostic feature of vascular rejection, is a frequent histological finding in kidney allografts (5, 8, 9). In this study, we found that intimal arteritis as part of histologic ABMR represents a risk for early development of TG regardless of the presence or absence of donor-specific anti-HLA antibodies. Therefore, a poor outcome of this phenotype is anticipated. On the other hand, we found that intimal arteritis as part of TCMR has a favorable kidney graft outcome when standard antirejection therapy is applied. Similar to our data, molecular assessment of kidney allografts revealed the early occurrence of isolated intimal arteritis of benign origin after transplantation (10–12).

Previously, vascular rejection, i.e., intimal arteritis, was characterized by a high rate of steroid-resistance and poor kidney allograft outcomes (4, 13). Just recently, the intimal arteritis was shown to be associated with DSA with the detrimental impact of this rejection phenotype on graft survival and was accepted as a diagnostic criterion for ABMR (5, 6).

Interestingly, in DSA– patients with intimal arteritis as part of histologic ABMR, the occurrence of TG was higher than in DSA– patients without intimal arteritis despite histologic ABMR. TG is a frequent morphological finding in chronic antibody mediated rejection, and thus, those patients are at the highest risk for premature kidney allograft loss (14).

In our study, the outcome of DSA negative patients with intimal arteritis along with MVI was similar to DSA+ patients with MVI, but without intimal arteritis. One of the possible explanations for this phenomenon is the hypothetical presence of non-HLA antibodies. The association of both MVI and intimal arteritis with anti-angiotensin II type 1 receptor antibodies has been discussed for more than a decade (15, 16). Besides anti-angiotensin II type 1 receptor antibodies, Delville et al. described the association of MVI and intimal arteritis with broader autoimmune reactivity measured *in vitro* by renal microvascular endothelial cells crossmatch assay (17). Very recently, NK cells were found to trigger MVI when a mismatch between donor HLA I and recipient inhibitory killer cell immunoglobulin-like receptor was present (18). This missing self- hypothesis may explain poor outcomes of DSA negative patients with microvascular inflammation. Whether such mechanisms are involved in intimal arteritis as well remains unclear.

Intimal arteritis as part of DSA– ABMR has not been entirely studied. A higher prevalence of AT1R positivity was found along with intimal arteritis. DSA– patients with AT1R positivity lost their grafts prematurely (19). On the other hand, a good prognosis of DSA negative patients with antibody mediated rejection histology was found (7). Graft survival of DSA negative patients with intimal arteritis was not specifically addressed in either of these studies.

There is a lack of studies dealing with graft outcomes in different phenotypes of vascular rejection, especially DSA– histologic ABMR with intimal arteritis, since the major Banff classification update in 2013 (6). Rabant et al. analyzed the outcome of patients with early isolated v-lesions and found better outcomes than in other phenotypes (10). Shimizu et al. observed similar outcomes in patients with intimal arteritis as part of both TCMR and ABMR; however, the study cohort was limited to 31 patients (20). Wu et al. compared the outcome of patients with intimal arteritis, classified both according to the grade of intimal arteritis and Banff classification and found that the grade plays a more important role than the Banff category (21). Salazar et al. reported nine out of 10 graft failures in patients with v-lesions (11). Patient numbers in all the mentioned studies above are small, which limits their conclusions. It is important to note that

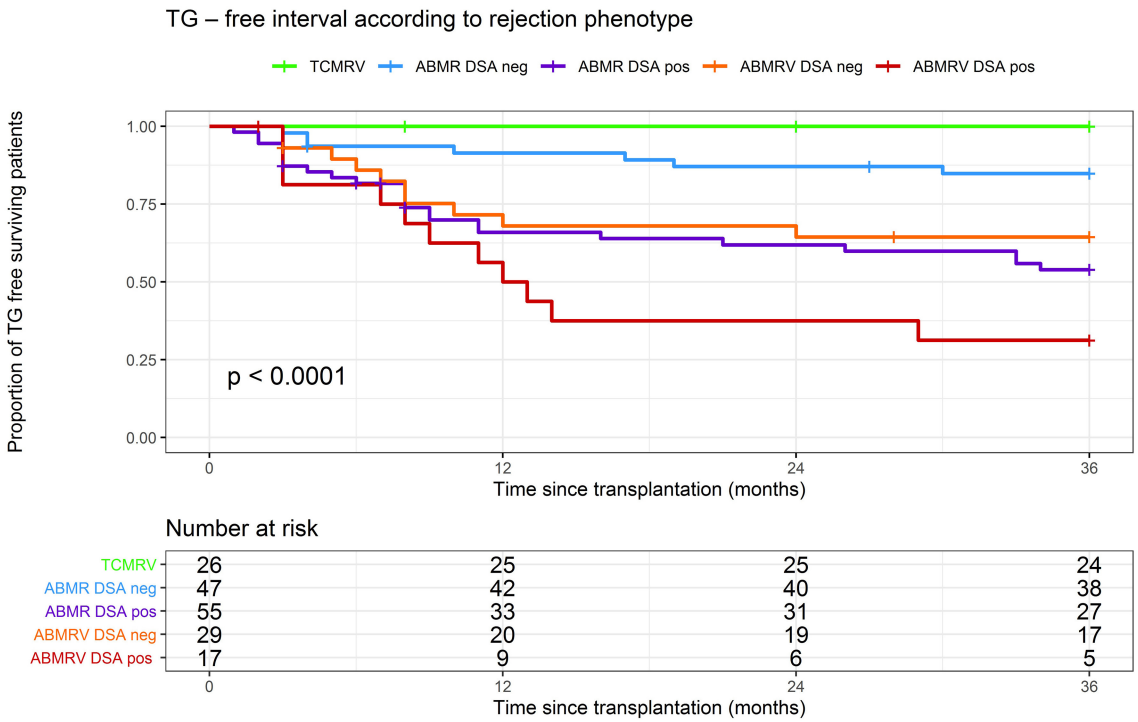

FIGURE 3 | Kaplan–Meier analysis of TG-free interval according to rejection phenotype.

TABLE 3 | Comparison of Banff scores between diagnostic and surveillance biopsies for patients with intimal arteritis.

	TCMRV			ABMRV/DSA–			ABMRV/DSA+		
	Diagnostic biopsy	**Rebiopsy**	***P*–value**	**Diagnostic biopsy**	**Rebiopsy**	***P*–value**	**Diagnostic biopsy**	**Rebiopsy**	***P*-value**
N	26	21		29	23		17	15	
g	25/1*/0/0	18/0/1/0	0.655	7/7/9/6	15/5/1/1	**0.001**	6/7/3/1	7/1/5/2	0.566
ptc	24/1/0/0	19/0/0/0	1.000	14/3/10/2	20/2/0/0	**0.015**	11/1/5/0	11/0/4/0	0.705
i	12/9/3/2	14/4/0/0	**0.005**	14/3/8/4	19/2/1/0	**0.021**	10/3/4/0	13/2/0/0	**0.045**
t	11/4/4/7	11/5/1/1	**0.010**	6/8/6/9	16/2/1/3	**0.018**	9/3/1/4	10/4/1/0	0.075
ti	11/9/4/2	13/4/1/0	**0.020**	3/8/13/5	14/3/4/1	**0.028**	6/4/3/4	5/6/1/3	0.928
v	0/19/6/1	18/0/1/0	**<0.001**	0/20/9/0	20/2/0/0	**<0.001**	0/14/2/1	12/3/0/0	**0.003**
ci	7/19/0/0	3/15/0/0	0.705	5/20/3/0	5/11/6/0	0.405	6/11/0/0	3/11/1/0	0.102
ct	4/21/1/0	0/19/0/0	1.000	1/24/3/0	3/13/6/0	0.480	0/17/0/0	0/14/1/0	0.317
cg	25/1*/0/0	19/0/0/0	0.317	26/1/2/0	16/4/2/0	0.180	15/2/0/0	8/4/2/1	**0.015**
cv	3/16/5/2	2/12/3/3	1.000	5/12/9/2	1/15/3/2	0.439	3/6/6/2	4/5/5/1	0.565
ah	1/21/4/0	3/10/5/1	0.739	3/17/8/1	3/14/5/0	0.527	4/6/7/0	1/9/5/0	0.608
C4d	20/5/0/1[#]	16/4/0/0	0.366	20/6/2/1	18/3/1/0	0.317	5/2/2/8	4/4/3/4	0.336
IF/TA	7/17/1/0	3/15/0/0	0.527	5/20/3/0	5/11/6/0	0.405	6/10/1/0	3/11/1/0	0.257

Differences were calculated by the Wilcoxon matched–paired signed–rank test.
**Suspicious for recurrence of the original disease.*
[#]ABO incompatible transplantation, assumption of accommodation.
Bold P-values represent statistic significance below 0.05.

the repeated updates of Banff classifications on ABMR make the interpretation of former studies problematic (22).

Limitations of our study were a short follow-up period which confined the number of study endpoints, and above all, we were not able to demonstrate the significant impact of vascular rejection in the absence of DSA on graft survival. The incomplete number of surveillance biopsies in the control groups was limiting

TABLE 4 | Cox regression assessing risk factors of development of transplant glomerulopathy (No. of events is 52) in histologic ABMR rejection categories (ABMR/DSA–, ABMR/DSA+, ABMRV/DSA–, ABMRV/DSA+).

Variable	Univariable analysis	*p*–value	Variable	Univariable analysis	*p*–value
	HR (95% CI)			**HR (95% CI)**	
ABMRV/DSA–	1.0 (0.5–2.0)	0.972	**Basiliximab**	0.4 (0.2–0.7)	**0.002**
ABMRV/DSA+	2.7 (1.4–5.2)	**0.004**	**rATG±PE/IVIG**	2.8 (1.4–5.5)	**0.002**
ABMR/DSA–	0.3 (0.1–0.6)	**0.001**	**Diagnostic biopsy**		
ABMR/DSA+	1.6 (1.0–2.8)	0.076	g>0	1.0 (0.5–2.1)	0.937
Donor (living)	0.5 (0.2–1.4)	0.179	ptc>0	1.4 (0.8–2.4)	0.256
PRA>20%	1.2 (0.7–2.1)	0.508	i>0	0.8 (0.4–1.5)	0.445
DSA positivity	2.7 (1.5–4.8)	**0.001**	t>0	1.3 (0.7–2.3)	0.351
HLA mm>3	1.4 (0.8–2.4)	0.244	ti>1	0.9 (0.5–1.6)	0.642
HD vintage> 3y	0.9 (0.5–1.5)	0.609	v>0	1.7 (1.0–2.9)	0.064
CKD diagnosis			ci>1	1.2 (0.4–3.9)	0.727
Diabetes	1.2 (0.6–2.6)	0.588	ct>1	1.2 (0.4–3.9)	0.727
Vascular	1.1 (0.6–2.2)	0.692	cg>0	NA	NA
Glomerulonefritis	0.7 (0.4–1.3)	0.277	cv>1	1.0 (0.6–1.7)	0.947
Hereditary	1.1 (0.6–2.1)	0.826	ah>1	1.1 (0.6–1.9)	0.724
Other	1.1 (0.5–2.3)	0.822	IF/TA>1	1.6 (0.6–4.3)	0.388
Retransplantation	1.8 (1.0–3.1)	**0.040**	C4d>1	1.7 (1.0–3.0)	0.068
	Multivariable analysis			**Multivariable**	
	HR (95% CI)			**HR (95% CI)**	
Retransplantation	0.8 (0.4–1.5)	0.395	**rATG ± PE/IVIG**	2.4 (1.0–5.6)	0.055
DSA positivity	2.1 (1.0–4.7)	0.064	**v>0**	2,1 (1.2–3.8)	**0.012**
			C4d>1	1.2 (0.6–2.3)	0.614

Bold P-values represent statistic significance below 0.05.

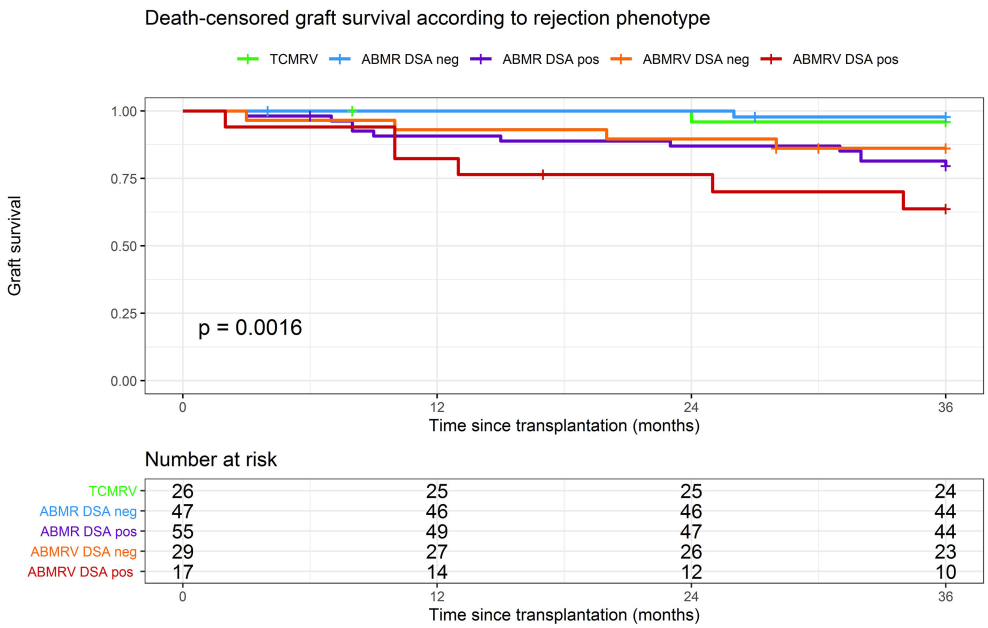

FIGURE 4 | Death-censored graft survival according to rejection phenotype.

direct comparison of all rejection phenotypes. The study did not involve the evaluation of non-HLA antibodies or transcriptomic data.

In conclusion, intimal arteritis along with MVI was found to represent a risk for the early development of TG regardless of the presence or absence of donor-specific anti-HLA

TABLE 5 | Cox regression assessing risk factors of graft failure (No. of events 23) in histologic ABMR rejection categories (ABMR/DSA–, ABMR/DSA+, ABMRV/DSA–, and ABMRV/DSA+).

Variable	Univariable analysis	*p*–value	Variable	Univariable analysis	*p*–value
	HR (95% CI)			**HR (95% CI)**	
ABMRV/DSA–	0.9 (0.3–2.7)	0.864	**Basiliximab**	0.2 (0.1–0.8)	**0.021**
ABMRV/DSA+	3.3 (1.3–8.6)	**0.011**	**rATG±PE/IVIG**	4.1 (1.2–14.1)	**0.021**
ABMR/DSA–	0.1 (0.0–0.7)	**0.020**	**Diagnostic biopsy**		
ABMR/DSA+	1.7 (0.8–4.0)	0.192	g>0	0.6 (0.2–1.5)	0.294
Donor (living)	0.3 (0.0–2.2)	0.239	ptc>0	1.6 (0.7–3.7)	0.295
PRA>20%	4.4 (1.8–10.8)	**0.001**	i>0	1.5 (0.6–3.4)	0.400
DSA positivity	3.9 (1.4–10.6)	**0.007**	t>0	1.3 (0.6–3.0)	0.574
HLA mm>3	1.5 (0.7–3.6)	0.334	ti>1	1.3 (0.6–3.2)	0.527
HD vintage> 3y	1.1 (0.5–2.5)	0.871	v>0	1.9 (0.9–4.6)	0.112
CKD diagnosis			ci>1	0.9 (0.1–6.7)	0.918
Diabetes	0.04 (0.0–7.0)	0.221	ct>1	0.9 (0.1–6.7)	0.918
Vascular	1.8 (0.7–4.5)	0.185	cg>0	0.04 (0.0–20.0)	0.314
Glomerulonefritis	1.3 (0.5–3.1)	0.570	cv>1	1.9 (0.8–4.3)	0.145
Hereditary	0.9 (0.3–2.6)	0.792	ah>1	1.5 (0.7–3.6)	0.312
Other	1.0 (0.3–3.2)	0.943	IF/TA>1	1.7 (0.4–7.6)	0.441
Retransplantation	4.0 (1.7–9.6)	**0.002**	C4d>1	2.5 (1.0–6.3)	**0.041**

Bold P-values represent statistic significance below 0.05.

antibodies. Therefore, it is likely that this phenotype reflects the presence of harmful endothelial injury of different origin than humoral alloimmunity.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data are stored as medical records of Institute for Clinical and Experimental Medicine, Prague, Czechia. Requests to access these datasets should be directed to Department of Nephrology, Institute for Clinical and Experimental Medicine.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Board of Institute for Clinical and Experimental Medicine approved the study under No.: 15-265191A. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MN: data gathering and processing and manuscript writing. PH: serum and sample storage and assessment and data processing. MK and LV: assessment of histological slides. KK and AS: HLA typing and anti-HLA antibodies evaluation. OV: manuscript writing and supervising. All authors contributed to the article and approved the submitted version.

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Predictive Potential of Flow Cytometry Crossmatching in Deceased Donor Kidney Transplant Recipients Subjected to Peritransplant Desensitization

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Recipient sensitization is a major risk factor of antibody-mediated rejection (ABMR) and inferior graft survival. The predictive effect of solid-phase human leukocyte antigen antibody testing and flow cytometry crossmatch (FCXM) in the era of peritransplant desensitization remains poorly understood. This observational retrospective single-center study with 108 donor-specific antibody (DSA)-positive deceased donor kidney allograft recipients who had undergone peritransplant desensitization aimed to analyze variables affecting graft outcome. ABMR rates were highest among patients with positive pretransplant FCXM vs. FCXM-negative (76 vs. 18.7%, $p < 0.001$) and with donor-specific antibody mean fluorescence intensity (DSA MFI) > 5,000 vs. < 5,000 (54.5 vs. 28%, $p = 0.01$) despite desensitization. In univariable Cox regression, FCXM positivity, retransplantation, recipient gender, immunodominant DSA MFI, DSA number, and peak panel reactive antibodies were found to be associated with ABMR occurrence. In multivariable Cox regression adjusted for desensitization treatment (AUC = 0.810), only FCXM positivity (HR = 4.6, $p = 0.001$) and DSA number (HR = 1.47, $p = 0.039$) remained significant. In conclusion, our data suggest that pretransplant FCXM and DSA number, but not DSA MFI, are independent predictors of ABMR in patients who received peritransplant desensitization.

Keywords: kidney, transplantation-kidney, HLA incompatibility, donor specific antibodies, rejection, flow cytometry, immunosuppression

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INTRODUCTION

Preformed antibodies directed against donor human leukocyte antigen (HLA) antigens represent a major obstacle in kidney transplantation, limiting both access to transplantation and kidney allograft survival (1, 2). It is widely accepted that kidney transplantation across donor-specific antibodies (DSA) identified either by solid-phase assays or flow cytometry crossmatch (FCXM) is associated with a higher risk of antibody-mediated rejection (ABMR) and inferior allograft outcomes, even in absence of positive complement-dependent cytotoxicity crossmatch (complement-dependent cytotoxicity crossmatch [CDC XM]) (3–7). Several transplant programs have implemented peritransplant desensitization regimens using T- and B-cell depleting antibody induction, peritransplant apheresis, and high-dose intravenous immunoglobulin (IVIg) to counteract the deleterious effects of preformed DSA (8). Despite intense strategies of desensitization, there is still an increased rejection risk, which may critically depend on the strength of the preformed DSA. Previously, the Viennese group used anti-thymocyte globulin (ATG) induction and peritransplant immunoadsorption (IA) as desensitization regimens in DSA-positive deceased donor kidney transplantation. The only predictor of antibody-mediated rejection found by this study was donor-specific antibody mean fluorescence intensity (DSA MFI) (9). FCXM may have several advantages over DSA MFI in terms of better predictive power to select grafts at risk of ABMR (10, 11). Moreover, kidney transplantation with a low level of DSA with or without a low positive B-cell FCXM was found to be associated with satisfactory outcomes in highly sensitized mostly living donor kidney transplant recipients who received depleting antibody induction and frequently also desensitization (12). In HLA incompatible deceased donor kidney transplantation with peritransplant desensitization, outcome predictors are poorly understood. Therefore, in this retrospective single-center observational cohort study, we assessed several variables to predict antibody-mediated rejection in those DSA positive deceased donor kidney transplant recipients who had undergone peritransplant desensitization.

PATIENTS AND METHODS

Study Design and Population

Our study was a single-center, cohort observational study with retrospective data analysis. A study flow chart is shown in **Figure 1**. All patients who underwent kidney transplantation from a deceased donor between January 2013 and April 2018 and had a positive DSA were included. Out of 1,153 allograft recipients who received a kidney transplant during the study period, 360 (31%) subjects had preexisting anti-HLA antibodies, and among those, 113 (9.8%) had one or more preformed DSA detected by solid-phase testing. The presence of circulating anti-HLA-A, -B, -C, -DR, and -DQ antibodies were annually screened using solid-phase testing. The arbitrary threshold for positivity was defined as 1000 MFI. Therefore, anti-HLA antibody specificities were accessible at the time of the transplant offer, and donor-specific antibodies were identified before transplantation based on historical Luminex assessment. Patients with DSA > 1,000 MFI and a negative current complement-dependent cytotoxicity crossmatch (CDC XM) were included (**Figure 1**). Immunodominant DSA was defined as the highest MFI from the last available pretransplant sera. Due to missing donor DQ typing, the anti-DQ antibodies were not traced as DSA and, therefore, excluded from the analysis. Patients were followed until the allograft loss or end of the follow-up; the median follow-up was 1,110 days. Histological diagnosis of ABMR was defined according to the latest Banff criteria (13). The design of this retrospective observational study was approved by The Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital while ensuring the anonymity and confidentiality of the data (listed as No. A-19-24).

Immunosuppression and Desensitization

All patients initially received triple-drug maintenance immunosuppression based on tacrolimus (Advagraf, Astellas, 0.2 mg/kg/day, target trough levels within the first 14 days at 8–15 ng/ml), mycophenolate mofetil (Cellcept, Roche, 2 g/day or generics) or enteric-coated mycophenolic acid (Myfortic, Novartis, 1440 mg/day), and tapered prednisone (initial dose 20 mg tapered to 5 mg at 3 months). All but two patients received rabbit anti-thymocyte globulin (Thymoglobuline, Genzyme, first dose of 1.5 mg/kg initiated before reperfusion, a total cumulative dose aimed at 5–7 mg/kg) induction immunosuppression. Patients received desensitization protocol mostly with plasmapheresis and IVIg, patients at highest risk received rituximab in addition. Details on desensitization strategies are given in **Figure 1**, **Table 3**. Further analyses were adjusted for desensitization strategy to account for the heterogeneity of applied treatments.

Recipients diagnosed with ABMR were treated with high dose steroids, plasmapheresis (1 plasma volume; 5–10 sessions per patient), and IVIg administration (0.5 g/kg) after each session. In the cases of refractory ABMR, bortezomib (Velcade, Johnson & Johnson) was administered as previously described in detail (11).

Pretransplant FCXM and Luminex

Donor splenocytes (50 μl, 5×10^4 cells) were incubated with patient or negative control sera (50 μl) for 30 min at 21°C. After washing three times in PBS (400 × *g*, 5 min), samples were incubated at 21°C for 30 min in an antibody cocktail – 5 μl anti-CD45-KO (Beckman Coulter, IN, USA), 5 μl anti-CD3-PE (Becton Dickinson, SJ, USA), 5 μl anti-CD19-PC5 (Beckman Coulter, IN, USA), and goat anti-human IgG-FITC (Jackson ImmunoResearch, West Grove, PA, USA), again washed, fixed (Cellfix), and measured on a Navios flow cytometer (Beckman Coulter, IN, USA). Fluorescein (FITC) fluorescence of patient

Abbreviations: ABMR, antibody-mediated rejection; CDC XM, complement-dependent cytotoxicity crossmatch; DD, deceased donor; DGF, delayed graft function; DSA, donor-specific antibody; eGFR, estimated glomerular filtration; FCXM, flow cytometry crossmatch; HD, haemodialysis; HLA, human leukocyte antigen; HLAi, HLA incompatible; IVIg, intravenous immunoglobulin; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MVA, multivariate analysis; PD, peritoneal dialysis; PRA, panel reactive antibodies; rATG, rabbit anti-thymocyte globulin; RTX, rituximab; FITC; fluorescein.

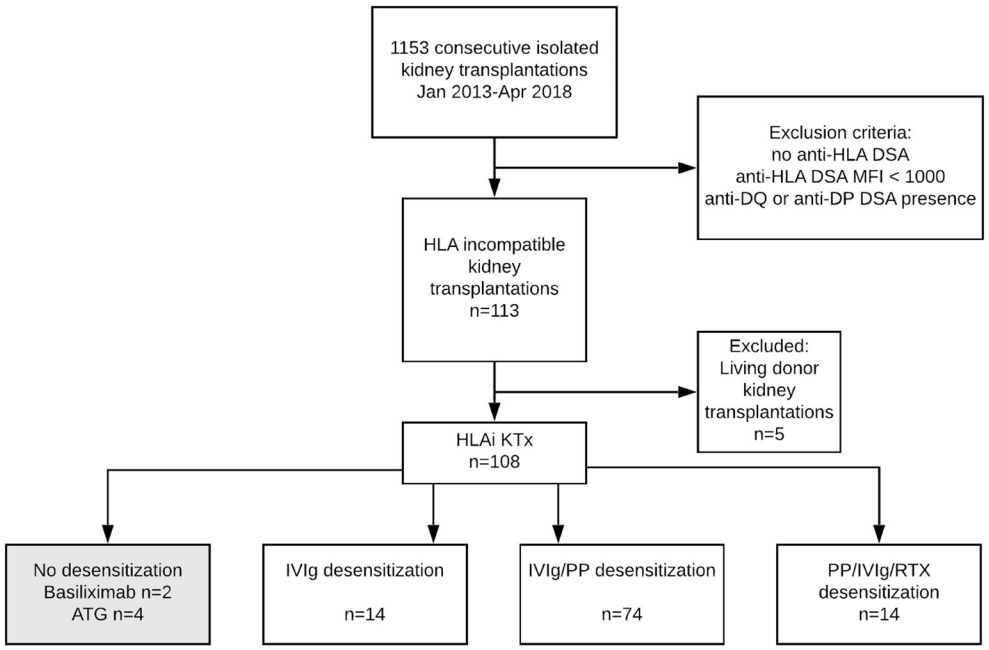

FIGURE 1 | Flow diagram of kidney transplant recipients included in the study and study cohort's definition. DSA, Donor-specific Antibodies; HLA, Human Leukocyte Antigen; KTx, Kidney Transplantation; ATG, Antithymocyte Globulin; IVIg, Intravenous Immunoglobulin; PP, Plasmapheresis; RTX, Rituximab.

samples was compared with the mean fluorescence of negative control samples. Cut off point was calculated as the ratio between the mean fluorescence intensity of samples and negative control sera. Cut off for T cells was 2 and for B cells was 2.5, respectively.

The FCXM positivity was defined as T-cell and/or B-cell FCXM positivity. The serum used for FCXM was obtained immediately pretransplant and was used for CDC XM.

The specificity of HLA antibodies was defined by LABScreen Mixed and Single Antigen (SAB) class I and class II beads (OneLambda Inc., CA, USA). The assay was performed according to the manufacturer's protocol. Samples were analyzed by the LabScan3D flowanalyzer (One Lambda Inc., CA, USA) using the HLA Fusion software (version no. 4.6). For the evaluation of DSA, beads with raw MFI values > 1,000 were considered to be positive. All sera were pretreated with EDTA in a validated laboratory procedure.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5, Version 5.03 (GraphPad Software, Inc., CA, USA) and IBM SPSS Statistics, Version 24 (International Business Machines Corp., NY, USA). Means and SDs or medians with min and max were used to describe continuous variables. Categorical variables are expressed as *n* and a percentage of the total. Survival analysis was performed by the Kaplan–Meier method, and with differences between groups compared using the log-rank test. The Kaplan–Meier curve was also used to express the ABMR-free interval, defined as the time between transplantation and biopsy-proven active ABMR. Death-censored allograft survival rates are also reported in this study. Univariable and multivariable Cox regression models were used to predict the odds of ABMR, the latter adjusted for applied desensitization treatment and all variables from univariable analysis with $p < 0.01$ (peak panel reactive antibodies (PRA), retransplantation, immunodominant DSA MFI, and DSA number). The area under the curve (AUC) for univariable and multivariable Cox regression was calculated using 10-fold cross-validation (14). A p-value of < 0.05 was considered statistically significant.

RESULTS

Baseline Characteristics

The study population consisted of 108 deceased donor allograft recipients subjected to peritransplant desensitization. Five living donor transplant recipients who underwent desensitization ahead of scheduled transplantation were excluded from analysis (**Figure 1**). The baseline characteristics of the study population are given in **Table 1**. Pretransplant immunological characteristics of recipients are shown in **Tables 2**, **3**. The majority of recipients had anti-HLA class I DSA ($n = 75$, 69%) whereas 13 (12%) of the recipients had class II DSA. Twenty patients (18.5%) had both DSA class I and class II DSA (**Table 2**). Pretransplant FCXM positivity was identified in 35 recipients (32.4%). Isolated T-cell FCXM positivity was observed in 3 out of 35 (8.6%), isolated B-cell FCXM positivity in 16 out of 35 (45.7%) and both T-cell and B-cell FCXM were observed in 16 out of 35 patients (45.7%), respectively. Sixty patients (55.6%) had only 1 preformed DSA, 37 (34.2%) had 2 DSAs, and 11 (10.2%) had 2–5 DSAs.

TABLE 1 | Baseline demographic and clinical characteristics of the study population.

Patient characteristics ($n = 108$)	
Recipient characteristics	
Age, years, median [min, max]	53 [23,79]
Sex male, *n (%)*	54 (50%)
ESRD causes	
Glomerulonephritis, ns, *n (%)*	48 (44.4%)
Interstitial nephropathy, *n (%)*	27 (25%)
Polycystic kidney disease, *n (%)*	11 (10.2%)
Hypertension, *n (%)*	7 (0.06%)
Other, *n (%)*	12 (11.1%)
Undetermined, *n (%)*	3 (0.03%)
Dialysis vintage, months, median [min, max]	40 [0,137]
Type of dialysis treatment	
Haemodialysis, *n (%)*	86 (79.6%)
Peritoneal dialysis, *n (%)*	11 (10.2%)
Combination of HD and PD, *n (%)*	9 (8.3%)
Preemptive transplantation, *n (%)*	2 (1.9%)
Donor characteristics	
Age, years, median [min, max]	52 [1,80]
Sex male, *n (%)*	62 (57.4%)
Donor type	
Deceased donor, *n (%)*	108 (100%)
ECD donor, *n (%)*	43 (39.8%)
Transplant baseline characteristics	
Retransplantation, *n (%)*	65 (60.2%)
1 prior graft, *n (%)*	47 (43.5%)
2 prior graft, *n (%)*	15 (13.9%)
3 prior graft, *n (%)*	3 (2.8%)
Cold ischemia time, hour, median [min, max]	16 [4,28]
DGF[a], *n (%)*	25 (23%)
Induction and desensitization	
IA/PP	88 (81.5%)
IVIg	99 (91.7%)
Rituximab	13 (12%)
ATG	106 (98.1%)
Basiliximab	2 (1.6%)

Data are presented as median [min, max] or number (%). ESRD, End-stage Renal Disease; HD, Haemodialysis; PD, Peritoneal Dialysis; ECD, Extended Criteria Donor; DGF, Delayed Graft Function; IA, Immunoadsorption; PP, Plasmapheresis; IVIg, Intravenous Immunoglobulins; ATG, Anti-Thymocyte Globuline. [a]Delayed graft function was defined as the need for dialysis during the first-week post-transplant.

TABLE 2 | Immunological characteristics of human leukocyte antigen incompatible kidney transplant recipients.

Immunological status	
PRA last, %, mean [SD]	21.3 [26.4]
PRA max, %, median [min, max]	31 [0,100]
HLA mismatch, median [min, max]	4 [0,6]
FCXM positivity, *n (%)*	35 (32.4%)
Pretransplant DSA	
Immunodominant class I	75 (69.4%)
Immunodominant class II	13 (12%)
Both DSA classes I and II	20 (18.5%)
Immunodominant DSA MFI, median [min, max]	3,344 [1,036, 20,793]
Number of DSA, median [min, max]	1 [1,5]

Data are presented as mean [standard deviation (SD)] or median [min, max] or n (%). PRA, Panel Reactive Antibodies; HLA, Human Leukocyte Antigen; FCXM, Flow Cytometry Crossmatch; DSA, Donor-specific Antibodies; MFI, Mean Fluorescent Intensity.

TABLE 3 | Pretransplant immunological characteristics of recipients receiving desensitization.

Immunological status	**IVIg ($n = 14$)**	**PP/IVIg ($n = 74$)**	**PP/IVIg/RTX ($n = 14$)**	***p*-value**
PRA max, %, mean [SD]	24 [27.9]	41.6 [31.6]	45.8 [32.9]	0.0997
HLA mismatch, mean [SD]	3.2 [0.7]	3.6 [1.4]	3.7 [1.1]	0.5077
FCXM positivity, *n (%)*	0 (0)	24 (32.4)	11 (78.6)	**<0.0001**
MFI, median [IQR]	1,948 [1,194, 2,727]	3,477 [2,150, 5,491]	5,872 [4,996, 10,938]	**<0.0001**
Number of DSA, mean [SD]	1.3 [0.5]	1.5 [0.7]	2.4 [1.4]	**0.0227**

Data are presented as mean [SD], median [IQR] or number (%). PRA, Panel Reactive Antibodies; HLA, Human Leukocyte Antigen; FCXM, Flow Cytometry Crossmatch; MFI, Mean Fluorescence Intensity; DSA, Donor-specific Antibodies; SD, Standard Deviation; IQR, interquartile range. Bold values indicates statistically significant.

Two patients had no documented sensitizing events. About 63/108 (58.3%) recipients experienced previous transplantation. A total of 69/108 (63.9%) patients had received the previous transfusion. About 46 of 54 (85.2%) women were previously pregnant.

Risk of ABMR

Biopsy-proven ABMR (active or chronic active) in indication or protocol biopsies was found in 38 out of 108 patients (35%) within the first 3 years posttransplant. The median time until the first ABMR occurrence was 11 days [min 5, max 1,078]. ABMR rates were highest among patients with immunodominant DSA MFI > 5,000 vs. < 5,000 [18 out of 33 (54.5%) vs. 20 out of 75 (26.6%), p = 0.01]. ABMR incidence was higher among patients with a positive vs. negative FCXM [26 out of 35 (74.3%) vs. 12 out of 73 (16.4%), $p < 0.001$], in patients with retransplantation vs. first transplantation [27 out of 63 (42.8%) vs. 11 out of 45 (24.4%), $p = 0.05$] and in patients with total DSA number > 2 vs. those with DSA number ≤ 2 [7 out of 11 (63.6%) vs. 31 out of 97 (32%), p = 0.04].

Three-year death-censored ABMR-free interval showed significantly shorter ABMR-free interval in patients with immunodominant MFI > 10,000 (log-rank $p = 0.0045$), with a higher number of DSAs (log-rank $p = 0.0045$) and in patients with pretransplant FCXM positivity (log-rank $p < 0.0001$, **Figure 2**).

In univariable Cox regression analysis, FCXM positivity (HR = 6.5, $p < 0.001$), retransplantation status (HR = 3.13, $p = 0.003$), recipient gender (HR = 2.1, $p = 0.026$), immunodominant DSA MFI (HR = 1.99, $p < 0.001$), DSA number (HR = 1.6, $p < 0.001$), and peak PRA (HR = 1.02, $p < 0.001$) were found to be associated with ABMR occurrence (**Table 4**).

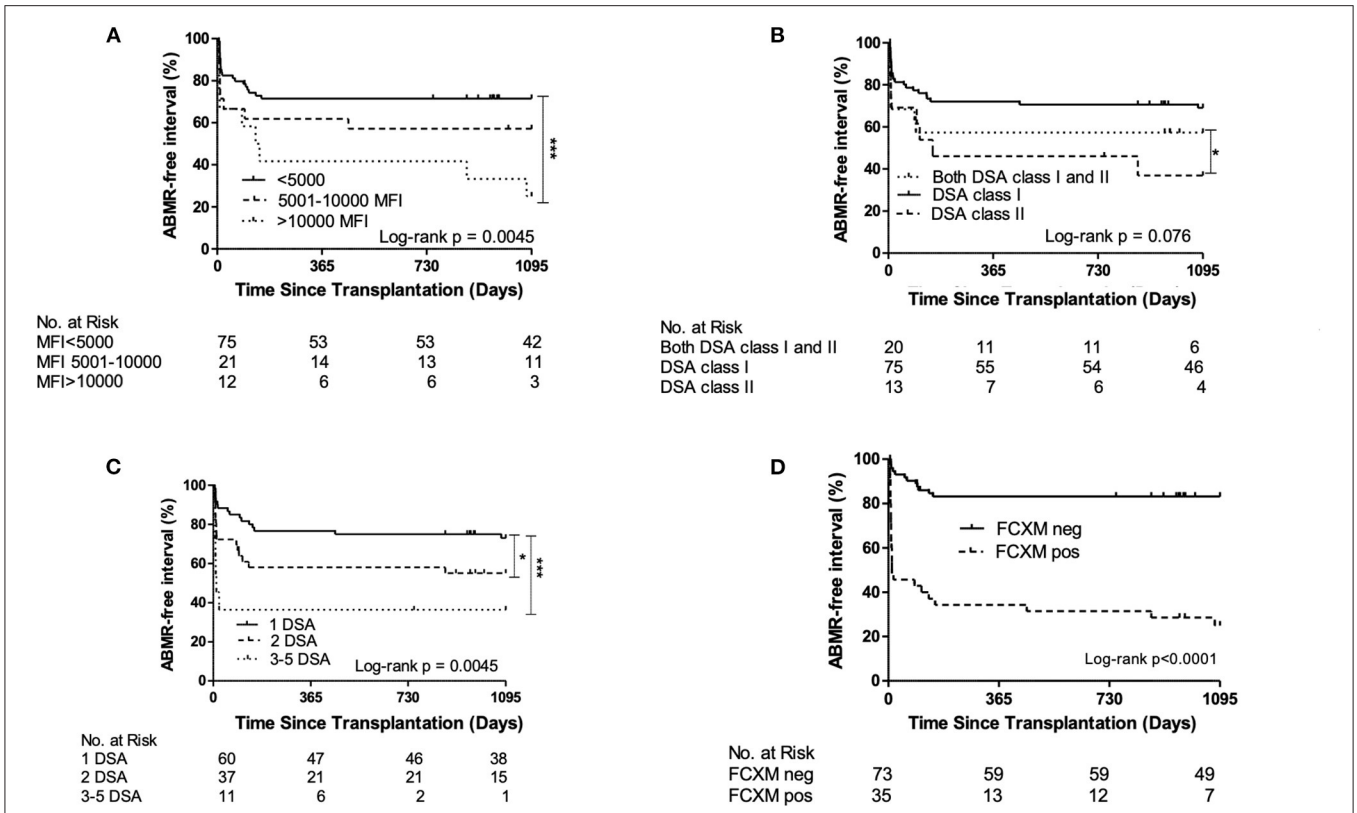

FIGURE 2 | Three-year death-censored antibody-mediated rejection (ABMR)-free interval displaying significantly higher incidence of ABMR in recipients with **(A)** donor-specific antibodies _(DSA) immunodominant mean fluorescence intensity (MFI) > 10,000, **(B)** DSA class II **(C)** higher DSA number and **(D)** patients with positive flow cytometry crossmatch (FCXM). *$p < 0.05$; ***$p < 0.001$.

The risk of ABMR in pretransplant positive FCXM remained significant also for patients with class I DSA positivity (HR = 9.63, 95% *CI* = 3.89–22.53, $p < 0.001$) and for those with MFI < 5,000 (HR = 6.7, 95% *CI* = 2.75–16.32, $p < 0.001$).

In multivariable Cox regression model adjusted for desensitization regimen, associations remained significant for FCXM positivity (HR = 4.6, $p = 0.001$) and DSA number (HR = 1.47, $p = 0.039$) (**Table 3**). Multivariable Cox model increased mean AUC (calculated in 10-fold crossvalidation) for pretransplant FCXM positivity from 0.7 to 0.8 (**Table 5**).

Flow cytometry crossmatch-positive patients had significantly higher immunodominant DSA MFI (**Figure 3**, $p < 0.0001$). This correlation was, however, not perfect (15, 16) as 15 out of 75 (20%) of FXCM-negative subjects had immunodominant DSA MFI > 5,000 (3 subjects with DSA-MFI > 10,000). Similarly, in FCXM-positive patients, 4 out of 33 (12%) had immunodominant DSA-MFI < 2,500.

Graft Survival

Three-year death-censored graft survival was shorter in patients with a positive FCXM ($p = 0.011$) and those with both DSA classes I and II ($p = 0.04$) or a higher number of DSA ($p < 0.001$) (**Supplementary Figure 1**).

DISCUSSION

Kidney transplantation across the HLA barrier is associated with an inferior allograft outcome. It is widely acknowledged that the presence of preformed DSA before transplantation increases the probability of ABMR occurrence (12–15). Strategies, namely, peritransplant desensitization, were implemented in many centers to prevent such an adverse outcome (17–19).

In this retrospective single-center analysis, we evaluated the potential of several clinical and immunological risk factors to predict ABMR in patients who had received deceased donor kidney allograft and in whom the peritransplant desensitization was applied due to the current presence of donor-specific antibodies. We found that pretransplant FXCM and the number of DSAs, but not MFI, are the most reliable tools for ABMR prediction.

It has been widely accepted that the prognostic value of DSA is limited (20, 21). First, Luminex-based DSA determination shows HLA antibody specificity to antigen but no information can be provided in regardsto epitope specificity (22, 23). Second, the Luminex method is unsuitable for exact value measurement because its MFI values are semiquantitative, not presenting the exact titer of antibodies; furthermore, the prozone effect must be taken into account, non-anti-HLA antibodies may interfere with the beads, and the previously

TABLE 4 | Risk factors of antibody-mediated rejection (ABMR) occurrence in donor-specific antibody plus patients in univariable and multivariable Cox regression model.

	Univariable Cox regression			Multivariable Cox regression		
	***p*-value**	**HR**	**95% *CI***	***p*-value**	**HR**	**95% *CI***
Recipient age, years	0.025	0.97	0.95–1.00			
Recipient gender, male	0.017	2.26	1.15–4.42			
Donor age, years	0.957	1.00	0.98–1.02			
Dialysis vintage, months	0.128	1.01	1.00–1.02			
Cold ischemia, hours	0.259	1.05	0.96–1.15			
Peak PRA	**<0.001**	**1.02**	1.01–1.03	0.139	1.01	0.99–1.02
HLA mismatch	0.178	1.18	0.93–1.51			
Retransplantation	**0.002**	**3.51**	1.60–7.66	0.177	1.87	0.75–4.63
Immunodominant DSA MFI (increase for each 5,000 MFI)	**<0.001**	**2.05**	1.44–2.92	0.870	0.96	0.61–1.52
DSA number	**0.001**	**1.62**	1.22–2.16	**0.025**	**1.52**	1.05–2.18
Both DSA classes I and II	0.363	1.44	0.66–3.14			
FCXM positivity	**0.000**	**7.31**	3.67–14.58	**<0.001**	**5.47**	2.22–13.49
Rituximab	**0.002**	**3.20**	1.51–6.79	0.891	1.06	0.48–2.34
IA/PP	0.122	2.27	0.80–6.39	0.373	0.56	0.15–2.02
IVIg	0.370	1.92	0.46–7.97	0.506	0.58	0.11–2.92
DSA DQ[a]	**<0.001**	**3.32**	1.67–6.59			

To final model, all significant variables from univariable Cox regression were entered and the model was adjusted to desensitization protocol. IA, Immunoadsorption; PP, Plasmapheresis; IVIg, Intravenous Immunoglobulins, PRA, Panel Reactive Antibodies; HLA, Human Leukocyte Antigen; FCXM, Flow Cytometry Crossmatch; MFI, Mean Fluorescence Intensity; DSA, Donor-specific Antibodies. [a]Subgroup analysis from available data (n = 40), not included in the multivariate analysis model. Bold values indicates statistically significant.

mentioned epitopes may be shared between the beads (24, 25).

In many centers, the FCXM assessment is not implemented as a 24/7 service, and therefore, there is a lack of information on whether FCXM outperforms DSA-based risk stratification. Thus, studies on FCXM are not consistent in respect of graft outcomes (10, 26, 27). Our data are similar to Couzi et al. (10), where higher rejection occurrence in patients with both DSA and FCXM positivity prior to transplantation was observed.

Our data from protocol biopsies suggest future long-term outcomes to be inferior as patients with positive pretransplant FCXM exhibit frequent subclinical rejections in 3-month protocol biopsies. However, it is well known that sensitized patients who have undergone desensitization and HLA incompatible living donor kidney transplantation have substantial survival benefits compared to those patients who did not undergo transplantation and those who waited for transplants from deceased donors (28). However, the overall incidence of ABMR in those patients was high when 24 out of 267 patients developed severe oliguric early ABMR treated with eculizumab or splenectomy and transplant glomerulopathy occurred later (29).

Data on HLA incompatible deceased donor kidney transplantation are scarce. In their pioneer work, the Vienna group evaluated the outcomes of 101 HLA incompatible kidney transplantation (9) who had undergone IA-based desensitization prior and after transplantation along with rabbit anti-thymocyte globulin. Most DSA+ patients had a negative CDC XM already before IA. Three-year death-censored graft survival in DSA+ patients was 79 and 33% of patients experienced ABMR. The authors described only a trend toward higher ABMR rates in positive baseline CDCXM while those patients with DSA MFI >15,000 experienced ABMR in 71%. Similarly to our study, Amrouche et al. (30) described in 95 patients, who received similar posttransplant desensitization due to DSA MFI levels >3,000 while negative complement-dependent cytotoxicity-negative crossmatch, satisfactory long-term outcomes: the 3-year

TABLE 5 | Area under curve for uni- and multi-variable Cox regression for ABMR risk in 10-fold crossvalidation.

	Mean AUC	**SD**
Univariable Cox model		
FCXM positivity	0.729	0.04
Peak PRA	0.668	0.02
Immunodominant DSA MFI	0.618	0.03
Retransplantation	0.626	0.04
Total DSA number	0.583	0.04
Multivariable Cox model		
(FCXM positivity, peak PRA, Immunodominant DSA MFI, retransplantation, total DSA number adjusted for desensitization treatment)	0.810	0.03

AUC, Area Under Curve; SD, Standard Deviation; PRA, Panel Reactive Antibodies; FCXM, Flow Cytometry Crossmatch; MFI, Mean Fluorescence Intensity; DSA, Donor-specific Antibodies.

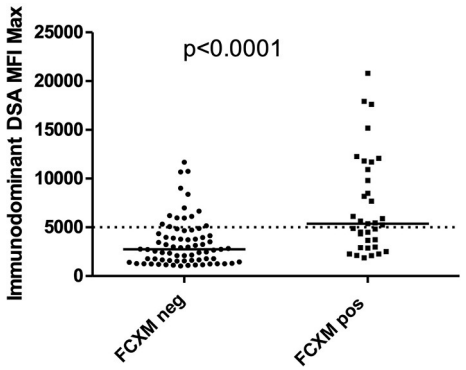

FIGURE 3 | Patients with FCXM positive have significantly higher immunodominant DSA MFI compared to patients with FCXM negative. Nevertheless, 14 out of 73 (20%) of FXCM negative and 19 out of 35 (54%) patients with FCXM positive had immunodominant donor-specific antibody mean fluorescence intensity (DSA MFI) > 5,000, 5 out of 35 (14%) patients with FCXM positive had immunodominant DSA MFI < 2,500.

death-censored allograft survival rates were 91%, and recipient survival rates were 93%, respectively. Those data are similar to our FCXM-negative cohort. Of note, the incidence of ABMR in the Amrouche study remained high and was detected in 32% of recipients which is similar to our FCXM negative cohort. Contrary to expectations, in our study, the FCXM positive cohort presented with far poorer outcomes in terms of ABMR incidence and 3-year graft outcomes. Therefore, it is likely that our HLA incompatible cohort was at a far higher risk in comparison with others. This fact also points out the necessity of more advanced risk stratification ahead of transplantation which may allow successful kidney transplantation even in patients with DSA MFI over 5,000. Our study shows the association of pretransplant FCXM with ABMR also in patients with MFI < 5,000 or with class I DSA. Therefore, it is likely that pretransplant FCXM positivity outperforms any DSA level from historical sera.

Based on the presented data, we have already modified a pre-transplant risk assessment in our center. In all kidney transplant recipients with present preformed DSA, the positive FCXM prior to transplant represents a veto for transplantation. Unpublished data suggest on far lower incidence of acute ABMR when this approach was implemented. It is, however, also likely that those patients with repeated positivity of FCXM prior to transplant are being trapped on the waiting list for a significant period of time, although the general waiting time to transplant is much shorter in comparison to other countries (31). Of note, described the poor outcome of HLA incompatible transplantation with pretransplant FCXM positivity justifies longer waiting time for more compatible donors as there is a lack of available organs and better allocation would finally increase the patient-years with functioning grafts. Some of those sensitized patients may also find their compatible donor in the case of kidney pair donation when a living donor is available (32).

The limitation of this study is the lack of DSA DP and DQ assessment, as donor DP and DQ typing were not available in the whole cohort.

In conclusion, our data suggest that pretransplant FCXM and DSA number, but not DSA MFI, are independent predictors of ABMR in patients who received peritransplant desensitization. We, therefore, suggest the implementation of FCXM assessment in a daily routine ahead of DSA positive deceased donor kidney transplantation when peritransplant desensitization is planned.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital (listed as No. A-19-24). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OV and GB designed the research. KO, GB, and OV wrote the manuscript. KO and PH collected the data. KO, OV, JS, AS, JM, and TM performed the research. KK provided the FCXM sera analysis. KO, PH, and JK participated in the data analysis and OV supervised the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.780636/full#supplementary-material

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Follow-Up of Blood Pressure, Arterial Stiffness, and GFR in Pediatric Kidney Transplant Recipients

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Pediatric renal transplant recipients (RTx) were studied for longitudinal changes in blood pressure (BP), arterial stiffness by pulse wave velocity (PWV), and graft function.

Patients and Methods: 52 RTx patients (22 males) were included; office BP (OBP) and 24 h BP monitoring (ABPM) as well as PWV were assessed together with glycemic and lipid parameters and glomerular filtration rate (GFR) at 2.4[1.0–4.7] (T_1) and 9.3[6.3–11.8] years (T_2) after transplantation (median [range]).

Results: Hypertension was present in 67 and 75% of patients at T_1 and T_2, respectively. Controlled hypertension was documented in 37 and 44% by OBP and 40 and 43% by ABPM. Nocturnal hypertension was present in 35 and 30% at T_1 and T_2; 24 and 32% of the patients had masked hypertension, while white coat hypertension was present in 16 and 21% at T_1 and T_2, respectively. Blood pressure by ABPM correlated significantly with GFR and PWV at T_2, while PWV also correlated significantly with T_2 cholesterol levels. Patients with uncontrolled hypertension by ABPM had a significant decrease in GFR, although not significant with OBP. Anemia and increased HOMAi were present in ∼20% of patients at T_1 and T_2.

Conclusion: Pediatric RTx patients harbor risk factors that may affect their cardiovascular health. While we were unable to predict the evolution of renal function based on PWV and ABPM at T_1, these risk factors correlated closely with GFR at follow-up suggesting that control of hypertension may have an impact on the evolution of GFR.

Keywords: hypertension, arterial stiffness, ABPM, PWV, transplantation

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INTRODUCTION

Pediatric kidney transplant recipients (RTx) have a decreased life expectancy. Although recent studies have shown an improvement in long term survival after RTx, it remains 20–25 years shorter compared to the general population (1). Cardiovascular disease (CVD) is the leading cause of mortality, accounting for 22–36% of all deaths (2, 3).

Hypertension (HT) is a common condition in RTx, with a prevalence reaching up to 80% (4). While major cardiovascular (CV) events are rare in RTx, changes in vascular wall structure may

nonetheless already be present in early stages of chronic kidney disease (CKD). Previous studies have confirmed a correlation between the severity of hypertension and cardiac morbidity as well as the evolution of graft function, suggesting that adequate control of blood pressure could improve cardiovascular health and kidney graft survival both in adults and children (4–6).

Twenty-four hour ambulatory blood pressure monitoring (ABPM) is the most suitable method for the diagnosis and follow-up of hypertension, as it can identify masked [elevated blood pressure (BP) occurring outside the clinical setting] and isolated nocturnal hypertension (hypertension during sleep only), as well as blunting of the normal nocturnal dip (6). Both nocturnal hypertension and non-dipping is associated with an elevated risk of CV events in adults (7–9), as well as in children (10–12).

Pulse wave velocity (PWV) is a validated marker of vascular damage in adult CKD patients, and a predictor of CV events. Such link is yet to be established in children. Elevated arterial stiffness is a common finding in pediatric end-stage renal disease (ESRD) patients (13, 14), contributing to overall morbidity and mortality.

The aims of the present study were to (i) assess the prevalence of hypertension as well as the presence of known metabolic CV risk factors and of increased arterial stiffness in a pediatric RTx cohort, (ii) describe longitudinal changes in ABPM and PWV, and (iii) identify associations between blood pressure, arterial stiffness, and graft function.

PATIENTS AND METHODS

Patients and Study Design

Of the eighty-seven patients controlled at our outpatient clinic, 52 RTx were available for the study. Inclusion criteria were a functioning allograft, with stable kidney function, without rejection episodes for at least 6 months prior to testing, absence of history of overt cardiovascular disease and an evaluable data set for both the first and second follow-up examinations.

Patients were assessed twice. A first cross-sectional checkup (T_1) was followed by a second after ∼6 years follow-up (T_2). At both visits, standard yearly examinations were performed including evaluation of biometric data, laboratory tests, ABPM and PWV measurements (see below for details). Doppler renal ultrasound was part of the annual assessment and no renal artery stenosis was observed.

Methods

Biometric data, etiology of ESRD, history of dialysis and transplantation, as well as immunosuppressive and antihypertensive medications were collected from medical charts. Height, weight, and BMI Z-scores were calculated using the CDC/WHO growth charts with overweight being defined as BMI >95 percentile (15, 16).

Laboratory data including creatinine, hemoglobin (Hgb), serum calcium (Ca), and phosphate (P), insulin resistance characterized by the HOMA index, as well as lipid profile at the time of follow-up were retrieved for analysis from the medical records.

HOMA index was calculated as fasting insulin multiplied by fasting glucose level divided by 22.5, as described by Matthews et al. (17). A cut-off level of >2.83 was used as described by Tresaco et al. (18). Diabetes mellitus (DM) was defined as a fasting glucose level >7 mmol/l or a 2-h plasma glucose level >11.1 mmol/l, based on the 2006 WHO criteria (19).

The revised Schwartz formula was used to calculate the glomerular filtration rate (GFR) (20). Proteinuria was defined as a urine protein/creatinine ratio greater than 200 mg/g (21).

Blood Pressure Measurements

Brachial blood pressure and heart rate were measured by a validated automated oscillometric device (Omron M4, Omron Healthcare, Kyoto, Japan) in sitting position using appropriate cuff sizes, with the mean of three measurements being used for analysis. OBP results are presented as absolute values and height-based Z-scores (22).

ABPM was performed by using a validated automated oscillometric device (ABPM-04, Meditech Kft., Budapest, Hungary) (23). BP was measured at 20-min intervals during the day, and every 30 min during the night (23, 24). The mean ambulatory BP for wake, sleep and 24-h cycles and the nocturnal dip were calculated according the 2017 guidelines (25, 26). ABPM data were expressed as Z-score for sex and height (27). Hypertension was defined as SBP and/or DBP equal or exceeding the 95th percentile for gender, height, or the use of antihypertensive medication.

Hypertensive children were further classified according to the control of hypertension. Uncontrolled hypertension was defined as SBP and/or DBP values exceeding the 95th percentile for height in patients with or without antihypertensive medication. In patients with controlled hypertension, both SBP and DBP were below the 95th percentile for height and patients taking antihypertensive medication. The normotensive group included patients with SBP or DBP below the 95th percentile without taking antihypertensive medication.

Nocturnal dipping was defined as a nocturnal decrease in BP measured by ABPM. Patients with dipping below 10% were considered as non-dippers (25, 27).

Patients with BP exceeding 95th percentile at night, but with normal blood pressure during the day were categorized as having isolated nocturnal hypertension (28, 29).

Masked hypertensives had normotensive OBP values, but had hypertension on ABPM, whereas patients with white coat hypertension had elevated OBP but had normotension on ABPM (30).

PWV Measurement

Carotid-femoral PWV measurement was performed by applanation tonometry with a PulsePen®™ (DiaTecne, Milan, Italy) device, as described previously (31). All participants were assessed in supine position. Arterial path length was determined

TABLE 1 | Patient characteristics at T_1 and T_2.

	T_1		T_2		*p*-value
Female	30	58%			
Male	22	42%			
Follow-up [years]	–		5.7	[4.6–9.3]	
Age [years]	13.6	[11.1–16.2]	18.89	[16.8–24.0]	* <0.001
Age at time of transplantation [years]	10.8	[8.5–12.7]			
Time since transplantation [years]	2.4	[1.0–4.7]	9.3	[6.3–11.8]	* <0.001
Cumulative time on dialysis [months]	11.0	[5.2–20.6]			
Number of second transplantations	5	10%			
Cadaver donor	46	88%			
Living related donor	6	12%			
Preemptive transplantation	9	17%			
CAPD	29	56%			
HD	9	17%			
CAPD & HD	5	10%			
Height Z score	−0.92	±1.39	−0.68	±1.59	0.06
Height <5pc	17	33%	12	23%	0.166
Weight Z score	−0.02	±1.17	−0.03	±1.41	0.9
BMI Z score	0.39	±0.88	0.48	±0.99	0.47

Continuous variables are presented as mean ± SD, time parameters are shown as median [IQR], categorical variables are expressed as number [percentage].
T_1, first follow-up visit; T_2, second follow-up visit; BMI, body mass index; CAPD, continuous ambulatory peritoneal dialysis; HD, hemodialysis; 5pc, 5th percentile.
**Significant p-values are indicated with an asterisk.*

by surface measurement, by subtracting the suprasternal-notch to carotid site distance from the suprasternal-notch to femoral site distance (31, 32). Aortic PWV was calculated as the distance of the carotid and femoral sampling sites divided by the time difference between the rise delay of the distal and proximal pulse according to the R wave belonging to the ECG qRs complex. Age-, sex-, and height-specific Z-scores were calculated using our previously established normative data (33).

Statistical Analysis

Statistical analyses were performed using IBM SPSS 26. Age is expressed as median and interquartile ranges. Continuous variables, reported as means and standard deviations, were compared with the Wilcoxon signed-rank test. Categorical variables were compared using McNemar's test. The Mann-Whitney test was used for comparing groups in the cohort.

Correlations between variables were assessed by linear regression analysis. A p-value of <0.05 was considered statistically significant.

RESULTS

Study Population

A total of 52 pediatric and young adult kidney transplant recipients (22 males) were included in the study. The median age [IQR] at T_1 and T_2 was 13.6 [11.1–16.2] years and 18.9 [16.8–23.9] years, respectively, with a follow-up of 5.7 [4.6–9.3] years.

Etiologies of kidney disease were congenital anomalies of the kidney and urinary tract ($n = 13$; 25%); focal segmental glomerulosclerosis (FSGS) ($n = 11$; 21%) (all patients had a genetically confirmed podocyte mutation); cystic kidney disease ($n = 7$, 13%); glomerulopathy ($n = 5$, 10%); nephronophtisis ($n = 5$; 10%); interstitial nephritis (3; 6%); acute tubular necrosis ($n = 1$; 2%); nephrocalcinosis ($n = 1$; 2%); Bardet-Biedl syndrome ($n = 1$; 2%); Denys-Drash syndrome ($n = 1$; 2%); cystinosis ($n = 1$; 2%); and unknown ($n = 3$; 6%).

Transplant recipients were on standard immunosuppression therapy with a calcineurin inhibitor (CNI) (tacrolimus or cyclosporine A) and mycophenolate mofetil. The dose of CNI inhibitors was adjusted to be in the target range and all patients were in the range at the time of the examinations. In addition, 60% of the patients were taking steroids at T_1 and 44% at T_2.

Patient characteristics at T_1 and T_2 are detailed in **Table 1**.

A trend in catch-up growth could be observed during the course of the study ($p < 0.06$), with no significant difference in weight and BMI-Z scores between T_1 and T_2. Of note, while there was an almost −1 SD deficit in height Z score at T_1, patient weight was appropriate for age, with a positive BMI Z score at both T_1 and T_2. Overweight was present in 4 (7.6%) and 6 (11.5%) patients at T_1 and T_2, respectively.

Relevant laboratory results are presented in **Table 2**.

GFR did not change significantly during follow-up. Approximately half of the patients had a GFR below 60 ml/min/1.73 m^2 at both time points. Anemia was present in 19 and 20% at T_1 and T_2, respectively. Nineteen percent of the patients had an increased HOMA index. Three patients had diabetes and a considerable proportion of patients had abnormal

TABLE 2 | Laboratory data at T_1 and T_2.

	T_1		T_2		*p*-value
GFR (ml/min/1.73 m²)	62.1	±30.7	60.4	±34.4	NS
GFR<60	25	48%	26	50%	NS
Hemoglobin (g/l)	129.2	±16.5	133.5	±27.3	NS
Hemoglobin <100	10	19%	10	19%	
Diabetes	3	6%	3	6%	NS
HOMA index >2.8	10	19%	10	19%	NS
Cholesterol (mmol/l)	4.32	±1.98	4.56	±1.71	0.06
Cholesterol >5.2	6	12%	11	21%	NS
Triglycerides (mmol/l)	1.56	±1.13	1.44	±1.32	NS
Triglycerides >1.1 mmol/l	24	46%	16	30%	NS
Proteinuria (#)	8	15%	19	36%	*0.007

Continuous variables are presented as mean ± SD. Categorical variables are expressed as number (percentage).
T_1, first follow-up visit; T_2, second follow-up visit; GFR, glomerular filtration rate; HOMA, Homeostasis Model Assessment - Insulin Resistance; NS, not significant.
**Significant p-values are indicated with an asterisk.*
#Urine protein/creatinine ratio 50–200 mg/mmol.

serum lipid levels. Proteinuria was present in 15 and 36% at T_1 and T_2, respectively; however, none of the patients had nephrotic range proteinuria at T_1 or T_2.

No abnormal values were observed in calcium and phosphate metabolism at the time of the study.

Antihypertensive Medication

Antihypertensive medication consisted of calcium-channel blockers (CCB) (T_1: $n = 27$; T_2: $n = 25$), beta-blockers (BB) (T_1: $n = 21$; T_2: $n = 24$), ACE-inhibitors (ACEi) or angiotensin receptor blockers (ARB) (T_1: $n = 9$; T_2: $n = 17$), diuretics (thiazide or indapamide) and alpha-adrenergic blocking agents (T_1: $n = 6$; T_2: $n = 10$). Mean number of antihypertensive medication was 1.3 ± 1.2 at T_1 and 1.5 ± 1.2 at T_2 ($p =$ NS).

Prevalence of Hypertension According to Office and ABPM Categories

All 52 patients had their OBP measurements recorded, while ABPM results were available for 37 patients. The prevalence of previously diagnosed hypertension in the whole cohort was 35 (67%) and 39 (75%) at T_1 and T_2, respectively ($p =$ NS). Controlled hypertension based on OBP measurements was 19 (37%) and 23 (44%) at T_1 and T_2 ($p =$ NS) (**Table 3**).

There was no significant difference between the OBP values of the whole cohort ($n = 52$) and those who also had ABPM measurements ($n = 37$) (data not shown).

Among those who had ABPM results, 26 (70%) and 31 (84%) had hypertension at T_1 and T_2, respectively ($p =$ NS). Controlled hypertension based on ABPM was present in 15 (40%) and 18 (49%) patients at T_1 and T_2, respectively ($p =$ NS).

Of those who had controlled hypertension at the first measurement, 9 (60%) were non-dippers. while this ratio was 7 (39%) at T_2 ($p =$ NS). The prevalence of non-dippers among uncontrolled hypertensives was 9 (81%) at T_1, and 8 (61%) at T_2 ($p =$ NS), respectively. Isolated daytime hypertension was present in 5 (14%) and 7 (19%) cases, whereas isolated nocturnal hypertension was present in 9 (24%) and 5 (14%) of cases at T_1 and T_2, respectively.

Using both ABPM and office results, 9 (24%) and 12 (32%) patients had masked hypertension, while white coat hypertension was present in 6 (16%) and 8 (21%) patients at T_1 and T_2, respectively.

Details relative to blood pressure and PWV results are shown in **Tables 4A,B**, respectively.

Blood Pressure

There was no significant change between T_1 and T_2 in either OBP or ABPM blood pressure Z-scores.

PWV Results

All children had carotid-femoral PWV measurements performed at both follow-up visits (**Table 4B**). While the absolute value of PWV increased significantly, there was no difference in Z scores at T_1 and T_2 (**Table 4B**).

Correlations

Correlations between blood pressure, GFR, PWV as well as blood pressure control and evolution of GFR and presented in **Tables 5A–C**.

Blood Pressure and GFR

There was no correlation between ABPM blood pressure measurements at T_1 and GFR values either at T_1 or at T_2. In contrast, all systolic and diastolic ABPM Z scores were closely correlated with GFR at T_2 (shown graphically in **Figure 1A**).

Dipper status did not affect kidney function. Of note, OBP values (either for the whole cohort or those with accompanying ABPM results) did not correlate with GFR whether at T_1 or at T_2.

Arterial Stiffness by PWV

There was no correlation between any of the blood pressure values (office or ABPM) and PWV-Z at T_1. However, there was a positive correlation between T_2 PWV-Z and all systolic and diastolic ABPM-Z scores (data shown for 24 h systolic and diastolic values), while office blood-pressure Z scores showed no correlation with PWV-Z at T_2 (shown graphically in **Figure 1B**).

There was no correlation between GFR and PWV-Z values. Regarding lipid measurements, T_2 PWV correlated with T_2 cholesterol ($R = 0.619$, $p < 0.001$).

Blood Pressure Control and GFR

While there was no significant difference between GFR values of patients with controlled and those with uncontrolled hypertension at the first follow-up, patients with uncontrolled hypertension at T_2 had a significant decrease in GFR (shown as change in GFR between T_1 and T_2) compared to controlled hypertensives. This difference was only present if ABPM values were considered, and not for OBP (shown graphically in **Figure 2**).

TABLE 3 | Blood pressure follow-up based on office blood pressures in the whole cohort ($n = 52$), and ABPM measurements ($n = 37$).

	Office BP				ABPM					Discordance between Office and ABPM results			
	T_1		T_2		T_1		T_2			T_1		T_2	
	n	**%**	***n***	**%**	***n***	**%**	***n***	**%**		***n***	**%**	***n***	**%**
Untreated HT	2	4%	3	6%	3	8%	0	0%	White coat HT	6	16%	8	21%
Uncontrolled HT	16	31%	16	31%	11	30%	13	35%	Masked HT	9	24%	12	32%
Controlled HT	19	36%	23	44%	15	40%	18	49%	**ABPM categories**				
Normotension	15	29%	10	19%	8	22%	6	16%	24 h HT	23	62%	25	67%
									Daytime HT	5	14%	7	19%
									Isolated nocturnal HT	9	24%	5	14%

T_1, first follow-up visit; T_2, second follow-up visit; ABPM, 24 h ambulatory blood pressure monitoring; HT, hypertension.

TABLE 4A | Blood pressure data at T_1 and T_2.

	T_1		T_2		***p*-value**
Office SBP	122	±14	126	±15	0.12
Office SBP-Z	0.92	±1.7	1.09	±2.0	0.83
Office DBP	74	±9	77	±11	*0.03
Office DBP-Z	0.36	±1.55	0.82	±1.85	0.075
24 h SBP	119	±11	123	±14	0.19
24 h SBP-Z	1.35	±1.40	1.28	±1.93	0.38
24 h DBP	68	±7	71	±11	0.18
24 h DBP-Z	0.38	±1.18	0.7	±2.05	0.90
Daytime SBP	123	±11	125	±16	0.49
Daytime SBP-Z	1.12	±1.30	0.91	±2.16	0.64
Daytime DBP	71	±7	74	±10	0.25
Daytime DBP-Z	−0.04	±1.21	0.13	±1.77	0.92
Nighttime SBP	113	±13	116	±16	0.20
Nighttime SBP-Z	1.72	± 1.44	1.73	±2.15	0.41
Nighttime DBP	61	±13	65	±12	0.15
Nighttime DBP-Z	1.07	± 1.91	1.42	±2.03	0.59

Office blood pressure: n = 52; ABPM: N = 37.
Continuous variables are presented as mean ± SD.
T_1, first follow-up visit; T_2, second follow-up visit; ABPM, 24 h ambulatory blood pressure monitoring; SBP, systolic blood pressure; DBP, diastolic blood pressure; SBP-Z, systolic blood pressure Z score; DBP-Z, diastolic blood pressure Z score.
**Significant p-values are indicated with an asterisk.*

TABLE 4B | Results of PWV measurements.

	T_1		T_2		***p*-value**
PWV absolute value	5.39	±0.9	5.82	±1.175	*0.03
PWV-Z	0.519	±1.041	0.40	±1.29	0.373

Continuous variables are presented as mean ± SD. PWV-Z, pulse wave velocity Z score.
**Significant p-values are indicated with an asterisk.*

TABLE 5A | Correlation between T_2 GFR and T_2 ABPM.

	R	***p*-value**
T_2 GFR		
T_2 24h SBP-Z	0.562	*0.0001
T_2 24h DBP-Z	0.444	*0.007

**Significant p-values are indicated with an asterisk.*

TABLE 5B | Correlation between T_2 PWV-Z and T_2 ABPM.

	R	***p*-value**
T_2 PWV-Z		
T_2 24h SBP-Z	0.437	*0.009
T_2 24h DBP-Z	0.523	*0.001

**Significant p-values are indicated with an asterisk.*

TABLE 5C | Comparison of renal function outcomes between controlled and uncontrolled hypertensives.

	Controlled	**Uncontrolled**	***p*-value**
ΔGFR expressed in ml/min/1.73m^2			
Office BP	1.55 ± 23.0	−2.4 ± 31.2	0.52
ABPM BP	3.0 ± 21.2	−14.3 ± 20.1	*0.03

Continuous variables are presented as mean ± SD.
T_1, first follow-up visit; T_2, second follow-up visit; GFR, glomerular filtration rate; SBP-Z, systolic blood pressure Z score; DBP-Z, diastolic blood pressure Z score; PWV-Z, Pulse wave velocity Z score; ΔGFR, change of GFR from T_1 to T_2; ABPM, 24 h ambulatory blood pressure monitoring.
**Significant p-values are indicated with an asterisk.*

DISCUSSION

Functional and structural arterial damage is already present in children with CKD, along with an increased risk of cardiovascular morbidity (6, 34–36). It has also been shown that RTx decreases the risk of CV, although remains approximately two magnitudes higher than in the normal population (5, 36, 37). In contrast to adults, hard endpoints of CV events are rare in RTx children, thus data are needed to establish the presence of cardiovascular risk factors and to assess the value of the various non-invasive measurements of cardiovascular health.

In this follow-up study, in addition to anthropometric data, we assessed the presence of several metabolic risk factors, the prevalence of hypertension and increased arterial stiffness and

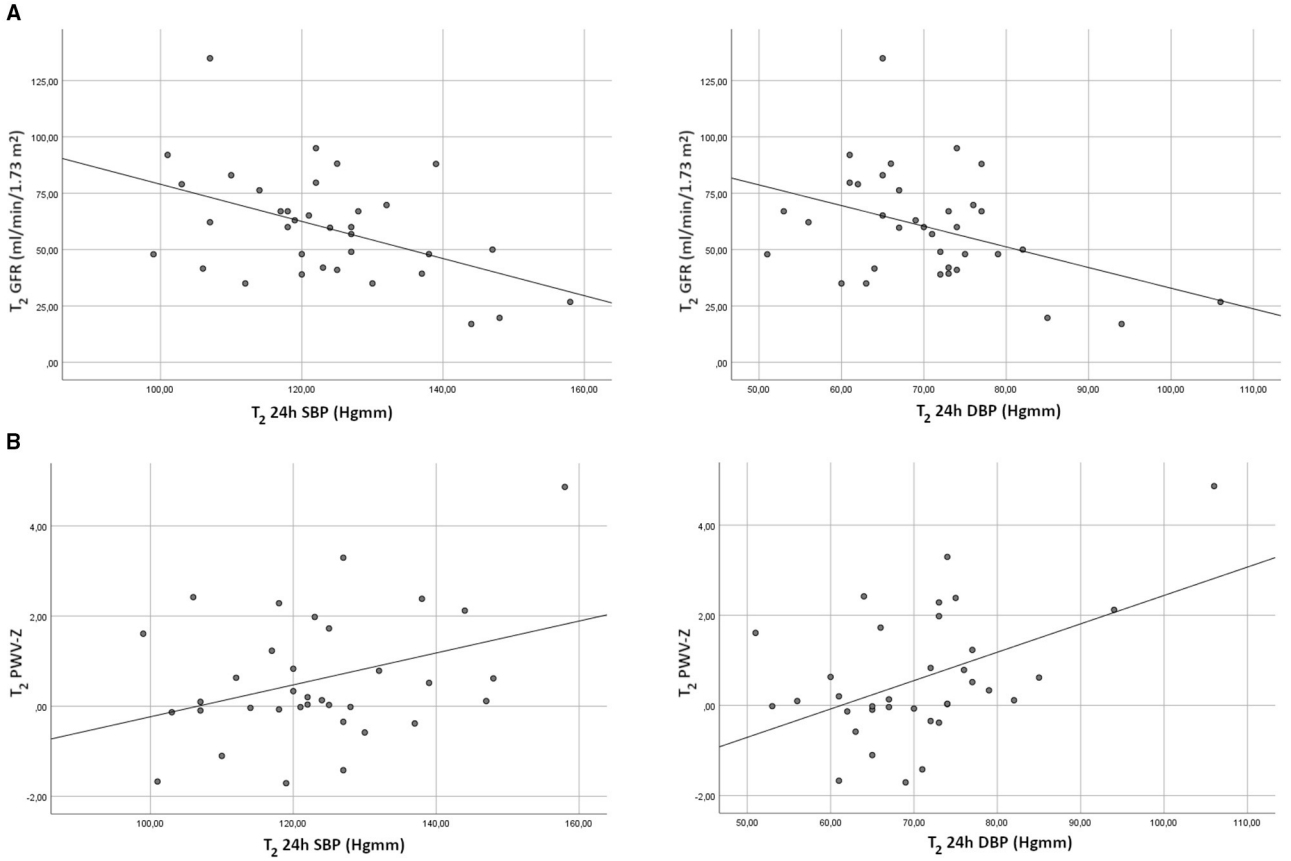

FIGURE 1 | (A) Correlation of 24 h ABPM blood pressure values and GFR at the second follow-up. GFR, glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure. **(B)** Correlation of 24 h ABPM blood pressure values and PWV-Z at the second follow-up. PWV, pulse wave velocity; SBP, systolic blood pressure; DBP, diastolic blood pressure.

decreased GFR to identify associations and longitudinal changes in a pediatric RTx population.

Our patients exhibited some growth deficit with a trend of catch-up growth observed during follow-up. The reason for the substantial growth deficit reported in earlier studies (38) comparatively to our patients' growth delay (averaging around −1 SD) may be that all patients with CKD in the current study were on growth hormone treatment prior to transplantation. Since GH was discontinued following RTx, the catch-up in height was the result of Tx rather than due to pharmacological therapy with growth hormone. Furthermore, both weight and BMI Z scores were in the normal range at both follow-up visits, which may be the result of proper dialysis treatment, regular dietary counseling, and control (39).

GFR remained at ∼60 ml/min/173 m^2 and did not deteriorate significantly during follow-up. However, approximately half of the patients had a GFR below 60 ml/min/1.73 m^2 on both visits.

The prevalence of risk factors such as anemia, diabetes and insulin resistance did not change significantly during follow-up. There was a trend toward an increase in total cholesterol and a significant increase in non-nephrotic range proteinuria in our cohort. Such correlation between cholesterol and PWV at T_2 is moreover in line with our previous report confirming that, after a median 2-year follow-up of renal transplant recipients, the correlation between cholesterol and PWV becomes significant (40).

Prevalence of Hypertension: Controlled—Uncontrolled

The relatively high rate of uncontrolled hypertension with both office and ABPM measurements in the present study population despite close clinical follow-up and personalized antihypertensive treatment was rather unexpected although in keeping with previous reports (41–43). In addition, with ABPM, we were able to confirm a high proportion of masked and white coat hypertension, as well as nocturnal and isolated nocturnal hypertension. Furthermore, the absence of nocturnal blood pressure dipping was high among controlled and even higher among uncontrolled hypertensive. These findings are similar to previous studies (9, 40, 44) showing the superiority of ABPM over conventional BP measurement techniques (4, 28–30).

The causes behind the relatively high rates of uncontrolled hypertension are multifactorial (3–5, 7, 28–30, 37). Kidney transplants may show decreased GFR, and in fact, more

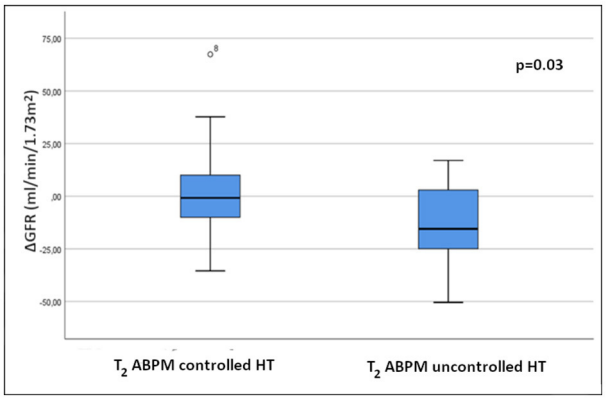

FIGURE 2 | Comparison of changes in GFR between controlled and uncontrolled hypertensive patients at the second follow-up. ΔGFR, GFR difference between T2 and T1 ($T2_{GFR}$ - $T1_{GFR}$).

than 50% of patients in our cohort had GFR values below 60. Immunosuppressants, namely CNIs and corticosteroids, may also contribute to increased blood pressure. However, our patients were within the target range for CNI values in both exams, and the steroid dose was minimized (2–4 mg methyprednisolone/day), with 40 and 56% of patients no longer receiving steroids at T1 and T2, respectively (see results, study population). We could not find any correlations between blood pressure, CNI levels or steroid consumption in the data analysis.

Adherence to blood pressure lowering medications is also an important issue. The study protocol did not include direct assessment of compliance, the fact that immunosuppressive drugs were in target range may indicate good adherence to antihypertensive therapy as well.

Finally, another factor contributing to hypertension could be recurrence of primary renal disease or post transplant glomerulopathy. Routine biopsies for screening these pathologies are not part of the protocol in our center. Since no clinical signs of *de novo* or recurrent glomerular disease was observed, we believe this may not be relevant to our study population.

Evolution of Arterial Stiffness

While the absolute value of PWV increased significantly, the height-controlled Z-score remained unchanged, highlighting the necessity to use appropriate, height-controlled Z-scores for comparison purposes in children (45, 46).

One of the major purposes of assessing *surrogate markers* of vascular health is not only to confirm changes but also to predict subsequent cardiovascular hard endpoints. ABPM-measured blood pressure and PWV are established markers and cardiovascular risk factors according to adult studies (47, 48). In the current assessment, neither blood pressure nor PWV at T_1 were able to anticipate the evolution of GFR during follow-up. However, there was a close correlation between arterial stiffness as well as ABPM blood pressure and GFR at T_2. In addition, uncontrolled hypertensives (by ABPM) exhibited a significant decrease in GFR at T_2 compared to the controlled group thus suggesting that controlling hypertension may impact the evolution of GFR on the long term (49–51). Once again, blood pressure measurement by ABPM revealed to be superior to OBP values since OBP values (whether in the whole cohort or in those with both office and ABPM results) did not correlate with GFR at T_2.

Limitations

This single-center follow-up study has some important limitations due to the relatively low sample size, related to the low prevalence of ESRD and transplantation in children. This limited availability also determines the limits of statistical analysis. Although followed at a regional transplant center, not all patients could be included in the study, hence the results are not unreservedly applicable to the entire RTx population. Furthermore, only a portion of the whole cohort had ABPM results. However, given that the OBP values of the ABPM sub-study did not differ from the entire cohort and that these OBP results failed to show the correlations observed with ABPM, we can still affirm the superiority of ABPM over OBP. Since the proteinuria was assessed semiquantitatively, no correlations could be calculated.

The fact that dipper status did not affect kidney function may be explained by the small number of patients in each category, which may be too low to reveal differences. This is also true for the subgroups of isolated nocturnal hypertension and white coat hypertension. Finally, the correlations found between ABPM, PWV, and GFR are not necessarily causal since decreasing GFR may also be the cause of uncontrolled hypertension and vice-versa.

CONCLUSION

In conclusion, our study provides additional data on the general CV health of RTx children more than 2 and 9 years after transplantation. Pediatric RTx patients harbor several cardiovascular risk factors that may affect their cardiovascular health. While we were not able to predict the evolution of renal function using surrogate markers such as PWV and ABPM blood pressure at T_1, these risk factors were closely correlated with renal function at follow-up, with control of hypertension having a significant impact on GFR evolution.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics (TUKEB 91/4-2008). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AV and AB organized the database performed the statistical analysis and wrote the first draft of the manuscript. OC, ÉK, KK, and ST wrote sections of the manuscript. AS and GR contributed to conception and design of the study and corrected the manuscript. All authors read and approved the submitted version.

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B Cell-Derived Extracellular Vesicles Reveal Residual B Cell Activity in Kidney Graft Recipients Undergoing Pre-Transplant Desensitization

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Background: Living-donor kidney transplant (LDKT) recipients undergoing desensitization for Human Leukocyte Antigen (HLA)-incompatibility have a high risk of developing antibody-mediated rejection (ABMR). The purpose of the study is to evaluate if residual B cell activity after desensitization could be estimated by the presence of circulating B cell-derived extracellular vesicles (BEVs).

Methods: BEVs were isolated by Sepharose-based size exclusion chromatography and defined as CD19+ and HLA-II+ extracellular vesicles. We analyzed stored serum samples from positive crossmatch LDKT recipients before and after desensitization at first post-transplant biopsy and at 12-month protocol biopsy ($n = 11$). Control groups were formed by hypersensitized patients who were not submitted to desensitization ($n = 10$) and by low-risk recipients ($n = 9$). A prospective validation cohort of 11 patients also included the analysis of B cells subpopulations in recipients' blood and lymph nodes recovered upon graft implantation, along with BEVs analysis before and after desensitization.

Results: We found out that CD19+ and HLA-II+BEVs dropped significantly after desensitization and relapse in patients who later developed ABMR was evident. We validated these findings in a proof-of-concept prospective cohort of 6 patients who received the same desensitization protocol and also in a control group of 5 LDKT recipients. In these patients, B cell subpopulations were also studied in recipients' blood and lymph nodes that were recovered before the graft implantation. We confirmed the significant drop in BEVs after desensitization and that this paralleled the reduction in CD19+cells in lymph nodes, while in peripheral blood B cells, this change was almost undetectable.

Conclusions: BEVs reflected B cell residual activity after desensitization and this could be a valid surrogate of humoral alloreactivity in this setting.

Keywords: B cells, kidney transplantation, desensitization, HLA-incompatibility, extracellular vesicles (EV), exosomes, plasma cells

INTRODUCTION

Patients with chronic kidney disease that are sensitized to HLA antigens have limited access to kidney transplantation, resulting to increased mortality in the waiting list. A possibility to overcome the HLA barrier is represented by desensitization before kidney transplantation. This regimen, which is usually based on plasma exchange, intravenous immunoglobulins, and anti-CD20 antibodies, is being done in order to reduce the quantity of circulating donor-specific antibodies (DSAs) and to deplete B cells (1–3). This option is associated with favorable results, as graft and patient survival are undoubtedly better when compared to hypersensitized patients on dialysis waiting for a compatible donor (4). However, despite the reasonable solid outcomes, incidence of antibody-mediated rejection (ABMR) is as high as 30–50% (5–7), and forces a transplant physician to enhance immunosuppression but with concerns of higher rate of infectious, neoplastic and cardiovascular complications (7, 8).

The effects of desensitization on DSA titer can be controlled using solid-phase techniques. However, how B cell biology is affected by this treatment remains unclear. One possibility is to think that circulating B cells represented a good estimate of treatment efficacy. However, while circulating B cells are easily depleted after a single dose of anti-CD20 antibody, a high proportion of B cells survived in lymph nodes with a switched-memory phenotype (9). To check activity and proliferation of residual B cells after desensitization, a fine-needle aspiration of bone marrow or lymph node biopsy is unpractical, apart from being invasive. Therefore, we hypothesized that the activity of B cells, which survived in lymphoid organs after desensitization, can be estimated by the presence of circulating B cell-derived extracellular vesicles (BEVs).

Extracellular Vesicles (EVs) are a heterogeneous population of cell-derived vesicles of various origins and sizes, including exosomes, microvesicles, and apoptotic bodies (10). They played a key role in intercellular communication, delivering signal molecules (proteins, nucleic acids, lipids, etc.) that can regulate immune functions (11). Concretely, EVs derived from immune cells are implicated in antigen presentation, immunoregulation, and viral transmission (12, 13). B cells actively secrete EVs upon proliferation stimuli, such as T-cell help via CD40 and IL-4 signaling (11, 13). Importantly, BEVs display markers of B cell (CD19, IgM, IgG) and of antigen-presenting cells origin (HLA-I, HLA-II, CD86) (11).

The possibility of circulating EVs to act as efficient biomarkers has been recently highlighted in different fields. In oncology, for example, tumor-derived circulating exosomes are associated with the burden of the primary mass (14). In kidney transplantation, mRNA transcripts from circulating exosomes are associated with rejection phenotypes (15). Our group recently highlighted the importance of transplant immunosuppression in EV content from colorectal cancer cell lines in the regulation of the pre-metastatic niche (16). It is reasonable to speculate that circulating BEVs reflect B cell proliferation in bone marrow and lymphoid organs, even though the circulating B cells are not detectable because of desensitization. To prove this hypothesis, we developed a retrospective and a prospective study in living-donor kidney transplant (LDKT) recipients who were undergoing a desensitization protocol based on anti-CD20 antibodies, plasma exchanges, and intravenous immunoglobulins.

MATERIALS AND METHODS

Study Population

The retrospective cohort included patients who had kidney transplantation procedure across a 10-year period (2006–2015). Demographics and clinical characteristics of both donors and recipients have been collected, along with immunological profiles of recipients, HLA-matching, and immunosuppressive treatment. Patients were divided into three groups according to the immunological risk: patients who received desensitization before transplantation for a positive crossmatch ("DS" group, $n = 11$), hypersensitized patients with a cPRA I+II $> 85\%$ not submitted to desensitization and without a DSA ("HS" group, $n = 10$), and a low-risk group with baseline cPRA I+II $< 10\%$ and no DSAs ("CT" group, $n = 9$). For those patients who were transplanted before 2008, the year in which Luminex study was routinely implemented in the transplant work-up, patients were assigned to the HS group if they had been re-transplanted and if they had lost their previous graft for rejection (two cases).

Induction was based on anti-thymocyte globulins, monoclonal anti-CD25 antibodies, or omitted according to the immunological risk. Maintenance was based on tacrolimus, mycophenolate, and steroids. Desensitization was based on anti-CD20 monoclonal antibodies (Rituximab, Mabthera, Roche, Basel, Switzerlad, 1 or 2 doses of 400 mg) and on plasma exchanges (total number according to DSA titer or repeated XM measurements), followed by intravenous immunoglobulins every two exchanges (Plangamma, 200 mg/Kg for session, Institute Grifols, Barcelona, Spain).

The three time-points examined in the retrospective study were as follows: (i) transplantation day; (ii) first biopsy (either for indication or for-protocol); and (iii) one-year after transplantation. In our institution, kidney biopsies are performed per-protocol at 3 and 12-months after kidney transplantation. Regarding the second time-point, we examined the sample of the first per-indication biopsy in those cases in which rejection developed before the 3-month per-protocol biopsy. In all the other cases, the 3-month per-protocol biopsy sample was studied. This choice was made in order to avoid the potential effects

of B cell depleting treatment on BEVs content of the second time-point. Pathological examination and diagnosis of ABMR were updated according to Banff 2017 criteria (17). Treatment of ABMR was based on anti-CD20 monoclonal antibodies (Rituximab, Mabthera, Roche, Basel, Switzerlad, 1 dose of 400 mg at the beginning and 1 dose at the end of the cycle of 400 mg) and 6 plasma exchanges, followed by intravenous immunoglobulins every two exchanges (Plangamma, 200 mg/kg for session, Institute Grifols, Barcelona, Spain).

Regarding the prospective cohort, all patients were transplanted from 2018 to 2019, with the same desensitization, induction, and maintenance protocol employed as in the retrospective cohort.

Extracellular vesicles were studied according to the MISEV guidelines of 2018 (18). A checklist with all the items can be retrieved in **Supplementary Material**.

Isolation of EVs by Sepharose-Based Size-Exclusion Chromatography

Stored serum samples at −80°C were thawed in ice. After two centrifugation steps at 2000 g, to eliminate cellular debris,1 ml of the serum was loaded on a Sepharose column, as previously described (19, 20). Concisely 10 ml syringes (Becton Dickinson; San Jose, United States) were stacked with 10 ml of Sepharose CL-2B (GE Healthcare; Uppsala, Sweden). Sepharose was previously washed with 0.32% sodium citrate PBS 0.22 μm-filtered. The tip of the syringe was stuffed with nylon stockings (40 denier). Immediately after sample load, it was followed by elution with 0.32% sodium citrate PBS 0.22μm-filtered and 18 fractions of 0.5 ml volume were sequentially collected. These fractions were analyzed through a spectrophotometer at 280 nm absorbance to estimate protein contents (NanodropTM, ThermoFisher; Waltham, MA, USA).

Characterization of EVs by Flow Cytometry

The following step was the coupling of fraction 6–13 (50 μl for sample) to 5 μl of latex beads (4 μm in diameter) for 15 min and then with 1 ml of BCB buffer (PBS 0.1% BSA). After 1 night rotation at room temperature, fractions were incubated in a 96-well plate with two primary antibodies against the EV-specific tetraspanins biomarkers CD9 and CD81 (final dilution 1:10) (20). After careful washing and decanting, wells were charged with 200 μl of PBS 0.22μm-filtered before flow-cytometry study with the FACS-Fortessa cytometer (BD Biosciences; San Jose, CA, United States) was performed. For every well, 10.000 beads for sample were examined and MFI was used to calculate the content of EVs in the different fractions (**Supplementary Figure 1**). Those fractions that proved to be enriched in EVs by high expression of CD9 and CD81 were later pooled and analyzed for markers of B cells (CD19 and HLA-II) (21). The MFI value for CD19 and HLA-II was normalized to the CD9 biomarker of exosomes analyzed in the same pool. All antibodies for exosome characterization are indicated in **Supplementary Table 1**.

Nanoparticle Tracking Analysis (NTA)

Size distribution and concentration of EVs were measured using the NanoSight LM10 instrument (Malvern, United Kingdom), equipped with a 638 nm laser and CCD camera (model F-033). Data were analyzed with the Nanosight NTA Software version 3.1 (build 3.1.46). Representative samples were evaluated in sterile-filtered PBS 1X ($n = 3$). Readings were taken in single capture or triplicates for 60 s at 30 frames per second, and through manual monitoring of temperature (**Supplementary Figure 2**).

Complement-Dependent Cytotoxicity and Flow Cytometry Cross-Match

Complement-Dependent Cytotoxicity (CDC) crossmatch was performed with peripheral blood mononuclear cells (PBMCs) of the donor, according to the NIH Technique. To rule out the presence of autoantibodies, all crossmatch tests were carried out with sera, with and without dithiothreitol treatment, in order to discard IgM. The T and B cell flow cytometry crossmatch was performed using freshly obtained PBMCs from the donor as well. The T and B cells were identified using mouse anti-human CD19 and CD3 antibodies. Goat antihuman F(ab')2 IgG(γ) was used to identify anti-HLA IgG attached to the cells (Clones indicated in **Supplementary Table 2**). The shift of the median channel fluorescence (SMCF) between the test sera and the negative control sera was used to assign positivity or negativity. The cut-off values were assigned according to the median + 3 standard deviations of 20 non transfused male Single Antigen test negative sera.

Assessment of DSAs and Calculated PRA by Luminex Technology

Anti-HLA antibodies were assessed before transplantation and during the per-protocol and the for-cause renal biopsy through Luminex-based technology (22). Briefly, antibodies were tested using Single Antigen Bead test (LIFECODES® Single Antigen, Immucor, Georgia, USA). An allele was considered positive if the MFI was over 1,500 and was x4 times higher than the Lowest Reactive Antigen (LRA) of the same locus. Calculated PRA (cPRA) was determined using a panel of 500 unrelated individuals from local population and was typed for A, B, C, DRB1, and DQB1 in high resolution (2 fields) by sequence-based typing.

Flow Cytometry Analysis of PBMC and Lymph Nodes

Peripheral blood mononuclear cells (PBMCs) were isolated from patient blood samples by centrifugation on a density gradient (400 g for 30 min in Ficoll-paque PREMIUM, GE Healthcare, Madrid, Spain). Lymph nodes were mashed and passed through a 70 μm nylon cell strainer (BD Falcon) and single-cell suspensions were obtained. Cell surface markers were stained with antibodies indicated in **Supplementary Table 3**, according to the instructions of the manufacturer. In all samples, Aqua Live/Dead fixable dead cell kit (Thermo Fisher Scientific, Waltham, MA, USA) was used unambiguously to remove dead cells. Flow cytometry analysis was performed on a FACS Canto II (BD Biosciences, Heidelberg, Germany). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Transmission Electron Microscopy (TEM)

A Holey Carbon support film on a 400-mesh copper grid was used. After the glow discharge, the sample was deposited onto the grid, which was mounted on a plunger (Leica EM-CPC) and blotted with Whatman No. 1 filter paper. The suspension was vitrified by rapid immersion in liquid ethane. The grid was mounted on a Gatan 626 cryo-transfer system and was inserted into the microscope. Images were obtained using a Jeol JEM 2011 cryo-electron microscope operated at 200 kV, recorded on a Gatan Ultrascan US1000 CCD camera, and were analyzed with a Digital Micrograph 1.8 ($n = 3$ per group).

Statistical Analysis

Data are presented as number and/or percentages, mean and standard deviation, or median and interquartile range, as indicated. Statistical comparison among groups has been performed through Student's *t*-test, Mann-Whitney, or one-way ANOVA with LSD *post-hoc* analysis, as appropriate depending on data distribution and number of groups. Correlation between continuous variables has been explored with Pearson's analysis. Time-dependent association with rejection of the biomarkers in the retrospective analysis has been analyzed with mixed model linear rejection. All statistical tests have been conducted with a 95% confidence interval, and a $p < 0.05$ has been considered significant and has been highlighted with an asterisk in the Figures. To carry out all the above-mentioned analysis, software SPSS v.20 (SPSS Inc., Chicago, IL, USA) and GraphPad v.5 (GraphPad Software, La Jolla, CA, USA) have been used. The local Ethical Committee approved the study.

TABLE 1 | Baseline characteristics (above) and B cell-derived extracellular vesicles (BEVs) assessment (below) in the three study groups.

	Control (CT) $n = 9$	Hypersensitized (HS) $n = 10$	Desensitized (DS) $n = 11$
Age (years)	41.9 ± 9.94	43.2 ± 6.86	45.4 ± 15.64
Sex (%males)	3/9 (33.3%)	4/10 (40.0%)	5/11 (45.5%)
Dialysis vintage (months)	20.8 ± 22.3	65.8 ± 52.3	49.3 ± 78.1
Hypertension (% yes)	8/9 (88.8%)	8/10 (80.0%)	9/11 (81.8%)
Diabetes mellitus (% yes)	2/9 (22.2%)	1/10 (10.0%)	3/11 (27.3%)
Previously transplanted (% yes)	2/9 (22.2%)	5/10 (50.0%)	6/11 (54.5%)
Donor age (years)	52.0 ± 14.2	55.3 ± 13.3	54.4 ± 10.3
Donor sex (%males)	5/9 (55.5%)	4/10 (40.0%)	4/11 (36.4%)
HLA A-B-DR incompatibilities	3.11 ± 1.90	2.20 ± 2.46	3.91 ± 1.57
cPRA I+II at baseline (%)	0	95.6 ± 3.8	78.4 ± 21.4
Induction (%)			
None	1/9 (11.1%)	/	/
Anti-CD25 antibodies	5/9 (55.5%)	1/10 (10.0%)	/
Lymphocyte-depleting agents	3/9 (33.3%)	9/10(90.0%)	11/11 (100.0%)
Creatinine +3 months (mg/dl)	1.22 ± 0.48	1.59 ± 0.49	1.55 ± 0.60
Creatinine +12 months (mg/dl)	1.23 ± 0.53	1.65 ± 0.58	1.69 ± 0.58
Desensitization parameters			
DSA (MFI)	/	/	6,130 ± 3,405
Rituximab (mg)	/	/	618.2 ± 208.9
Plasma exchanges (*n*)	/	/	5 [2–7]

HLA, Human Leukocyte Antigen; cPRA, calculated Panel-Reactive Antibodies.

RESULTS

Baseline Characteristics of the Studied Population

B cell-derived extracellular vesicles (BEVs) were extracted from stored serum samples of 30 LDKT recipients in the following time-points: transplantation day, first biopsy (either per-protocol or per-cause), and 12-month protocol biopsy. This population included the study group of 11 desensitized patients for a positive cross-match (DS group), while control groups were represented by 10 hypersensitized patients (defined as baseline cPRA I+II > 85%) not submitted to desensitization (HS group), and nine low-risk patients (defined as baseline cPRA I+II < 10%, CT group). In the DS group, before starting the desensitization protocol, a stored serum sample was also available and studied in seven of the 11 patients. One patient of the DS group died of metastatic colon cancer before completing the 12-month follow-up. Baseline characteristics revealed no differences among groups in terms of age, sex, diabetes, hypertension, and donor characteristics. Calculated PRA (cPRA) I+II at baseline was higher in the HS and DS group as expected. Causes for desensitization in the DS group were a positive flow-cytometry crossmatch in 8 patients and DSA in 3 patients. Of the 11 patients, 10 had a detectable DSA by Luminex before transplantation (mean MFI 6,130 ± 3,405) and the only patient without a DSA had a positive flow-cytometry crossmatch for both T and B cells. Induction was most frequently based on lymphocyte-depleting agents in the HS and DS group, while baseline immunosuppression was based on tacrolimus, mycophenolate, and prednisone in all groups (**Table 1**).

Circulating BEVs Can Be Detected During the First Year After Kidney Transplantation

Isolation and characterization of microparticles were consistent with EV surface biomarkers (CD9 and CD81) and with the diameter according to flow-cytometry (**Supplementary Figure 1**), NTA analysis (**Supplementary Figure 2**) and electronic Microscopy (**Supplementary Figure 3**). BEVs were defined as either CD19+ or HLA-II+ EVs and were detected all along the first year after the kidney transplantation in the three groups (CT, HS, and DS) (**Figure 1**), without significant difference at ANOVA analysis (**Table 2**). However, when comparing the DS group with the similar risk controls (HS group), there was a tendency toward lower CD19+ EVs at transplantation ($p = 0.068$, **Figure 1A**), at first biopsy ($p = 0.101$, **Figure 1B**), and lower HLA-II+ EVs at first biopsy ($p = 0.053$, **Figure 1E**).

BEVs Drop After Desensitization and Relapse During ABMR

Antibody-mediated rejection (ABMR) during the first year after kidney transplantation occurred in seven of the 11 patients of the DS group. Of the seven rejections in the DS group, four were discovered at 3-month protocol biopsy, and three

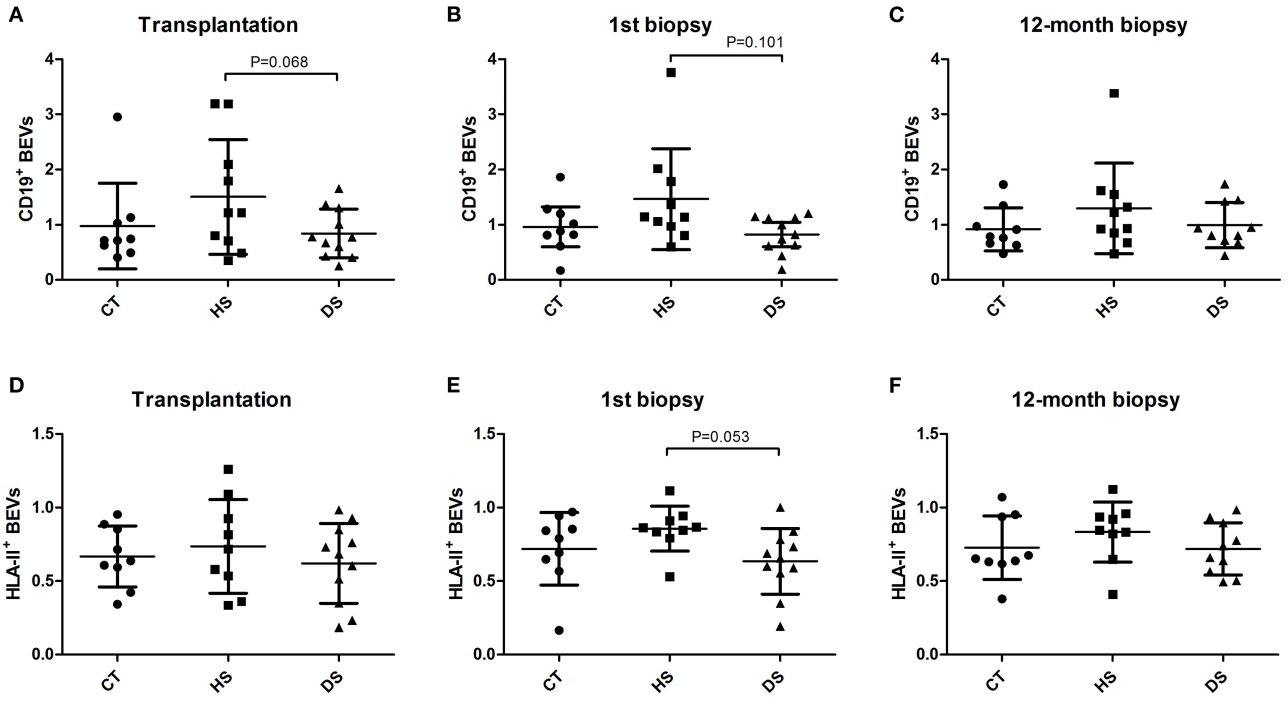

FIGURE 1 | B cell-derived Extracellular Vesicles (BEVs) evolution along the first year after kidney transplantation at the time of Transplantation **(A,D)**, first biopsy **(B,E)** and 12-month protocol biopsy **(C,F)**. BEVs ($CD19^+$ and $HLA\text{-}II^+$ EVs) were analyzed in the study groups in the three time-points. transplantation day, first biopsy, and 12-month protocol biopsy (ANOVA and LSD *Post-hoc* analysis).

TABLE 2 | BEVs assessment (below) in the three study groups at transplantation, first renal biopsy, and at 12-month protocol biopsy.

	Control (CT) $n = 9$	Hypersensitized (HS) $n = 10$	Desensitized (DS) $n = 11$	*P*-value (ANOVA)
Transplantation (BEVs)				
$CD19^+$ EVs	0.97 ± 0.77	1.50 ± 1.04	0.84 ± 0.44	0.148
$HLA\text{-}II^+$ EVs	0.66 ± 0.20	0.73 ± 0.31	0.62 ± 0.27	0.639
First renal biopsy (BEVs)				
$CD19^+$ EVs	0.96 ± 0.47	1.46 ± 0.91	0.88 ± 0.40	0.102
$HLA\text{-}II^+$ EVs	0.71 ± 0.24	0.85 ± 0.15	0.64 ± 0.22	0.097
12-month renal biopsy (BEVs)				
$CD19^+$ EVs	0.91 ± 0.39	1.29 ± 0.50	0.99 ± 0.40	0.337
$HLA\text{-}II^+$ EVs	0.72 ± 0.21	0.83 ± 0.20	0.71 ± 0.17	0.403

were discovered before in a per-cause renal biopsy. Within this group, DSAs were present in two of seven patients with ABMR (MFI of 3,654 and 4,077, respectively) and in two of four patients without ABMR (MFI of 9,066 and 6,613, respectively).

We observed a significant drop in both CD19+ and HLA-II+ EVs after desensitization (CD19+ EVs 1.27 ± 0.30 before and 0.59 ± 0.25 after desensitization, $p = 0.003$, and HLA-II+ EVs 0.75 ± 0.06 before and 0.47 ± 0.22 after desensitization, $p = 0.024$) (**Figures 2A,B**). Within the DS group, patients with active ABMR had higher CD19+ and HLA-II+ EVs both at first biopsy ($n = 7$) and at 12 months ($n = 5$) (**Table 3** and **Figures 2C,D**). All the cases of ABMR were treated except the patient with neoplasia. Only one patient experienced full recovery after treatment, while the other patients progressed to chronic ABMR at 12-month protocol biopsy. Evolution of CD19+ EVs after transplantation proved to be significantly associated with rejection ($p = 0.002$), while DSAs were not ($p = 0.186$), according to mixed-model linear rejection analysis (**Figures 2E,F**).

Survival of CD19+CD20- B Cells in Lymph Nodes After Desensitization

We developed a prospective validation study to assess the B cell subpopulations from PBMC and the external iliac lymph node recovered upon surgery, along with the circulating BEVs. In this

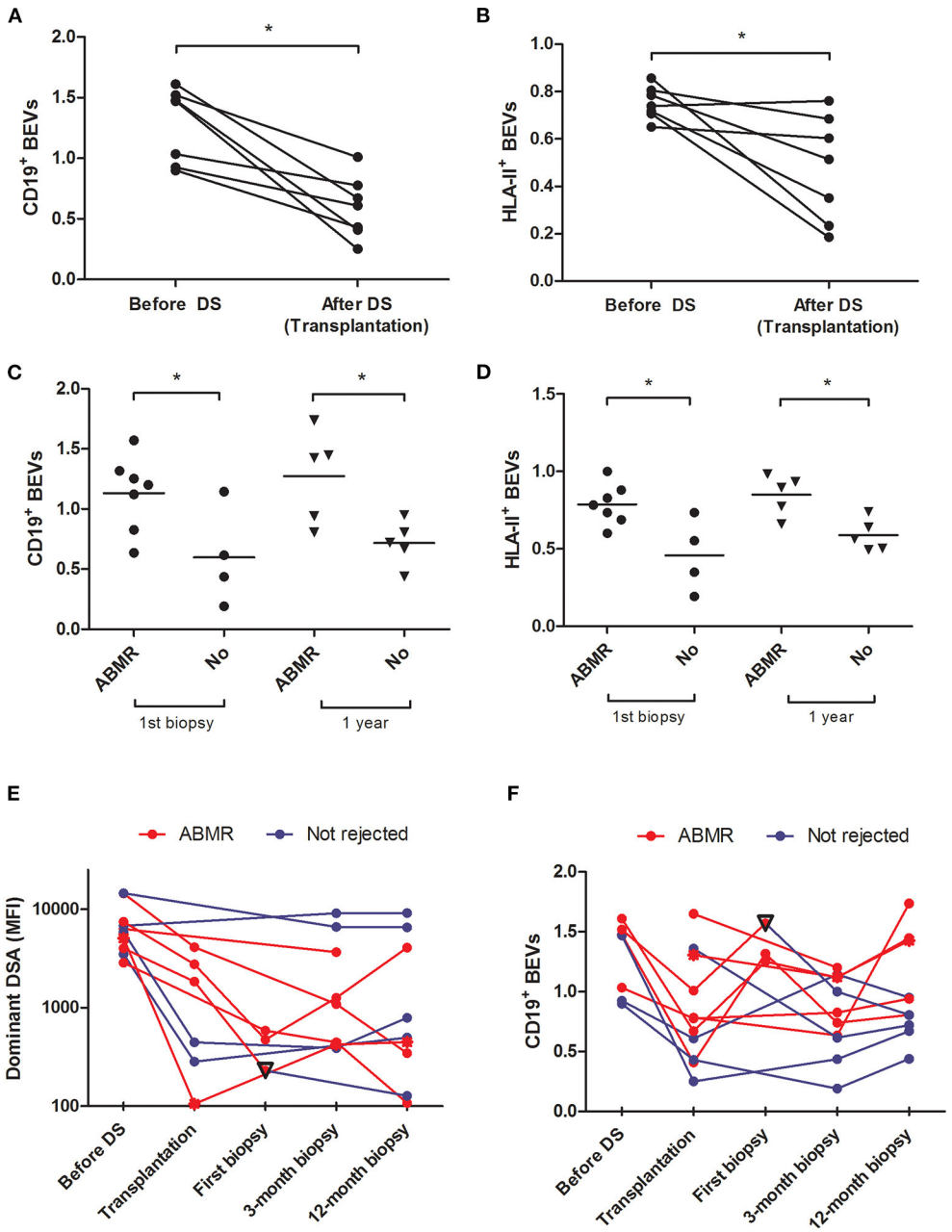

FIGURE 2 | Analysis of BEVs in patients undergoing desensitization and their relationship with antibody-mediated rejection (ABMR)–BEVs expressed as $CD19^+$ and $HLA\text{-}II^+$ exosomes before and after desensitization in the DS group [**(A,B)** paired samples *t*-test] and according to whether or not patients developed ABMR at first biopsy or at 12 months after kidney transplantation [**(C,D)** Student's *t*-test, **Table 3**]. Evolution of patients according to whether they have rejected (red line) or not (blue line) during the first year is highlighted for MFI of the DSA **(E)** and $CD19^+$BEVs **(F)**. Among patients with ABMR, the only patient who did not progress to chronic ABMR and had a normal kidney at following biopsies is highlighted with a reverse triangle.

cohort, we studied 11 patients of which six were submitted to the same desensitization protocol as above, while five low-risk recipients represented the control group. Baseline characteristics of this prospective population are described in **Table 4**, without any significant difference between the two groups.

There was a total depletion of CD3-CD20+ B cells from both periphery and lymph node after desensitization. The CD3-CD19+ B cells were almost depleted after desensitization in blood (**Figure 3C**, $p = 0.024$), but they were still present in the lymph node, without statistical difference compared to the control group at the time of transplantation (**Figure 3D**, $p = 0.762$, and representative sample in **Figure 4A**). On the other side, CD19+CD20+ B cells were also undetectable in lymph nodes in the DS group,

TABLE 3 | BEVs evolution in patients submitted to desensitization according to the clinical outcome.

	n	CD19+ EVs	*P*-value	HLA-II+ EVs	*P*-value
First biopsy					
No rejection	4	0.59 ± 0.40	0.036	0.45 ± 0.23	0.014
ABMR	7	1.13 ± 0.31		0.78 ± 0.13	
12-month biopsy					
No rejection	5	0.71 ± 0.18	0.021	0.58 ± 0.10	0.008
ABMR	5*	1.27 ± 0.38		0.84 ± 0.13	

*1 missing value (patient death).
ABMR, antibody-mediated rejection.

TABLE 4 | Baseline characteristics of the prospective validation study cohort.

	Desensitized *n* = 6	Control *n* = 5	*P*-value
Age (years)	51.2 ± 12.7	45.2 ± 20.0	0.562
Sex (% males)	6 (100.0%)	4 (80.0%)	0.455
Dialysis vintage (months)	0 [0–48]	8 [3–17]	0.840
Hypertension (% yes)	5 (83.3%)	5 (100.0%)	1
Diabetes mellitus (% yes)	1 (16.7%)	0 (0.0%)	1
Previously transplanted (% yes)	1 (16.7%)	1 (20.0%)	1
Donor age (years)	54.7 ± 4.96	56.6 ± 4.21	0.510
Donor sex (% males)	0 (0.0%)	0 (0.0%)	1
HLA A-B-DR incompatibilities	4.00 ± 2.44	3.00 ± 2.23	0.501
cPRA I+II at baseline (%)	0 [0–100]	0 [0–46]	0.714
Creatinine +3 months (mg/dl)	1.49 ± 0.48	1.52 ± 0.37	0.926
Creatinine +6 months (mg/dl)	1.44 ± 0.28	1.77 ± 0.59	0.420
Desensitization parameters			
Isoaglutinins IgG	4 [2–32]		
Isoaglutinins IgM	4 [2–64]		
Rituximab (mg)	1,000.0 ± 903.3		
Plasma exchanges (*n*)	4 [1–7]		

The desensitized group also included ABO-incompatible recipients, so data about pre-transplant isoaglutinins IgG and IgM had been added.
HLA, Human Leukocyte Antigen; cPRA, calculated Panel-Reactive Antibodies.

while a substantial presence of CD19+CD20- B cells still persisted (**Figure 4B**).

Circulating BEVs Correlate With Residual CD3-CD19+ B Cells in Lymph Nodes That Display a Switched-Memory Phenotype

We confirmed the significant drop in BEVs after desensitization, being CD19+ EVs 1.04 ± 0.29 before and 0.80 ± 0.11 after ($p = 0.024$) and HLA-II+ EVs 0.86 ± 0.28 before and 0.73 ± 0.11 after desensitization ($p = 0.024$), respectively (**Figures 3A,B**). There was a significant correlation between CD19+ EVs and the percentage of CD3-CD19+ B cells in the lymph node ($r = 0.839$, $p = 0.037$) at the moment of transplantation, while there was no correlation between circulating CD19+ PBMCs and CD3-CD19+ B cells in the lymph node ($r = -0.197$, $p = 0.708$).

More precise phenotype of B cells revealed that after desensitization, there was a loss of naïve B cells (CD27-IgD+) in lymph nodes, while a substantial proportion of switched-memory B cells (CD27+IgD-) was still alive (**Figure 5**).

DISCUSSION

Desensitization before LDKT represents a reasonable option for highly sensitized patients in terms of graft and patient survival (4). However, ABMR incidence can be as high as 30–50% (5–7). This suggests that the biomarkers currently used to check proper desensitization (DSAs, circulating B cells, flow-cytometry crossmatch) only partially reflect the complex biology of humoral alloimmunity. In order to check B cell proliferation in secondary lymphoid organs and bone marrow after desensitization, we propose the use of B cells-derived EVs.

B cell-derived extracellular vesicles (BEVs) are generated by B cells upon differentiation and proliferation stimuli (13) and could be a major source of EVs *in vivo* (11). In large B cell lymphomas, exosome-derived miRNAs are associated with chemotherapy resistance (17). The value of circulating EVs as diagnostic tool has been highlighted in oncology (14) and in kidney transplantation (15, 16). A recent report also found out that urinary CD3+ EVs are associated with cellular rejection (23). To date, there are no studies that specifically assessed BEVs in kidney transplant recipients.

In our experience, we observed the presence of circulating BEVs in normal and hypersensitized kidney transplant recipients, before and within the first year after transplantation, with a non-significant trend toward higher concentration of BEVs in the hypersensitized group (**Table 2** and **Figure 1**).

In patients submitted to pre-transplant desensitization, we observed a significant reduction in BEVs upon treatment completion (**Figures 2A,B**) with a significant rebound in patients who later developed ABMR (**Table 3** and **Figures 2C,D**). In this prospective study, we observed a complete depletion of CD3-CD20+ both in periphery and in the lymph node. B cells were also assessed as CD3-CD19+ and we also observed that these were almost completely cleared from circulation (**Figure 4A**). In lymph nodes, however, we observed a switch from CD19+CD20+ to CD19+CD20- phenotype in desensitized patients (**Figure 4B**), with a switched-memory phenotype (**Figure 5**) and a statistical association with circulating CD19+ EVs. Differences, albeit moderate, were statistically significant and also showed that the evolution of CD19+ EVs was associated with the development of rejection while the DSAs were not (**Figures 2E,F**).

Taken together, these findings confirmed that a desensitization protocol based on anti-CD20 antibodies, intravenous immunoglobulins, and plasma exchanges is associated with residual alloimmunity that cannot be detected with the currently available clinical tools. As we observed a significant increase in circulating BEVs in patients experiencing ABMR, we propose that this reflects proliferation and

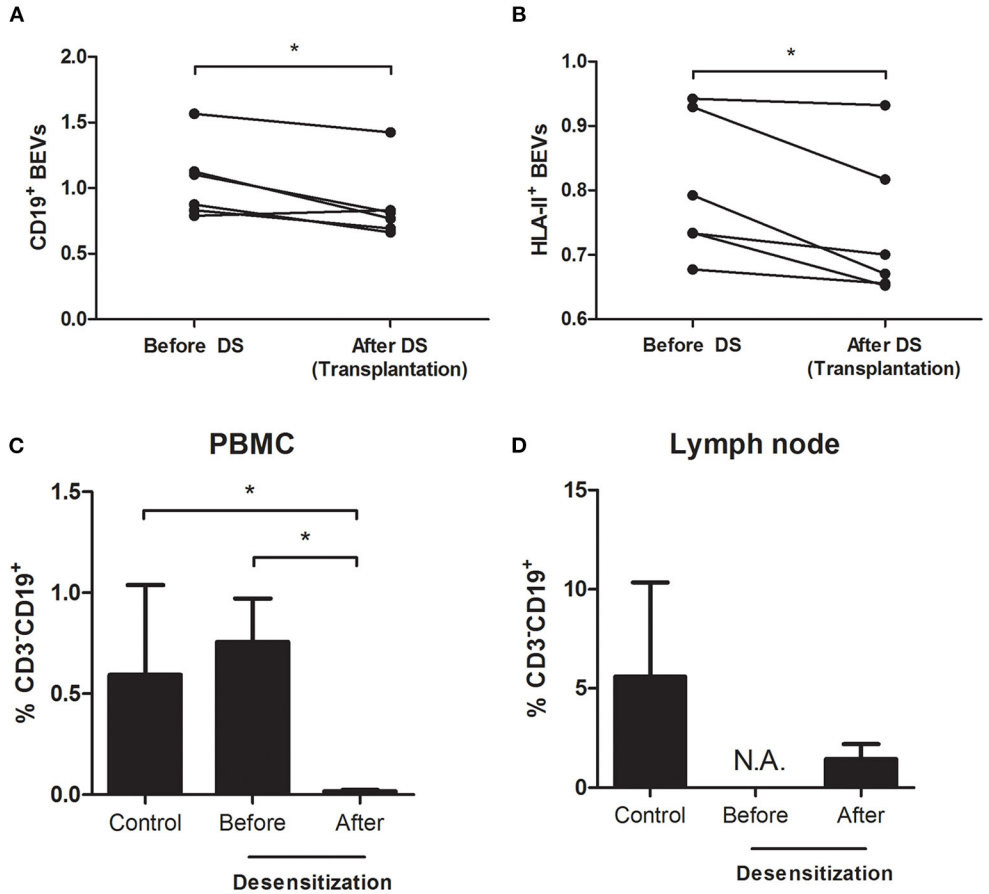

FIGURE 3 | Analysis of BEVs and B cells from peripheral blood mononuclear cells (PBMCs) and lymph node in the prospective validation cohort–$CD19^+$ and HLA-II^+ BEVs before and after desensitization [**(A,B)** paired-samples t-test]. At baseline, there is no difference in percentage of circulating B cells ($CD3^-CD19^+$) between the two groups of patients. Upon desensitization, there is a depletion in circulating $CD3^-CD19^+$ cells [**(C)** Student's t-test] but a relevant proportion of them persisted alive in the lymph node **(D)**.

differentiation of alloreactive B cells resident in secondary lymphoid organs and bone marrow that would not be detectable otherwise.

However, some points need to be addressed before drawing firm conclusions, given the small sample size analyzed in this preliminary experience and the generating-hypothesis nature of this work. First, while in the DS group, there was a striking difference in BEVs before and after desensitization and between patients who rejected or not, we did not observe significant differences between the DS and the other groups (**Figure 2**). This may suggest that the relative changes in BEVs can be meaningful in patients treated with B cell targeted therapies, but at present, this cannot be applied to other population who receive standard-of-care induction. Second, the two biomarkers that we used to define BEVs (CD19 and HLA-II) were not specific of only a cell population. The CD19 is expressed by B cells in most of their differentiation steps and HLA-II is also expressed by other antigen-presenting cells such as monocytes-macrophages and dendritic cells. Third, as B cells lose CD19 expression along their differentiation, CD19+ EVs may not reflect the activity of plasma cells, which are ultimately responsible for DSA production. However, it seems that non-myelomatous plasma cells still express CD19, even though in a heterogeneous way between individuals (24, 25), while there is more information about EVs secretion by myelomatous plasma cells (26, 27).

In conclusion, in patients undergoing desensitization before kidney transplantation, we observed a significant decrease in circulating BEVs after treatment. These were associated with the presence of surviving B cells in the lymph nodes. Patients who developed ABMR have experienced a significant rebound in circulating BEVs, suggesting proliferation and differentiation of not-circulating alloreactive B cells. The next steps will be to analyze BEVs kinetic and function not only in desensitized patients but also in low and high-risk kidney transplant recipients. Circulating BEVs may demonstrate to reveal a part of the humoral response that cannot be assessed yet in clinical practice,

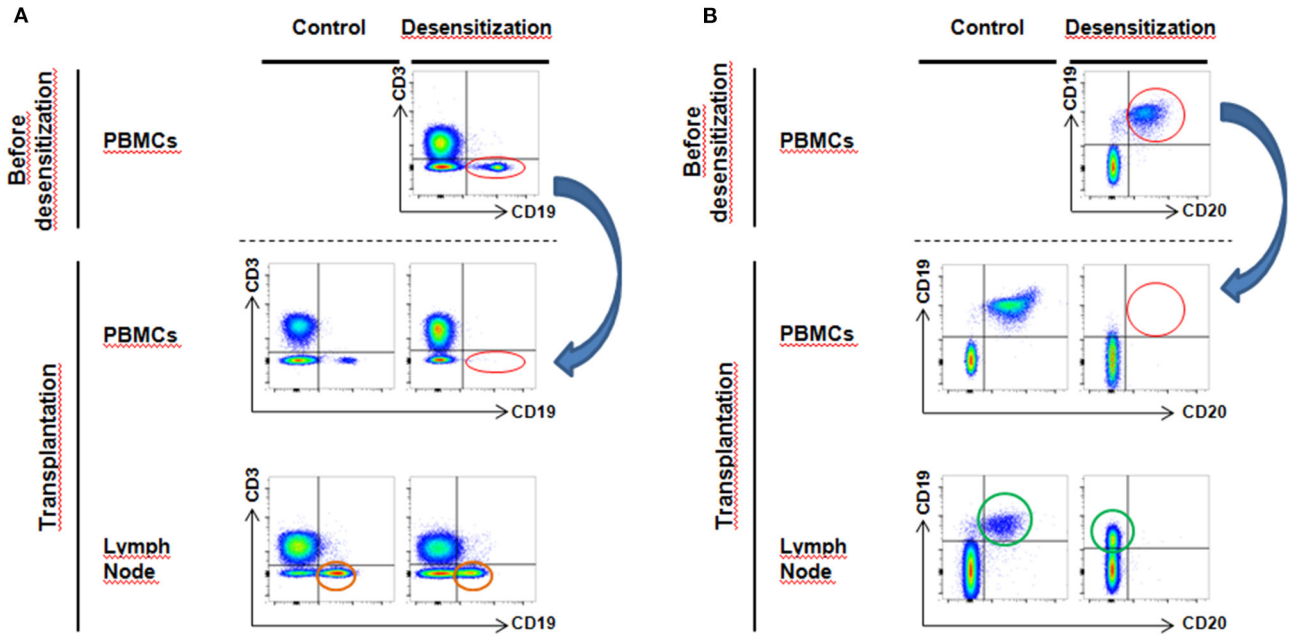

FIGURE 4 | Flow cytometry analysis of circulating and lymph node B cells–**(A)** After desensitization, $CD3^{-}CD19^{+}$ B cells disappear from blood circulation (red circle, blue arrow). However, in lymph nodes at the moment of transplantation, we still observe a substantial proportion of surviving $CD3^{-}CD19^{+}$ B cells (orange circle). **(B)** Desensitization eliminates all $CD19^{+}CD20^{+}$ B cells from peripheral circulation (red circle, blue arrow). However, at transplantation, we observe that the $CD19^{+}CD20^{+}$ population lose CD20 expression compared to the control group (green circle, $CD19^{+}CD20^{-}$).

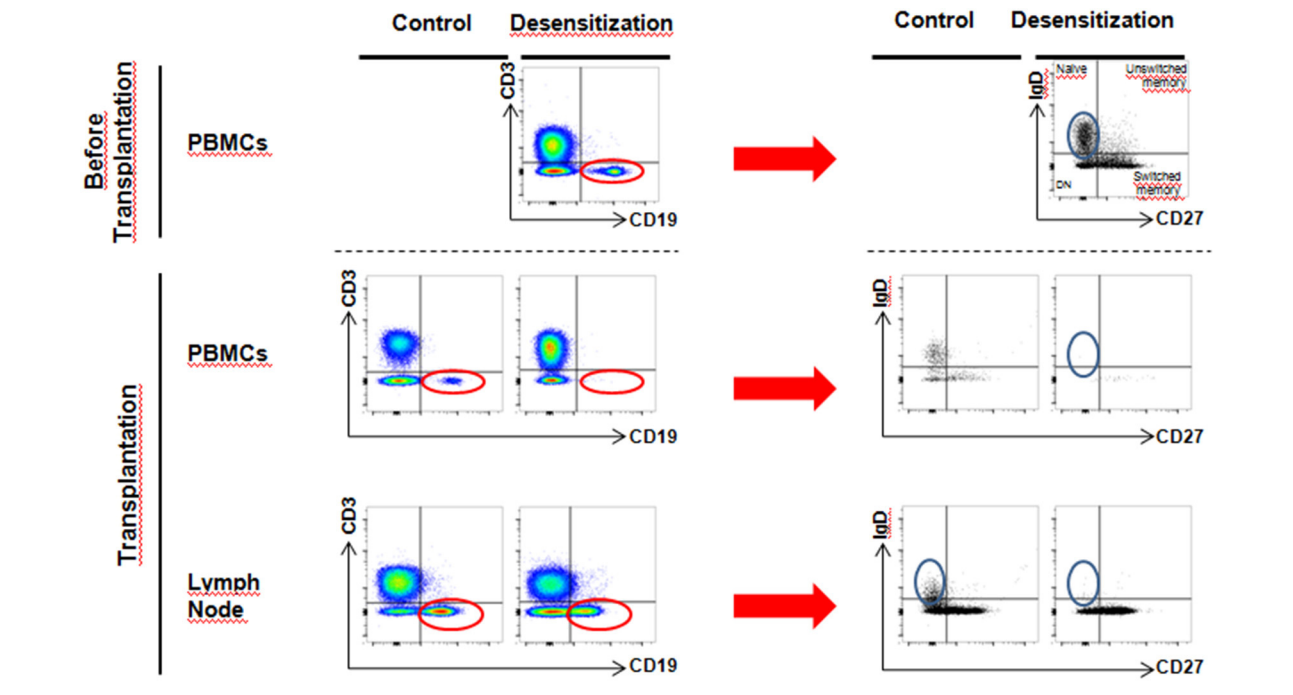

FIGURE 5 | More detailed phenotype analysis of circulating and lymph node B cells–By selection of $CD3^{-}CD19^{+}$ cells (red circle) and further analysis of phenotype, we observed a loss of naive B cells ($CD27^{-}IgD^{+}$) upon desensitization in the lymph node (blue circle), while $CD27^{+}IgD^{-}$ cell population still persisted (switched memory phenotype).

i.e., alloreactive B cells resident in primary and secondary lymphoid organs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by HCB/2016/0394. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DC was involved in study conception, laboratory experiments, data analysis, and writing of the manuscript. VT was involved in laboratory experiments and data analysis. JR, MR-B, and EB-M were involved in study conception, laboratory experiment, and data analysis. ML-R and NH-G were involved in laboratory experiments. FB, JM, and AH was involved in study conception and data analysis. PV-A and GP were involved in data analysis and writing of the manuscript. LP and MM were involved in data analysis. FO, JC, and FD were involved in data analysis and revision of the final draft of the manuscript. IR was involved in study conception, data analysis, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.781239/full#supplementary-material

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Machine Retrograde Perfusion of Deceased Donor Kidneys: A Prospective Study

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Objective: To maximize the utilization of potential kidneys, improving perfusion and preservation techniques is necessary.

Methods: We investigated the safety and efficacy of retrograde machine perfusion of kidneys from deceased donors. A total of 30 kidneys were included and all the grafts were preserved in the Kidney Transporter machines. A total of 15 kidneys that received retrograde perfusion (RP) were selected as the RP group ($n = 15$) and their counterparts received standard antegrade perfusion (AP) as the control group ($n = 15$).

Results: All the recipients were followed up for 6 months. Renal resistance in the RP group remained stable during the perfusion. There was no primary nonfunction. No difference in the incidence of delayed graft function was found in both groups (3 in RP vs. 2 in AP, $p = 0.62$). The RP group had lower serum creatinine (RP vs. AP, 102.20 vs. 138.67, $p = 0.05$) and blood urea nitrogen (RP vs. AP, 6.44 vs. 8.71, $p = 0.05$) than that in the AP group at 6 months. Both the groups had comparable estimated glomerular filtration rate and cystatin C within 6 months.

Conclusion: This novel technique may be an effective and safe alternative for kidney preservation.

Keywords: deceased donor kidneys, retrograde perfusion, kidney transplantation, LifePort, organ recovery

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INTRODUCTION

Kidney transplantation is the treatment of option for end-stage renal disease (ESRD) (1). However, there is still a major discrepancy between the kidney available for transplantation and the actual demand, resulting in an increasing number on the waiting list (2). Efforts should be made to utilize any potential kidney grafts. Besides living donation, kidneys from deceased, old, and "marginal or expanded" donors are the essential source to expand the donor pool (3). However, organs from these donors are associated with higher rates of being discarded, especially when they are not well perfused. Therefore, novel preservation techniques should be adopted and increase the utilization of these organs. Hypothermic machine perfusion (HMP) answers this call and mounting evidence has indicated that HMP had reduced delayed graft function (DGF), better recovery, and kidney function compared with static cold storage (SCS) (4).

The current standard practice of HMP involves perfusion of cold preservation solution into the kidney via a cannula connected to the renal artery. In procurement, we may encounter multiple

renal arteries, artery spasm, or intraoperative damage to the arteries. In these circumstances, the conventional antegrade perfusion (AP) is not a proper technique because it might lead to unsatisfied kidney perfusion and inferior clinical outcomes after transplantation (5–8), even organ discarded. Previous studies have indicated that retrograde perfusion (RP) through the inferior vena cava in some cardiothoracic surgery can protect abdominal organs and kidneys (9–11). Han et al. even showed the feasibility and efficacy of RP in kidney graft from rabbits, sheep, and pigs (11, 12). Inspired by the aforementioned findings, we utilized HMP with RP technique to perfuse kidneys from deceased donors. In this study, we reported the short-term results of these novel techniques.

PATIENTS AND METHODS

Patients

This study is a prospective observation of kidney transplants from deceased donors in West China Hospital, Sichuan University (ClinicalTrials.gov ID: NCT04569682). The institutional review board approved the study protocol and authorized data collection and we obtained the consensus from all the participants. All the kidney grafts were procured from donation after brain death (DBD) between January 1, 2020, and August 1, 2020, with conventional perfusion through a lower segment of the abdominal aorta. When the procurement was completed, kidneys were immediately placed in the ice water for vascular clip on the table preparation. All the right renal veins were lengthening with inferior vena cava for surgical convenience. After that, all the kidneys were perfused with HMP in the LifePort Kidney Transporter machines (Organ Recovery Systems Incorporation, Itasca, Illinois, USA) until operation (13). A total of 30 kidneys were randomized to receive AP and RP. Consequently, their recipients were selected into the AP group ($n = 15$) and the RP group ($n = 15$). Demographic data of both the donors and recipients, human leukocyte antigen (HLA) mismatch, warm ischemia time (WIT), cold ischemia time (CIT), perfusion time (PT), urine output, DGF (defined as requiring dialysis in the first week), serum creatinine (Scr), blood urea nitrogen (BUN), estimated glomerular filtration rate (eGFR), and cystatin C (CysC) at postoperative day (POD) 1, 2, 3, 4, 7, 14, 21, 30, 60, 90, 120, 150, and 180 were collected. Ultrasonic arterial resistance 1 week after the operation was compared as well. No executed prisoners were used as donors and participants were neither paid nor coerced in this study.

Hypothermic Machine Perfusion

All the kidneys were preserved by the LifePort Kidney Transporter machines (Organ Recovery Systems Incorporation, Itasca, Illinois, USA). The technique for RP was described as follows: a catheter was inserted into the renal vein and the RP was performed with a pulsatile flow of kidney preservation solution-1 (14) at 1 to 8°C (**Figure 1**). The initial perfusion pressure was set at 15 mm Hg. If the perfusion went well, perfusion pressure was gradually reduced to 12 mm Hg with 1 mm Hg lower every 10 min. Otherwise, the pressure was maintained at 15 mm Hg. The initial perfusion pressure was set at 30 mm Hg in the AP group and all the kidneys were preserved with machine perfusion until transplantation. The machine perfusion time, pressure, flow, and resistance index were recorded and analyzed.

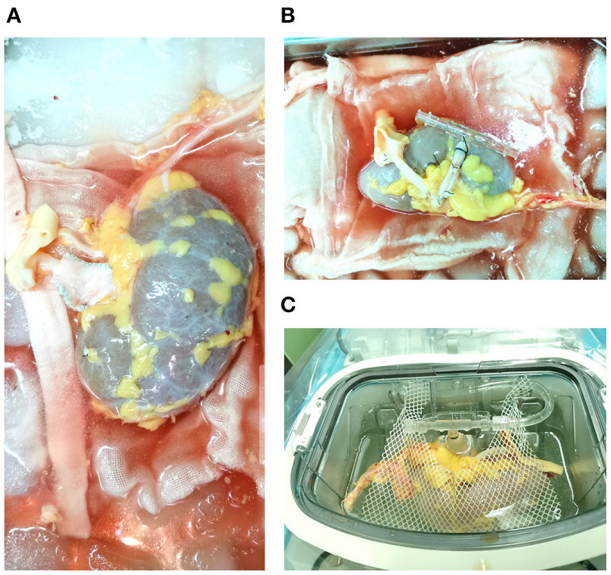

FIGURE 1 | Donor kidney perfused by the retrograde technique. **(A)** Lengthening the right renal vein for kidney graft; **(B)** A catheter was inserted into the renal vein; **(C)** kidney graft was perfused by the retrograde technique in the LifePort Kidney Transporter machines.

Statistical Analysis

Continuous data were represented as the mean ± SD and assessed by the Student's *t*-test and the Mann–Whitney U test to evaluate the differences between the AP and the RP groups. Categorical data were expressed as frequency and analyzed with the chi-squared test. $p < 0.05$ was considered as statistically significant. All the statistical analyses were performed using the SPSS software package (version 24) (SPSS Incorporation, Chicago, Illinois, USA) and the GraphPad Prism version 8 (GraphPad Software Incorporation, San Diego, California, USA).

RESULTS

Characteristics of Donors and Recipients

The demographic data of donors are given in **Table 1**. Among them, 5 (33.33%) donors had hypertension and 2 (13.33%) donors had diabetes mellitus. A total of 10 (66.67%) donors died of cerebral hemorrhage and 5 (33.33%) donors had experienced cardiac arrest. The average length of intensive care unit (ICU) stay was 4.53 days. Terminal serum creatinine was 121.42 μmol/l and the urine output per hour was about 164.50 ml/h. All 15 donors had uneventful intraoperative courses.

The characteristics of recipients are shown in **Table 2**. Two recipients had received peritoneal dialysis and the others had received hemodialysis. The dialysis duration was 54.07 months and 46.80 months for the RP group and the AP group, respectively. All the patients received primary

TABLE 1 | Donor characteristics.

Donor characteristics	Mean ± SD or *n* (%)	Range
Age (years)	50.67 ±10.90	19–66
Gender (%)		
Male	9 (60.00)	
Female	6 (40.00)	
BMI (kg/m^2)	23.29 ± 2.72	17.58–29.30
Hypertension (%)		
Yes	5 (33.33)	
No	10 (66.67)	
Diabetes (%)		
Yes	2 (13.33)	
No	13 (86.67)	
Cardiac arrest (%)		
Yes	5 (33.33)	
No	10 (66.67)	
Donor cause of death (%)		
Cerebral hemorrhage	10 (66.67)	
Accident	4 (26.67)	
Anoxia	1 (6.66)	
Length of stay in ICU (days)	4.53 ± 2.82	2.50–11.00
Terminal Scr (umol/L)	121.42 ± 73.09	47.25–284.50
Urine output (ml/h)	164.50 ± 53.75	109.25–300.50

BMI, body mass index; ICU, intensive care unit; Scr, serum creatinine.

TABLE 2 | Recipient characteristics.

Characteristics	RP group	AP group	*P*
Number, n	15	15	NS
Sex, M/F	8/7	11/4	0.45
Age, years	45.33 ± 11.34	39.73 ± 8.58	0.14
BMI, kg/m^2	20.60 ± 1.77	20.25 ± 3.24	0.72
Type of dialysis (HD/PD)	14/1	14/1	NS
Dialysis duration, months	54.07 ± 32.20	46.80 ± 24.89	0.50
Recipient retransplant	0	0	NS
Mean PRA, %	0	0	NS
HLA-MM (mean ± SD)	4.27 ± 1.03	3.93 ± 0.80	0.33
Lymphocytotoxicity test, %	2	2	NS
Induction agent, (%)			0.14
rATG	6 (40.0)	10 (66.7)	
Basiliximab	9 (60.0)	5 (33.3)	
Immunosuppression, n (%)			NS
CsA + MMF + S	0 (0)	1 (6.7)	
FK + MMF + S	15 (100)	14 (93.3)	

RP, retrograde perfusion; AP, antegrade perfusion; M, male; F, female; BMI, body mass index; HD, hemodialysis; PD, peritoneal dialysis; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; CysC, cystatin C; BUN, blood urea nitrogen; PRA, panel reactive antibody; HLA-MM, human leukocyte antigen-mismatch; rATG, rabbit antithymocyte globulin; CsA, cyclosporine A; FK, tacrolimus; MMF, mycophenolate mofetil; S, steroid; NS, no statistically significant.

kidney transplantation and standard triad immunosuppressive regimen, with no difference in induction therapy and HLA-mismatch (HLA-MM) between the two groups (RP vs. AP, 4.27 vs. 3.93, $p = 0.33$).

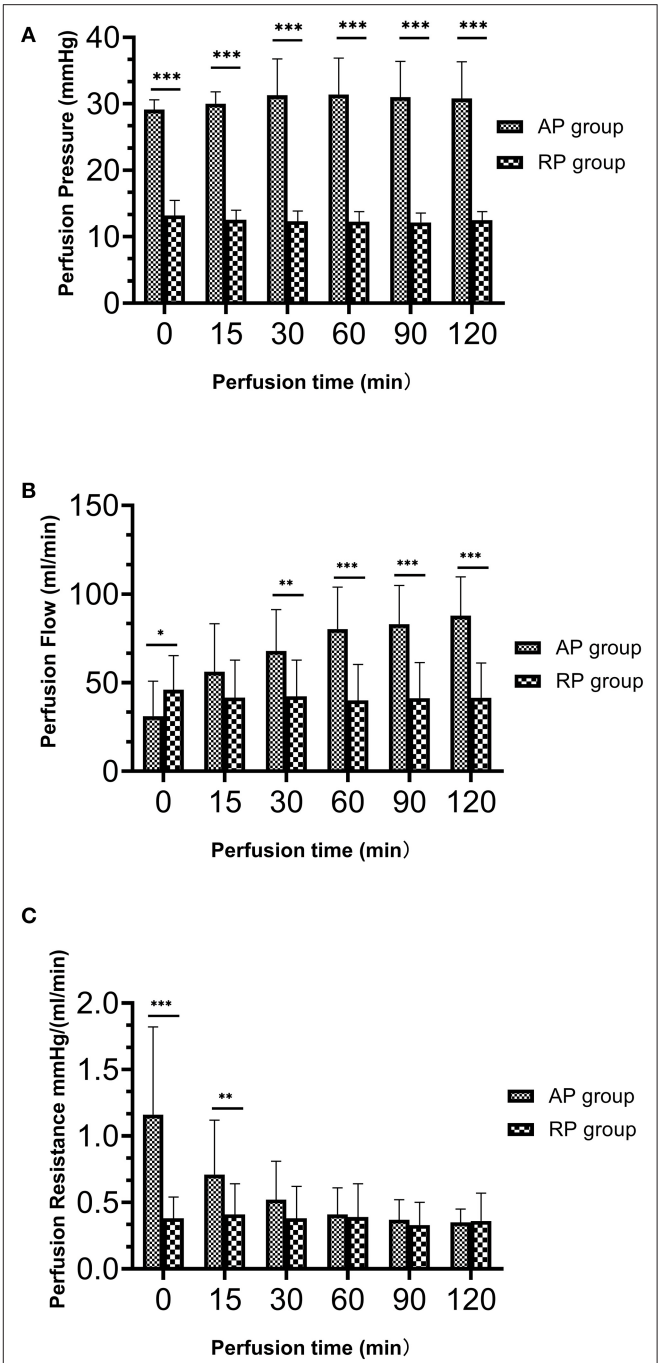

FIGURE 2 | The dynamic perfusion parameters during hypothermic machine perfusion between both the groups. **(A)** Perfusion pressure (mm Hg); **(B)** Perfusion flow (ml/min); **(C)** Perfusion resistance [mm Hg/(ml/min)] (*$p = 0.05$ between two groups; **$p > 0.01$ but < 0.05 between two groups; ***$p < 0.01$ between two groups.

Parameters of Perfusion

Details of the perfusion parameters are shown in **Figure 2** and **Table 3**. There was no difference in WIT, CIT, and PT in both groups. Due to the artificial setting, the AP group had significantly higher initial and maintenance perfusion pressure

TABLE 3 | The parameters of perfusion in two groups.

Variables	RP group	AP group	*P*
Number (n)	15	15	NS
Warm ischemia time, min	2.79 ± 0.62	2.79 ± 0.62	NS
Cold ischemia time, h	10.71 ± 3.99	9.30 ± 3.77	0.33
Perfusion time, h	6.64 ± 3.87	5.25 ± 3.95	0.34
Initial perfusion pressure, mmHg	13.20 ± 2.27	29.13 ± 1.46	<0.01
Initial perfusion flow, ml/min	45.93 ± 19.37	31.07 ± 19.78	0.05
Initial perfusion resistance, mmHg/(ml/min)	0.38 ± 0.16	1.16 ± 0.66	<0.01
Perfusion pressure (2h), mmHg	12.47 ± 1.30	30.80 ± 5.53	<0.01
Perfusion flow (2h), ml/min	41.53 ± 19.62	87.80 ± 21.83	<0.01
Perfusion resistance (2h), mmHg/(ml/min)	0.36 ± 0.21	0.35 ± 0.10	0.84
Terminal perfusion pressure, mmHg	12.40 ± 1.50	30.40 ± 5.65	<0.01
Terminal perfusion flow, ml/min	42.07 ± 21.23	90.53 ± 24.12	<0.01
Terminal perfusion resistance, mmHg/(ml/min)	0.30 ± 0.16	0.32 ± 0.10	0.59

RP, retrograde perfusion; AP, antegrade perfusion; NS, no statistically significant.

TABLE 4 | The clinical outcome of kidney transplantation in both groups.

Variables	RP group	AP group	*P*
Number	15	15	NS
DGF, (%)	3 (20.00)	2 (13.33)	NS
PNF, (%)	0 (0)	0 (0)	NS
Suspected acute rejection, (%)	1 (6.67)	3 (20.00)	0.60
Wound infection, (%)	0	0	NS
Urinary fistula, (%)	0	0	NS
Hospital stays, days	21.87 ± 8.13	19.73 ± 4.80	0.39
Urine output at Pod 30, ml	2203.33 ± 205.69	2230.00 ± 671.83	0.88
Scr at Pod 30, umol/L	120.47 ± 44.54	131.07 ± 44.53	0.60
eGFR at Pod 30, ml/(min 1.73m^2)	63.57 ± 22.84	58.91 ± 19.63	0.55
Cys-c at Pod 30, mg/L	1.63 ± 0.48	1.62 ± 0.53	0.97
BUN at Pod 30, mmol/L	8.82 ± 4.22	8.72 ± 3.47	0.95
Scr at Pod 60, umol/L	108.00 ± 28.27	143.13 ± 85.68	0.14
eGFR at Pod 60, ml/(min 1.73m^2)	69.21 ± 19.98	58.08 ± 21.68	0.18
Cys-c at Pod 60, mg/L	1.47 ± 0.33	1.67 ± 0.56	0.22
BUN at Pod 60, mmol/L	7.04 ± 2.73	8.47 ± 3.26	0.20
Scr at Pod 90, umol/L	107.47 ± 24.85	141.20 ± 64.55	0.07
eGFR at Pod 90, ml/(min 1.73m^2)	69.65 ± 16.71	59.06 ± 26.68	0.25
Cys-c at Pod 90, mg/L	1.48 ± 0.27	1.78 ± 0.76	0.16
BUN at Pod 90, mmol/L	6.40 ± 2.02	8.81 ± 4.01	0.05
Scr at Pod 180, umol/L	102.20 ± 16.21	138.67 ± 66.73	0.05
eGFR at Pod 180, ml/(min 1.73m^2)	71.60 ± 11.43	63.57 ± 23.04	0.28
Cys-c at Pod 180, mg/L	1.40 ± 0.18	1.75 ± 0.67	0.06
BUN at Pod 180, mmol/L	6.44 ± 1.51	8.71 ± 3.99	0.05

POD, postoperative day; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; CysC, cystatin C; BUN, blood urea nitrogen; DGF, delayed graft function; PNF, primary non-function; NS, no statistically significant.

than that in the RP group ($p < 0.01$). At the beginning of perfusion (PT = 10 min), the RP group had higher perfusion flow than the AP group (RP vs. AP, 45.93 vs. 31.07, $p = 0.05$), but the RP group had significantly lower terminal perfusion flow (RP vs. AP, 42.00 vs. 90.67 ml/min, $p < 0.01$). The RP group had lower initial perfusion resistance [RP vs. AP, 0.36 vs. 1.37 mm Hg/(ml/min) at PT 10 min, $p < 0.01$], while no difference was found between the two groups in terminal perfusion resistance [RP vs. AP, 0.30 vs. 0.32 mm Hg/(ml/min), $p = 0.59$]. During the perfusion, the resistance in the RP

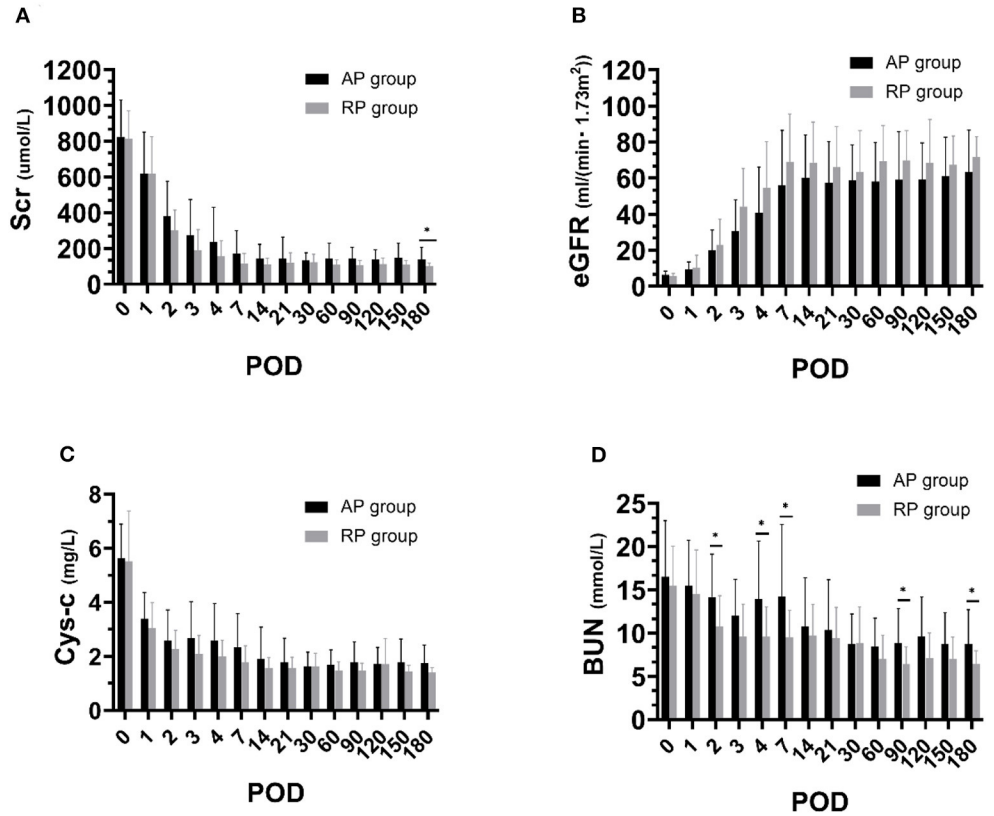

FIGURE 3 | Renal function during postoperative 6 months between both the groups. **(A)** Serum creatinine (Scr); **(B)** Blood urea nitrogen (BUN); **(C)** Estimated glomerular filtration rate (eGFR); **(D)** Cystatin C (CysC) (*$p < 0.05$ between two groups).

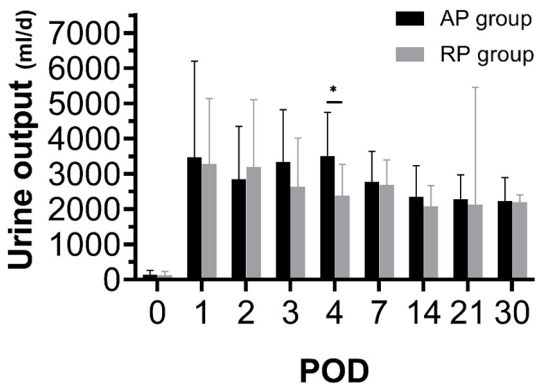

FIGURE 4 | Twenty-four hours urine output during postoperative 1 month between both the groups (*$p < 0.05$ between two groups).

group was relatively stable (**Figure 2C**, **Supplementary Table S1**, **Supplementary Figure S1**).

Transplantation Outcomes

All the patients were followed up for 6 months. Postoperative information is given in **Table 4**. There was no primary non-function (PNF) in both groups. Three cases had DGF in the RP group and 2 cases had DGF in the AP group. DGF in the RP group lasted for 1 to 2 days, with 1 or 2 sessions of dialysis. Similarly, DGF occurred in the AP group lasted 1 or 3 days (**Supplementary Table S2**). As indicated in **Figures 3**, **4**, **Table 4**, **Supplementary Figure S2** and **Supplementary Table S3**, we found that patients who received RP perfused kidney had comparable urine output, Scr, CysC, BUN, and eGFR at any time point in the first month to those receiving AP perfused grafts. In postoperative 6 months, we found that the RP group had lower Scr (RP vs. AP, 102.20 vs. 138.67, $p = 0.05$) and BUN (RP vs. AP, 6.44 vs. 8.71, $p = 0.05$) than the AP group. There was no statistically significant difference between eGFR and CysC in POD 180 ($p > 0.05$). One clinically suspected acute rejection episode occurred in the RP group and three clinically suspected acute rejection episodes occurred in the AP group and all received methylprednisolone pulse therapy and recovered. There was no significant difference in the length of hospital stay between the two groups (RP vs. AP, 21.87 vs. 19.73, $p = 0.39$). There was no surgical-related complication such as wound infection, urinary leakage, or ureter stricture during the follow-up.

At 1 week, all the allografts received the evaluation of ultrasound and there was no difference in renal, segmental, interlobar, and arcuate arterial resistance index (**Supplementary Table S4**). In the RP group, we divided

the RP perfused grafts into two subgroups according to the perfusion resistance at 2 h (group 1, perfusion resistance < 0.4; group 2, perfusion resistance ≥ 0.4). In subgroup analysis, no difference was found in the arterial resistance index (**Supplementary Table S5**). Similarly, as shown in **Supplementary Table S6**, both the groups had a comparable renal function.

DISCUSSION

In this study, we first used RP machine perfusion for the preservation of kidneys from a deceased donor and found that kidneys receiving RP had a comparable incidence of DGF, urine output to the AP perfused allograft. Interestingly, although both the groups had comparable eGFR, we found that allografts perfused by RP had lower Scr and BUN than those receiving AP perfusion.

In organ procurement, we may come across renal artery injury, anatomical variation, and malformations of the arteries. These kidneys might not be well perfused through traditional arterial-to-venous perfusion (AP), which might increase the discard rate (15). Each renal segment was supplied by a segmental artery as an end-artery. In the back-table preparation, for kidneys with multiple arteries, effective perfusion of the whole kidney requires separate cannulation and flushing of each renal artery, which was time-consuming and laborious and the perfusion was not satisfying. Renal veins had greater diameters, less variation than renal arteries, and no venous valves in the renal venous system (16). Most importantly, there was extensive communication between segments on the venous side. In view of the anatomical difference between arteries and veins, it seemed to be possible to perfuse from veins to arteries and evidence from the animal study has demonstrated that renal perfusion could be carried out by retrograde blood flow from the efferent artery to the afferent artery (17).

Wilhelm et al. (18) had used the RP technique *in-situ* perfusion of dog model for the first time. Until the late 1980s, Rolles et al. (19) had carried out a clinical study on retrograde oxygen perfusion of renal grafts. Although this technique had not been further applied in transplantation due to the improvement of preservation solutions, it provided us with the feasibility of RP. An animal study found that 24 h RP of rabbit kidneys revealed good morphological changes (12). To further verify its feasibility and safety, Han et al. (11) conducted a porcine renal autotransplantation and found no difference in the renal function between the AP and the RP groups at day 7. Of note, Han et al. (15) compared the AP and RP in kidneys with damaged or variant arteries and found comparable graft survival at 1, 3, and 5 years. In another case series study, Hobeika et al. (20) also reported no difference in eGFR between the RP and the AP groups. These studies have indicated that RP of the kidney is feasible and safe.

In this study, we found no difference in the incidence of DGF, urine output, and renal function in the first month between the AP and RP groups. However, we found that the RP group had lower Scr and BUN than the AP group at 6 months, indicating that retrograde machine perfusion of allograft was not inferior to the conventional technique. Experience from lung transplant had indicated that retrograde flush could remove residual microthrombi after antegrade flush (21) and microthrombi were often found in kidneys from a deceased donor and these grafts may have a higher incidence of DGF and inferior early function (22). These facts raised the hypothesis that the RP technique might also help to remove microthrombi in the kidneys and improve organ perfusion and function preservation, while further evidence is required to verify it.

Initially, we set the perfusion pressure for RP at 15 mm Hg and found that the perfusion pump worked well and the solution could go smoothly into all the kidneys. The normal pressure in the renal vein is 10 mm Hg and higher venous pressures have been associated with impaired renal function (23). Thus, we planned to gradually lower the perfusion pressure down to 10 mm Hg. To the best of our knowledge, the lowest pressure for the perfusion pump to work in RP was 12 mm Hg. Therefore, we gradually decreased the pressure to 12 mm Hg every 10 min with 1 mm Hg lower. For some cases, we maintained the pressure at 15 mm Hg because when we lowered the pressure by 1 mm Hg, the perfusion pump failed to work. Interestingly, although the perfusion pressure and flow were much higher in the AP group, the perfusion resistance of the AP group gradually decreased and become very close to that of the RP group after 2 h. Of note, the perfusion resistance for most cases remained stable from the beginning to the end of perfusion in the RP group. Previous studies had indicated that perfusion resistance of allografts undergoing HMP (AP technique) was considered as a measure of organ quality (24) and the resistance often took a long time to obtain, usually more than 2 h. Therefore, our results suggested that retrograde machine perfusion might make a quicker assessment of kidney quality than the conventional perfusion technique.

In addition, most studies set the threshold of 0.4 mm Hg/ml/min for the perfusion resistance (AP technique) and found that resistance greater than 0.4 was associated with increased graft failure, even not being used for transplantation (25). In this study, we categorized the patients in the RP group according to the perfusion resistance at 2 h and found that the renal function in patients receiving kidneys with resistance <0.4 was similar to those patients receiving kidneys with resistance ≥ 0.4. Additionally, evidence from AP has shown that early renal Doppler ultrasound intrarenal resistive index was associated with detrimental pathological changes (26) and can help to predict long-term graft function (27). Our results found that kidneys with resistance < 0.4 had a comparable renal resistance index at Day 7, suggesting that resistance of 0.4 obtained from the RP technique is not a proper cutoff value for organ quality assessment and more evidence is required to find a resistance threshold of clinical significance.

There are several limitations to this study. First, we reported our early experience of RP techniques and the included cases were limited, which may be underpowered to detect the difference between the AR and RP groups. Second, we only included kidneys from DBD donors, and how the RP work in kidneys from cardiac death donor or those with acute kidney injury remained unknown. In addition, after perfusion satisfied

by cannulating the renal vein to LifePort, we usually removed the joint part of the renal vein before the operation, which may lead to a shorter renal graft vein and increase the difficulty of vascular anastomosis, especially for retransplant or obese patients, since it has not happened in this study. Finally, we did not obtain biopsy data that whether RP could preserve the microstructure efficiently is still at issue. Finally, due to the short follow-up period, the long-term effect of RP is not clear. Therefore, a prospective trial with a greater number of participants and long-term follow-up is necessary to prove the equivalence or superiority of retrograde machine perfusion in kidney preservation.

CONCLUSION

Machine perfusion with RP technique is effective and safe in preserving kidneys from deceased donors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ conceived and designed the study and prepared the first draft of the manuscript. ZJ performed the statistical analysis. TL analyzed the data. TS interpreted the data and revised the manuscript. All authors approved the final manuscript and contributed intellectually important content of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.785953/full#supplementary-material

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Living Donor Kidney Transplantation in Patients With Donor-Specific HLA Antibodies After Desensitization With Immunoadsorption

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Due to the current organ shortage, living donor kidney transplantation is increasingly performed across HLA (human leukocyte antigen) or ABO antibody barriers. There is still uncertainty about the risk of antibody-mediated rejection (AMR) episodes, which may limit long-term graft survival. From March 2007 to December 2016, 58 sensitized living donor kidney transplant candidates were identified and 38 patients eventually included in the study: 36 patients (95%) had pre-transplant and pre-desensitization Luminex-detected donor-specific HLA antibodies (DSA), and 17/36 patients (47%) in addition had a positive crossmatch result. Two patients had no detectable DSA but a positive CDC B-cell crossmatch result. Patients were treated with pre- and post-transplant apheresis and powerful immunosuppression including the anti-CD20 antibody rituximab ($N = 36$) in combination with thymoglobulin ($N = 20$) or anti-IL2 receptor antibody ($N = 18$). The results of the 38 successfully desensitized and transplanted patients were retrospectively compared to the results of 76 matched standard-risk recipients. Desensitized patients showed patient and graft survival rates similar to that of standard-risk recipients ($P = 0.55$ and $P = 0.16$, respectively). There was a trend toward reduced death-censored graft survival in desensitized patients ($P = 0.053$) which, however, disappeared when the 34 patients who were transplanted after introduction of sensitive Luminex testing were analyzed ($P = 0.43$). The incidence of rejection episodes without borderline changes were in desensitized patients with 21% similar to the 18% in standard-risk patients ($P = 0.74$). Thirty-six patients had pre-transplant HLA class I and/or II DSA that were reduced by 85 and 81%, respectively, during pre-transplant desensitization ($P < 0.001$ for both). On day 360 after transplantation, 20 of 36 (56%) patients had lost their DSA. The overall AMR rate was 6% in these patients, but as high as 60% in 5 (14%) patients with persistent and *de novo* DSA during year 1; 2 (40%) of whom lost their graft due to AMR. Eleven (31%) patients with persistent DSA but without *de novo* DSA had an AMR rate of 18% without graft loss while one patient lost her graft without signs of AMR.

Our desensitization protocol for pre-sensitized living donor kidney transplant recipients with DSA resulted in good graft outcomes with side effects and rejection rates similar to that of standard-risk recipients. Adequate patient selection prior to transplantation and frequent immunological monitoring thereafter is critical to minimize rejection episodes and subsequent graft loss.

Keywords: desensitization, immunoadsorption, donor-specific antibody (DSA), antibody-mediated rejection (AMR), kidney transplantation

INTRODUCTION

The increasing number of patients with chronic kidney disease and the ongoing organ shortage have led to efforts to increase the number of living donor transplants. One possibility is living donor kidney transplantation across the human leukocyte antigen (HLA) barrier after desensitization prior to transplantation. A study by Montgomery et al. showed that the overall survival rate of patients who were desensitized for living donor kidney transplantation was significantly higher than the survival rate of patients waiting for a compatible allograft from a deceased donor (1). These results were later confirmed in a larger multicenter study from the United States, but did not hold up when the same analysis was performed for patients transplanted in the United Kingdom (2, 3). Several protocols exist for desensitization of kidney transplant recipients, that are all based on rapid reduction of HLA antibodies before transplantation and strong immunosuppression to permanently suppress *de novo* HLA antibody formation thereafter. Immunoadsorption (IA) has been shown to be effective in rapidly removing HLA antibodies before transplantation. Bartel et al. published encouraging results from 68 HLA-sensitized deceased donor kidney transplant recipients desensitized by IA (4). We demonstrated that HLA antibody removal by IA was effective in 10 recipients of crossmatch-positive living donor kidney transplants (5). A larger analysis of 23 HLA-sensitized recipients from our center confirmed the excellent results with a graft and patient survival rate of 100% at two years and a low rate of treatment-related adverse events and rejection episodes (6).

Since 2006, we have been consistently using this desensitization protocol and have gained broader experience with desensitization in a total of 58 patients, 38 of whom were eventually included in this study. The aim of this study was to compare the results of these 38 successfully desensitized patients with the results of 76 matched standard-risk recipients. The primary outcome measures were graft and patient survival while the secondary outcome measures included effectiveness of antibody-removal by desensitization with immunoadsorption, graft function, biopsy-proven rejection episodes, complications, and the course of donor-specific HLA antibodies (DSA) after transplantation. In addition, we aimed to determine the impact of the introduction of the highly sensitive Luminex assay to our routine in 2009 on the outcomes of these HLA antibody-incompatible transplants.

MATERIALS AND METHODS

"Heidelberg Algorithm" Criteria and Patient Selection

Patients transplanted under the "Heidelberg Algorithm" by December 2016 were considered for inclusion in the study. The "Heidelberg Algorithm" was developed in 2005 and applied since April 2006 to identify and treat patients on the Heidelberg waiting list who are at particularly high risk for AMR after transplantation (**Supplementary Table 1**) (7–9). Based on results from the *Collaborative Transplant Study*, patients were considered at increased risk if they had a CDC-PRA-DTT $\geq$ 85% (current or past), HLA class I and II antibody positivity in ELISA screening, or HLA class I antibody positivity in ELISA screening at re-transplantation (donor-independent criteria), or a positive CDC B-cell crossmatch in re-transplant recipients with HLA class II antibody positivity in ELISA screening, or a positive CDC T-cell crossmatch (donor-dependent criteria). In addition, recipients of living donor kidneys were considered as high-risk if they had a DSA $\geq$ 1,000 MFI (after April 2009) (7–9). These patients were treated and monitored according to a specific algorithm that, for living donor kidney transplant recipients, included pre- and post-transplant desensitization, powerful antibody induction therapy and immunosuppression and close post-transplant antibody monitoring together with protocol biopsies (**Supplementary Table 1**).

Desensitization Therapy

IA was performed with Peptid-GAM-coated Globaffin columns that specifically bind IgG1, 2, and 4 and intermediately strong IgG3 and weakly IgM antibodies (Fresenius Medical Care, Bad Homburg, Germany) on an ADAsorb device (medicap clinic GmbH, Ulrichstein, Germany) together with an AS.TEC 204 centrifuge (Fresenius Medical Care). Treatment was repeated before transplantation until the CDC- and ELISA-crossmatch results became negative. In addition, DSA had to be negative in ELISA screening and below 1,000 MFI in Luminex testing (from March 2009). IA was applied on alternate days. Anticoagulation during IA consisted of 1,500 units of heparin per hour together with sodium citrate (ACD-A, Fresenius Kabi AG, Bad Homburg, Germany) at an infusion rate of 1:23 (citration infusion: blood flow). In patients with risk of bleeding, especially after surgery, the treatments were conducted without heparin and a sodium citrate infusion rate of 1:16. Resulting hypocalcemia was treated with calcium gluconate (10%, B. Braun, Melsungen, Germany). In patients with proven or suspected DSA of the IgM isotype,

plasmapheresis was used additionally to account for the reduced IgM removal efficacy of IA.

After transplantation, apheresis treatments were continued in all patients on alternate days until good allograft function was achieved with a serum creatinine of <2 mg/dL and the DSA remained negative in ELISA screening and below a cut-off of 1,000 MFI in Luminex single antigen testing (from March 2009).

Immunosuppression and Infection Prophylaxis

In sensitized patients, immunosuppression with tacrolimus (target trough levels month 1: 10–15 μg/L, month 2: 10–12 μg/L, month 3: 8–10 μg/L, beyond year 1: 5–8 μg/L), enteric-coated mycophenolate sodium (720 mg twice daily) and methylprednisolone (20 mg during desensitization, 250 mg on day 0, tapered to 20 mg on day 9 after surgery) was started with the initiation of IA. Induction therapy was applied with thymoglobulin ($N = 20$, 2–7 infusions of 1.5 mg/kg body weight with a target lymphocyte count of ∼0.2/nL within the first 14 days after transplantation), or basiliximab ($N = 18$, 20 mg on days 0 and 4 after transplantation). From May 2009, the induction therapy was changed from basiliximab to thymoglobulin in all sensitized patients due to an increased frequency of T cell-mediated rejection episodes. Basiliximab continued to be given in those patients who possessed low-level DSA that were identified in Luminex testing only but who had a negative crossmatch result (and a soluble CD30 (sCD30) concentration below 80 ng/mL, from October 2016).

In addition, rituximab was administered at a single dose of 375 mg/m^2 body surface after the last IA treatment on day-1 before surgery ($N = 36$).

Standard risk patients received immunosuppression with cyclosporine A (target trough levels month 1: 180–200 μg/L, month 2: 150–180 μg/L, month 3–year 1: 120–150 μg/L, beyond year 1: 100–120 μg/L) together with enteric-coated mycophenolate sodium and methylprednisolone at the same dose as in sensitized recipients. Induction therapy was conducted with basiliximab on days 0 and 4 after transplantation.

Prophylaxis with valganciclovir was performed for 3 months in cytomegalovirus-positive patients and/or recipients of a cytomegalovirus-positive organ. Fungal prophylaxis consisted of 1 mL of nystatin four times daily for 3 months. Pneumocystis jirovecii prophylaxis was conducted by

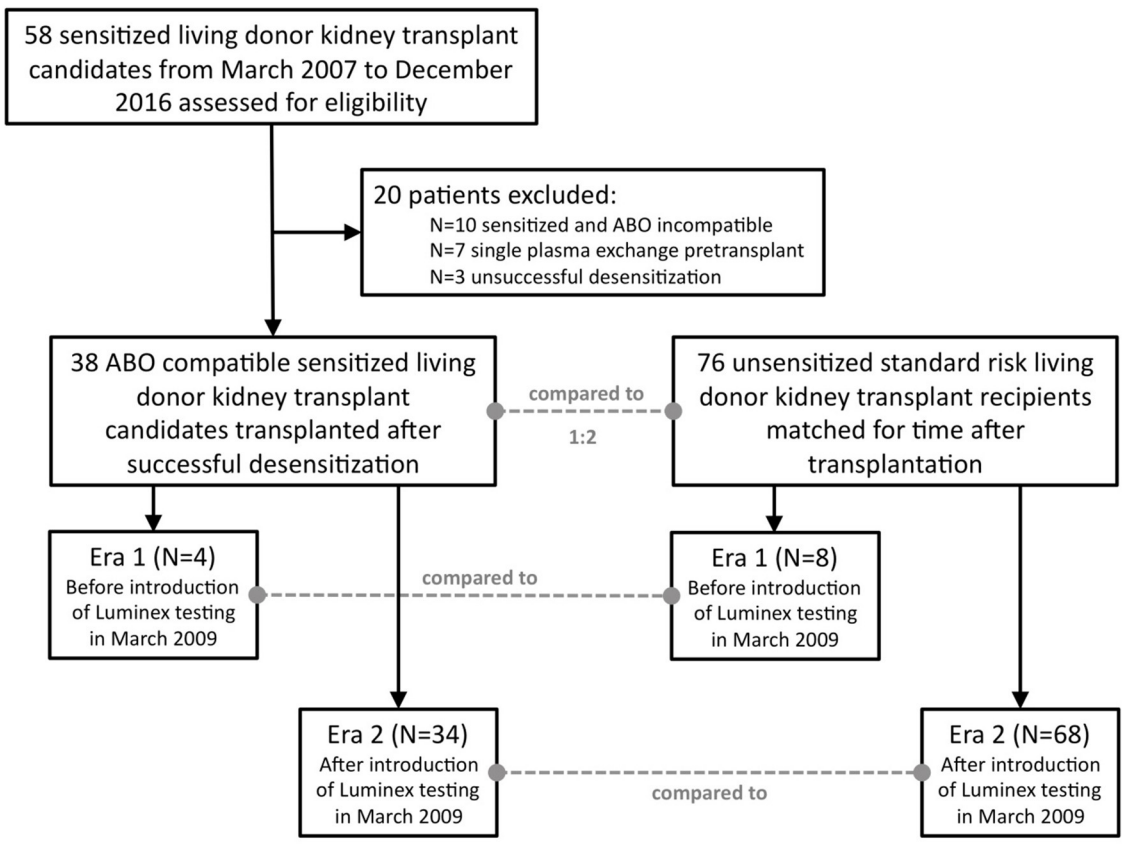

FIGURE 1 | Flow chart for patient selection. Fifty-eight sensitized patients were screened, and 38 patients eventually included in the analysis. Each sensitized patient ($N = 38$) was matched with two standard risk recipients ($N = 76$) for time after transplantation, i.e., the first standard risk recipient transplanted before and the first transplanted after surgery of the desensitized candidate. Patients were further stratified according to the date of transplantation, either before March 2009 or thereafter. In March 2009, highly sensitive Luminex testing was introduced into clinical routine at our center.

TABLE 1 | Baseline patient characteristics.

	Standard risk (*N* = 76)	Desensitized (*N* = 38)	*P* value
Recipient characteristics			
Female sex, N (%)	26 (34)	18 (47)	0.22
Age, median (range)	39 (16–70)	44 (20–62)	0.34
Caucasian race, N (%)	75 (99)	38 (100)	1.0
Cause of ESRD, N (%)			0.62
Diabetes	3 (4)	0 (0)	
Hypertension	7 (9)	2 (5)	
Glomerulonephritis	35 (46)	14 (37)	
Pyelonephritis	7 (9)	3 (8)	
ADPKD	10 (13)	9 (24)	
Other	10 (13)	6 (16)	
Unknown	4 (5)	4 (11)	
Comorbidities*, N (%)			
Diabetes	3 (4)	7 (18)	0.015
Hypertension	57 (75)	27 (71)	0.66
Cardiovascular event	5 (7)	2 (5)	1.0
N of previous tx, N (0/1/2)	70/6/0	23/11/4	<0.001
Mode of pre-tx dialysis, N (%)			0.24
HD	48 (62)	30 (79)	
PD	8 (11)	3 (8)	
Preemptive tx	20 (26)	5 (13)	
Years on dialysis before last tx, median (range)	0.8 (0–17)	1 (0–9)	0.71
Donor characteristics			
Female sex, N (%)	44 (58)	21 (55)	0.84
Age, median (range)	50 (27–77)	50 (25–75)	0.67
Related donor, N (%)	51 (67)	15 (39)	0.008
Pre-tx immunological parameters			
CDC T-Cell PRA %, median (range)	0 (0–5)	0 (0–98)	<0.001
HLA-A+B+DR mismatches, N (%)			
0–1	12 (16)	3 (8)	0.38
2–4	48 (63)	30 (79)	0.13
5–6	16 (21)	5 (13)	0.44
CDC-XM result positive, N (%)	2 (3)[a]	19 (50)	<0.001
B-cell	1 (1)	12 (32)	<0.001
T-cell	0 (0)	1 (3)	0.33
U+B-cell	0 (0)	3 (8)	0.035
U+B+T-cell	1 (1)	3 (8)	0.11
Luminex-DSA positive, N (%)	15 (20)	36 (95)	<0.001
Class I	4 (5)[b]	22 (58)	<0.001
Class II	9 (12)[c]	9 (24)	0.011
Both	2 (3)[d]	5 (13)	0.033
sCD30 positive, N (%)			
sCD30	31 (41)	17 (45)	0.84
sCD30 and DSA	0 (0)	16 (42)	<0.001
Procedures and follow-up			
Pre-tx immunoadsorption			
Patients, N (%)	0 (0)	38 (100)	<0.001
Treatments, median (range)	0 (0)	8 (4–22)	<0.001
Pre-tx plasma exchange			
Patients, N (%)	1 (1)	12 (32)	<0.001
Treatments, median (range)	0 (0–1)	0 (0–6)	<0.001
Post-tx immunoadsorption or plasma exchange			
Patients, N (%)	1 (1)	36 (95)	<0.001
Treatments, median (range)	0 (0–2)	4 (0–18)	<0.001
Induction therapy, N (%)			
No induction	7 (9)	0 (0)	0.093
Anti-CD20 rituximab	1 (1)	36 (95)	<0.001
Basiliximab	68 (89)	18 (47)	<0.001
Thymoglobulin	1 (1)	20 (53)	<0.001
Initial calcineurin inhibitor, N (%)			
Cyclosporine	55 (72)	1 (3)	<0.001
Tacrolimus	21 (28)	37 (97)	<0.001
Post-tx hospital stay (days), median (range)	13 (9–57)	18 (10–57)	<0.001
Follow-up (months), median (range)	51 (11–121)	43 (7–97)	0.20

**Comorbidities at time of transplantation (diabetes and arterial hypertension with treatment indication, cardiovascular event defined as s/p stroke or PCI/CAB surgery).*
[a] Unspecific, most likely due to autoantibodies.
[b] Below 1,000 MFI.
[c] Below 1,000 MFI (N = 7), or considered unspecific/irrelevant (N = 2).
[d] HLA antibodies were identified only retrospectively (before Luminex era).
ADPKD, autosomal-dominant polycystic kidney disease; DSA, donor-specific human leukocyte antigen (HLA) antibodies; ESRD, end-stage renal disease; HD, hemodialysis; N, number; PD, peritoneal dialysis; tx, transplant; XM, crossmatch.

alternate day administration of trimethoprim (160 mg) and sulfamethoxazole (800 mg) for 6 months.

Immunology

CDC crossmatches were performed with unseparated peripheral blood mononuclear cells as well as isolated donor T and B lymphocytes using the standard CDC technique without anti-human immunoglobulin enhancement. In addition, a solid-phase ELISA crossmatch assay (AbCross, Biotest, Dreieich, Germany) was used. PRA screenings were performed using CDC and ELISA techniques. DSA of the IgG isotype against HLA antigens were determined by ELISA and, since March 2009, in addition by Luminex technologies using the AbIdent kits of Biotest (Dreieich, Germany), and the LABScreen Single Antigen kit of One Lambda (Canoga Park, CA, USA), respectively. For the detection of DSA of the IgM isotype by Luminex, 1:100 diluted PE-conjugated $F(ab')_2$ fragments of donkey anti-human IgM, Fc antibodies (Dianova, Hamburg, Germany) were used. HLA typings of donors and recipients were performed using PCR-SSP and sequencing. The HLA alloantibodies were measured after transplantation on days 7, 30, 180, 360, and every 6 months thereafter. Additional testing was performed if deterioration of allograft function was noted. We and others use different cut-offs for the determination of DSA before and after transplantation (10). While our pre-transplant cut-off is 1,000 MFI, post-transplant DSA are considered relevant at a cut-off of 500 MFI to capture low level *de novo* DSA and because *in vivo* antibody adsorption in the allograft may lead to falsely low antibody reactivity. Since October 2016, pre-transplant and pre-desensitization serum samples were further tested for sCD30

using human sCD30 Instant ELISA (eBioscience, San Diego, CA) according to the manufacturer's instructions. An sCD30 concentration ≥80 ng/mL before transplantation was used as cut-off for positivity.

Statistical Analysis

The primary outcome measure was graft and patient survival while secondary outcome measures included effectiveness of antibody-removal by desensitization with immunoadsorption, graft function, biopsy-proven rejection episodes, complications, and the course of DSA after transplantation. In addition, the impact of the introduction of the highly sensitive Luminex assay to clinical routine in 2009 on the outcomes of HLA antibody-incompatible transplants was determined.

Data are given as median and range, mean and standard error of the mean, or number and percent. Statistical analyses were performed using SPSS Statistics 28 (IBM). For group comparisons *t*-test and Mann-Whitney-*U*-test were used, chi-square test for categorial variables.

Reduction of immunoglobulins or DSA was calculated with the Wilcoxon matched-pairs signed rank test. Graft survival was calculated according to the Kaplan-Meier method.

RESULTS

Patient Selection and Baseline Demographics

Fifty-eight HLA-sensitized living donor kidney transplant candidates who met the "Heidelberg Algorithm" criteria and were desensitized between March 2007 and December 2016 were identified and screened for eligibility (**Supplementary Table 1**; **Figure 1**). Twenty patients were excluded from the analysis due to transplantation in the co-presence of a major ABO incompatibility ($N = 10$), a single pre-transplant plasmapheresis treatment only due to very low DSA levels ($N = 7$), or an unsuccessful desensitization ($N = 3$). The 38 successfully desensitized patients met at least one criterion of the "Heidelberg algorithm": 36 patients (95%) had pre-transplant and pre-desensitization DSA, and 17/36 patients (47%) in addition had a positive crossmatch result. Two patients (number 22 and 23) had no detectable DSA but a positive CDC B-cell crossmatch result.

Results of 38 desensitized living donor kidney transplant recipients were analyzed and retrospectively compared to results of 76 standard-risk living donor kidney recipients matched for the time of transplantation. Patients were further divided into two different groups derived from two different eras, before (*N*

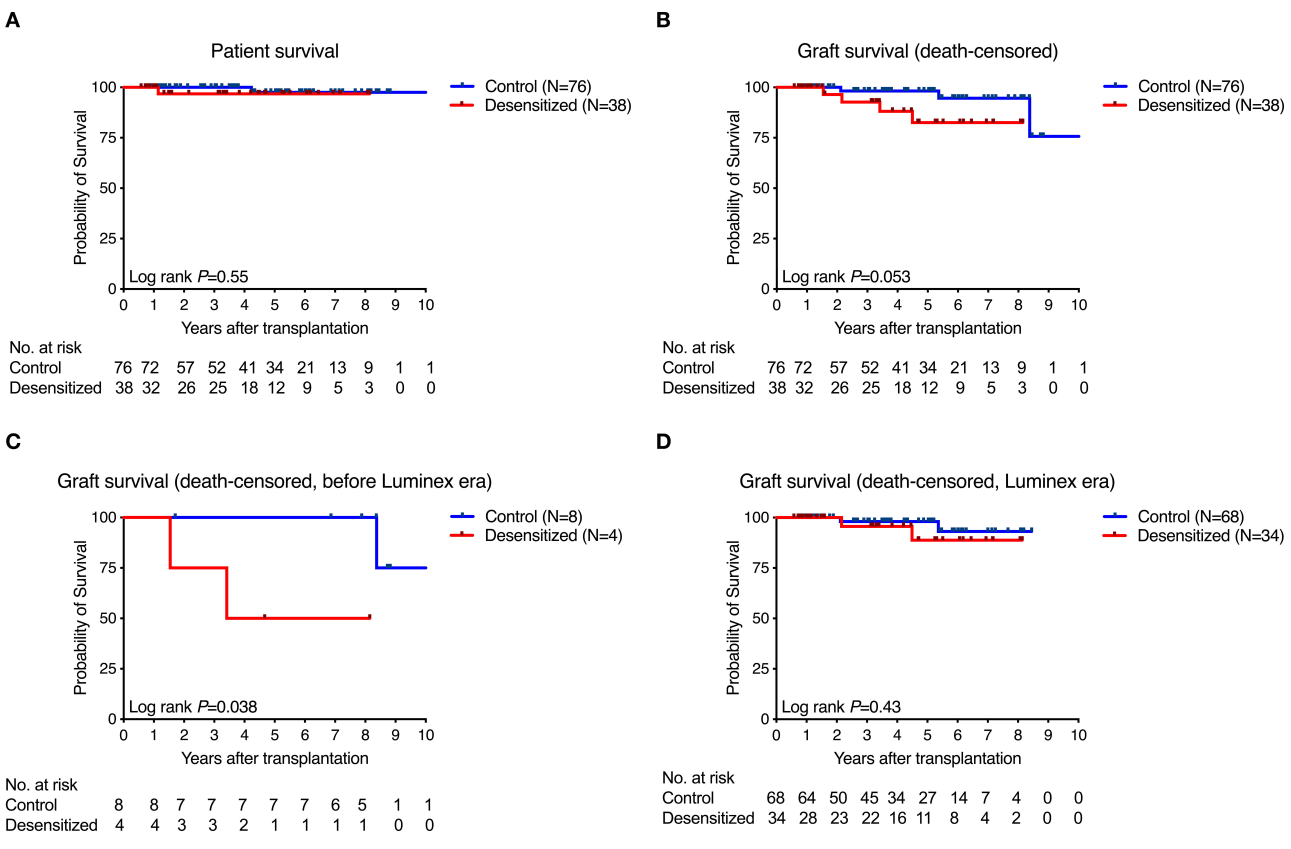

FIGURE 2 | Patient and graft survival. Patient survival did not significantly differ between groups **(A)** while there was a trend toward reduced death-censored graft survival **(B)** that was driven by results obtained from the era before Luminex testing became available in clinical routine **(C)**. In contrast, death-censored graft survival in both groups was nearly identical in the Luminex era **(D)**.

= 4) and after (N = 34) the introduction of highly sensitive Luminex testing in March 2009, and analyzed separately.

Most demographic data were comparable between the desensitized and standard-risk recipients (**Table 1**). In addition to the degree of sensitization, significant differences between the groups were a higher number of previous transplants (P = 0.01), a lower number of related donors (P = 0.01), a longer postoperative hospital stay (18 vs. 13 days) presumably due to the more complex procedure ($P < 0.001$), and the pre- and post-operative treatment ($P < 0.001$) in desensitized patients. The majority of desensitized patients received immunosuppression with tacrolimus except for one patient who received cyclosporine A due to tacrolimus intolerance. In contrast, 72% of standard-risk recipients received cyclosporine A treatment according to center protocol for immunological low-risk recipients at that time.

Graft Survival and Function

Figure 2 shows patient and graft survival. Patient survival did not significantly differ between desensitized and standard-risk control patients (P = 0.55; **Figure 2A**). There was a trend toward reduced death-censored graft survival in desensitized patients (P = 0.053, **Figure 2B**) that was mainly driven by reduced survival in the early era before highly sensitive Luminex testing was introduced into clinical routine at our center (P = 0.038; **Figure 2C**). In the Luminex era (from March 2009), however, death-censored graft survival did not significantly differ between the two groups (P = 0.43; **Figure 2D**).

Serum creatinine, MDRD-GFR and protein-to-creatinine ratio was also not significantly different between the two groups. At day 360 after transplantation, median serum creatinine was 1.36 mg/dL in desensitized and 1.38 mg/dL in standard-risk patients (P = 0.88). The respective numbers for MDRD-GFR were 55 and 54 mL/min (P = 0.38), and for protein-to-creatinine-ratio 11 and 14 g/mol creatinine (P = 0.35).

Rejection Episodes

The incidence of rejection episodes without borderline changes were with 21% not significantly different in desensitized patients from the 18% rate in standard-risk patients (P = 0.74, **Table 2**). AMR were more frequent in desensitized than in standard-risk recipients (16 vs. 7%, P = 0.12), without reaching statistical significance.

Infectious and Surgical Complications

Table 2 summarizes the infectious and surgical complications. Viral infections, such as cytomegalovirus infection (P = 0.18) or polyoma virus replication (P = 0.24), tended to be more frequent in desensitized compared to standard-risk recipients. No significant differences were found for bacterial (P = 0.71) or fungal infections (P = 0.16). Surgical complications, such as lymphoceles, were also not significantly different between both groups (P = 0.12) while a higher frequency of bleeding complications was observed in desensitized patients (P = 0.004), most likely due to the perioperative desensitization therapy.

Desensitization and HLA Antibodies

After a median of 8 pre-transplant IA treatments, total IgG was reduced by 98%, total IgM by 70%, HLA class I DSA by 85% and HLA class II DSA by 81% ($P < 0.001$ for all; **Figure 3**).

TABLE 2 | Rejection and complications.

	Standard risk (N = 76)	Desensitized (N = 38)	P value
Allograft rejection (BANFF 2017)			
At least one rejection episode (excluding Borderline changes), N (%)	14 (18)	8 (21)	0.74
TCMR, N (%)	9 (12)	3 (8)	0.75
TCMR IA	7 (9)	3 (8)	1.0
TCMR IB	2 (3)	0 (0)	0.56
TCMR II/III	0 (0)	0 (0)	1.0
AMR, N (%)	5 (7)	6 (16)	0.12
Delayed graft function, N (%)[a]	1 (1)	1 (3)	0.62
Infectious complications			
Viral, N (%)			
Polyoma virus replication[b]	3 (4)	4 (11)	0.24
BKVAN[c]	1 (1)	2 (5)	0.22
CMV[d]	13 (17)	10 (26)	0.18
Bacterial, N (%)			
Urosepsis	6 (8)	4 (11)	0.57
Pneumonia	11 (14)	8 (21)	0.44
Wound infection	3 (4)	1 (3)	0.70
CVC-associated infection	6 (8)	3 (8)	0.31
Fungal, N (%)	4 (5)	3 (8)	0.16
Surgical complications			
Lymphocele[e], N (%)	14 (18)	7 (18)	0.12
Bleeding[f], N (%)	11 (14)	15 (39)	0.004

[a] *Dialysis within the first week after transplantation, except single dialysis for hyperkalemia.*
[b] *>10,000 copies/mL.*
[c] *SV-40-positive.*
[d] *>1,000 copies/mL.*
[e] *Requiring intervention.*
[f] *Requiring intervention or blood transfusion, AMR: antibody-mediated rejection.*
BKVAN, BK virus-associated nephropathy; CMV, cytomegalovirus; CVC, central venous catheter; TCMR, T cell-mediated rejection; N, number.

After desensitization and before transplantation, all DSA were completely eliminated, according to the antibody detection technique at the respective time point. **Table 3** summarizes the outcomes of desensitized patients according to their pre- and post-transplant presence of DSA. Two of 38 patients (number 22 and 23) had no DSA but a positive B cell crossmatch result. These patients showed primary graft function, no AMR, and no graft loss or death during clinical follow-up. Of 36 patients with pre-transplant DSA in the range of 500 to 999 MFI (N = 3) or 1,000 to 17,682 MFI (N = 33), 20 patients (56%) completely had lost their DSA on day 360 after transplantation. Only 1 of these 20 patients (6%) with a pre-transplant DSA of 2,698 MFI against HLA DRB1*03:01 (DR17) experienced AMR early after transplantation, and lost her graft more than 4 years after transplantation after cardiac surgery and infectious complications. Eleven patients showed persistence of DSA that had already been identified pre-transplant. Two of them (18%) suffered from AMR without graft loss and one patient lost her graft without signs of AMR. AMR was as high as 60% when the 5 patients were analyzed who had persistent DSA on day 360 together with *de novo* DSA development at some time during the

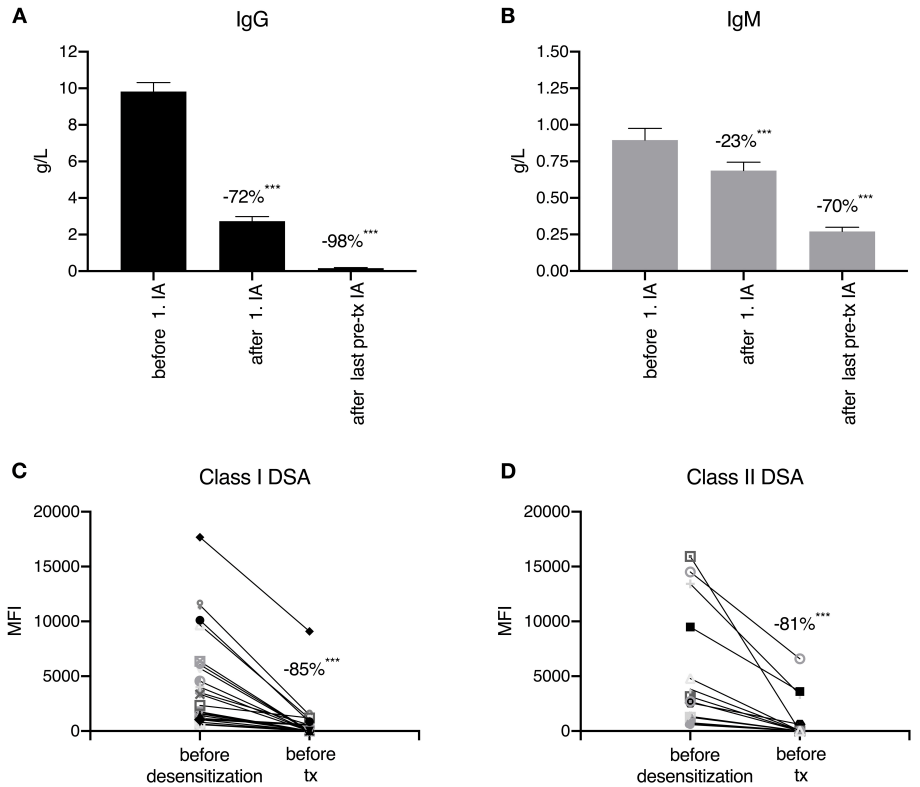

FIGURE 3 | Immunoglobulin **(A,B)** and donor-specific human leukocyte antigen antibody **(C,D)** reduction during desensitization. After the first immunoadsorption (IA) session, total IgG was reduced by 72% **(A)** and total IgM by 23% **(B)**. After a median of 8 IA treatments, the reduction in IgG and IgM reached 98 and 70%, respectively. During pre-transplant desensitization (immunoadsorption and plasmapheresis), human leukocyte (HLA) class I donor-specific HLA antibodies (DSA) were reduced by 85% and HLA class II DSA by 81%. ***$p < 0.001$.

first year after transplantation; two of these five patients lost their allograft. Compared to patients with loss of DSA, patients with persistent DSA had a higher MFI before desensitization (4,034, range 1,426–15,918 vs. 1,701, range 596–17,682, $P < 0.001$). Another two patients had (transient) *de novo* DSA without persistent DSA that disappeared during further follow-up with no AMR or graft loss.

Sixteen patients (44%) with pre-transplant DSA also had an sCD30 value of ≥80 ng/mL prior to transplantation as an indication of a pre-activated immune system. Nine of these 16 patients (56%) had persistent DSA and 4/16 patients (25%) suffered from AMR compared to only 7/20 (35%, $P = 0.31$) and 2/20 patients (10%, $P = 0.34$), respectively, with an sCD30 value below 80 ng/mL ($P = 0.38$). Most importantly, graft loss in patients with pre-transplant DSA was observed in 4/16 patients (25%) who were sCD30 positive, while only 1/20 patients (5%) with an sCD30 value below the cut-off experienced graft loss ($P = 0.15$) translating in a sensitivity of 80% and a NPV of 95%.

DISCUSSION

Several desensitization strategies have been published that allow transplantation across the HLA-antibody barrier. Most published protocols have used plasmapheresis and intravenous immunoglobulins, with graft survival rates ranging from 77% to 94% depending on the degree of sensitization, and concomitant AMR rates up to 15% (8, 11–15). Our group has developed a strategy to eliminate preexisting DSA by immunoadsorption, allowing safe transplantation even in highly sensitized recipients. Thorough pre-transplant risk stratification and effective antibody elimination combined with post-transplant antibody monitoring reduced AMR rates and improved graft survival. We present here the results of 38 desensitized patients transplanted at our center from 2007 to 2016 and compare them with 76 standard-risk patients who were matched for time after transplantation.

Desensitized patients had patient and graft survival rates that were not significantly different from those of standard-risk recipients, whereas desensitized patients showed a trend toward lower death-censored graft survival ($P = 0.053$). However, this trend disappeared when the 34 patients transplanted after the introduction of the sensitive Luminex-SAB assay were analyzed. The Luminex-SAB procedure, which allows more sensitive detection of DSA, was introduced into pre-transplant identification as part of the "Heidelberg algorithm" starting in April 2009 (9). An MFI value above 1,000 was classified as a risk factor for immunologic graft loss. More recently, our group has introduced another biomarker to

TABLE 3 | Donor-specific HLA antibody results and outcomes.

Pat.	Before desensitization	After desensitization[a]	Day 30	Day 180	Day 360	Event
1	B*07:02 (10,111)	B*07:02 (861)[b]	-	-	B*07:02 (740)	GL
	B*40:01 (B60) (7,282)	B*40:01(B60) (849)[b]	-	-	-	
2	DRB1*01:01 (3,996)	-	-	DRB1*01:01 (703)	DRB1*01:01 (1,037)	AMR,
	DQB1*05:01 (3,048)	-	DQB1*05:01 (1,110)	DQB1*05:01 (1,033)	DQB1*05:01 (1,273)	GL
	DPB1*02:01 (9,486)	DPB1*02:01 (3,611)[b]	DPB1*02:01 (6,832)	DPB1*02:01 (8,625)	DPB1*02:01 (10,661)	
			DPA1*01:03 (6,832)	DPA1*01:03 (9,696)	DPA1*01:03 (11,387)	
3	A*03:01(1,432)	-	-	-	-	
	A*29:01 (2,919, IgM: 476)	-	-	-	-	
4	A*03:01 (813)	-	-	-	-	
5	B*44:03 (17,682)	B*44:03 (9,096)	B*44:03 (1,138)	B*44:03 (524)	-	
6	DQ7 (14,509)	DQ7 (6,588)[c]	DQ7 (13,363)	DQ7 (18,529)	DQ7 (11,938)	AMR, GL
	DQA1*05:05 (13,581)	DQA1*05:05 (6,557)[c]	DQA1*05:05 (13,349)	DQA1*05:05 (18,529)	DQA1*05:05 (2675)	
			A*32:01 (689)	A*32:01 (771)		
7	A*01:01 (2,335)	A*01:01 (1,199)	A*01:01 (4,040)	A*01:01 (5,736)	A*01:01 (2,109)	AMR
			DRB1*01:01 (2,717)	DRB1*01:01 (6,248)	DRB1*01:01 (1,792)	
				C*05:01 (3,368)	C*05:01 (600)	
				DQB1*05:01 (885)		
				DQB1*06:03 (636)		
				DQA1*01:03 (769)		
8	A*24:02 (1,304)	-	-	-	-	AMR
	B*18:01 (9,676)	B*18:01 (1,138)	B*18:01 (1,370)	B*18:01 (808)	B*18:01 (566)	
	B*37:01 (2,516)	-	-	-	-	
	DRB1*16:01 (4,798)	-	DRB1*16:01 (1,526)	DRB1*16:01 (937)	DRB1*16:01 (1,454)	
	DRB5*01:01 (DR51) (1,653)	-	-	-	-	
	DQB1*06:02 (1,156)	-	-	-	-	
9	C*12:03 (IgM: 1,760)	-	-	-	-	
10	A*02:01 (1,050)	-	-	-	-	
11	DRB1*13:01 (IgM: 686)	-	DRB1*13:01 (IgM: 679)	-	-	
12	A*01:01 (1,579)	-	-	A*01:01 (1,010)	A*01:01 (779)	AMR
13	B*18:01 (4,012)	-	-	-	-	
	DRB1*03:01 (DR17) (553)	-	-	-	-	
	DQB1*02:01 (13,433)	-	-	-	-	
	DQA1*05:01 (13,433)	DQA1*05:01 (3,319)	DQA1*05:01 (898)	-	-	
14	A*02:01 (3,355)	-	A*02:01 (1,268)	-	-	
15	DRB1*03:01(DR17) (2698)	-	-	-	-	AMR, GL
16	DQB1*02:01 (15,918)	-	DQB1*02:01 (9,756)	DQB1*02:01 (13,107)	DQB1*02:01 (13,583)	
	DQA1*05:01 (14,587)	DQA1*05:01 (2,135)	DQA1*05:01 (6,822)	DQA1*05:01 (12,174)	DQA1*05:01 (12,729)	
17	C*07:02 (IgM: 596)	-	-	-	-	
18	A*02:01 (6,355)	-	-	-	-	
	A*69:01 (3,191)	-	-	-	-	
	B*44:02 (3,956)	-	-	-	-	
		-	-	-	C*05:01 (5,875)	
19	DRB1*03:01(DR17) (2,568)	DRB1*03:01(DR17) (600)	DRB1*03:01(DR17) (878)	-	DRB1*03:01(DR17) (1,277)	
20	B*51:01 (1,717)	-	-	-	-	
	DRB1*07:01 (752)	-	-	-	-	

(Continued)

TABLE 3 | Continued

Pat.	Before desensitization	After desensitization[a]	Day 30	Day 180	Day 360	Event
21	A*01:01 (11,485)	A*01:01 (1,464)	A*01:01 (6,865)	-	A*01:01 (5,253)	
22		-	-	-	-	
23		-	-	-	-	
24	DRB1*13:01 (3,828)	-	-	-	-	
	DRB3*01:01(DR52) (776)	-	-	-	-	
25	A*23:01 (1,426)	-	A*23:01 (568)	-	A*23:01 (1,464)	
			C*17:01 (591)			
			DRB1*07:01 (515)			
26	A*24:02 (2,891)	-	A*24:02 (511)	-	-	
	C*12:03 (6,064)	-	C*12:03 (512)	-	-	
	DPB1*15:01 (588)	-	DPB1*15:01 (600)	-	-	
27	A*01:01 (1,684)	-	-	-	-	
28	C*05:01 (1,683)	-	C*05:01 (570)	-	C*05:01 (582)	Death
29	A*32:01 (4,562)	-	A*32:01 (3,597)	-	A*32:01 (5,774)	
			B*08:01 (680)			
30	A*26:01 (IgM: 822)	-	-	-	-	
	B*13:02 (IgM: 1,466)	-	-	-	-	
	C*02:02 (IgM: 520)	-	-	-	-	
	C*06:02 (IgM: 1,591)	-	-	-	-	
31	B*58:01 (645)	-	-	-	-	
	DQB1*06:09 (1,266)	-	-	-	-	
	DQA1*01:02 (1,266)	-	-	-	-	
32	DQA1*03:01 (3,139)	-	-	DQA1*03:01 (2,040)	DQA1*03:01 (1,759)	
	DQ8 (2,539)	-	-	-	-	
33	DQB1*06:03 (1,339)	-	-	-	-	
	DQA1*01:03 (1,339)	-	-	-	-	
34	A*02:01 (IgM: 1,061)	A*02:01 (IgM: 529)	-	-	-	
	C*04:01 (IgM: 562)	-	-	-	-	
35	C*14:02 (5,728)	-	C*14:02 (587)	-	C*14:02 (1,717)	
36	A*02:01 (IgM: 1,164)	A*02:01 (IgM: 597)	-	-	-	
		C*03:04(Cw10) (IgM: 705)				
		DQB1*05:01 (IgM: 865)				
37	B*51:01 (IgM: 559)	-	-	-	-	
	C*04:01 (IgM: 1,642)	-	-	-	C*04:01 (755)	
38	B*73:01 (3,505)	B*73:01 (566)	B*73:01 (1,139)	B*73:01 (4,028)	B*73:01 (3,180)	

76–100% reduction
51–75% reduction
26–50% reduction
25% reduction to 25% increase
26–50% increase
51–75% increase
>75% increase
de novo DSA

[a] *After desensitization, some of the donor-specific HLA antibodies lie above the predefined threshold of 1.000 MFI. Unless otherwise indicated (footnotes b and c), these antibodies were identified only retrospectively during reanalysis when EDTA inactivation was replaced by heat inactivation.*
[b] *Before routine Luminex testing, antibodies were identified retrospectively.*
[c] *Before routine donor typing for HLA-C, -DQ, -DP locus antigens, antibodies were identified retrospectively.*
AMR, antibody-mediated rejection; DSA, donor-specific human leukocyte antigen (HLA) antibodies; GL, graft loss.

further improve pre-transplant risk assessment. Since 2016, the immune activation marker sCD30 has been integrated into our algorithm to identify patients at high immunological risk before transplantation. Several studies in deceased donor transplant recipients have shown that determination of sCD30 with a cut-off value of 80 ng/mL before transplantation is beneficial, as patients with the co-presence of DSA (MFI $\geq$ 1,000) and sCD30 ($\geq$80 ng/mL) before transplantation were shown to be at a significantly higher risk for AMR and graft loss than patients with DSA but without sCD30 positivity (10, 16). These patients may require a more intensive induction regimen including thymoglobulin as well as special care after renal transplantation. The present study in living donor transplant recipients shows a similar trend as in previous studies. Desensitized patients with both DSA- and sCD30-positivity before transplantation were at a higher risk for graft loss compared to patients without sCD30 positivity, however, without reaching statistical significance.

Living kidney transplant recipients were treated with repeated IA until the CDC crossmatch became negative and measured DSA were below the 1,000 MFI threshold. A median of 8 IA treatments before transplantation reduced IgG by 98% and IgM by 78% in sensitized patients. IA can treat large plasma volumes, which may improve antibody removal compared with desensitization with plasmapheresis: 95% of all patients studied were successfully desensitized and transplanted, compared with the much lower frequency of only 80% reported when plasmapheresis and intravenous immunoglobulins were used (17). Another advantage of IA is its specificity: bleeding complications in terms of transfusion requirement or intervention occur at a higher rate of up to 70% with multiple plasmapheresis treatments than with our desensitization protocol (17). Compared with newer desensitization strategies such as desensitization with imlifidase, immunoadsorption allows better risk assessment before transplantation. DSA that can be easily removed by IA before transplantation are more likely to be lost after transplantation. Conversely, antibodies that cannot be removed by IA may persist after transplantation and damage the renal graft (18). In contrast, imlifidase degrades IgG, and DSA are lost for several days regardless of the level of re-synthesis, making risk assessment impossible (19). Another advantage of IA compared to imlifidase is the fact that it can be used several times and even for weeks or months, while imlifidase may be given only once (20).

In previous studies, graft survival at two years was only about 50% when patients were desensitized prior to transplantation (21). Importantly, when IA is used as the main method of desensitization, antibody rebound after treatment must be considered. Therefore, according to our protocol, we performed repeated IA after transplantation in addition to the immunosuppression described above until good graft function was achieved (e.g., serum creatinine $<$ 2.0 mg/dl). Interestingly, DSA remained below a threshold of 1,000 MFI in 6 of 10 sensitized patients during the post-transplant observation period in a previously published cohort (5). In the present study, 20 of 36 patients (56%) had persistently negative DSA on day 360 after transplantation.

The use of strong immunosuppression may lead to negative side effects such as infectious complications. However, in the present study, no significant difference was observed, neither with respect to bacterial infections nor with respect to viral infections such as cytomegalovirus or polyoma infections. Thus, the present study demonstrates that treatment with repeated IA is feasible without severe infectious complications and without the need to replace immunoglobulins (22). Since April 2009, two depleting antibodies, thymoglobulin and rituximab, have been used in combination. In the present study, there is no evidence of increased infectious complications, but probably the numbers are too small. In the field of ABO-incompatible renal transplantation, more infectious complications have been observed probably due to the use of rituximab (23). Therefore, studies analyzing possible infectious side effects in sensitized renal transplant recipients are needed.

Limitations of the present study include the retrospective and monocentric character of the study with a rather small number of patients who experienced only a limited number of adverse events such as AMR and graft loss due to AMR as well as differences in the immunosuppressive regimens between sensitized (mostly tacrolimus as calcineurin-inhibitor) and standard risk (mostly cyclosporine A as calcineurin-inhibitor) patients.

In conclusion, both pre-transplant characterization of the immune status of sensitized kidney transplant recipients by measuring B-cell (DSA) and T-cell (sCD30) activity and post-transplant monitoring (DSA and non-DSA) play an important role in the management of living kidney transplantation across the HLA barrier. The algorithm we have described for identifying sensitized living kidney transplant recipients, consistent desensitization with repeated IA, and the peri-graft management described lead to good graft outcomes with side effects comparable to those of standard-risk recipients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FK, CSü, and CM designed the study. FK and LPdS performed the study. CSp, LB, CN, MS, JB, LP, HT, CSo, AM, and MZ contributed important patients. FK, CSü, LPdS, and CM analyzed the data and wrote the manuscript. All authors contributed to the final version of the manuscript and approved it.

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SUPPLEMENTARY MATERIAL

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Ameliorating Metabolic Profiles After Kidney Transplantation: A Protocol for an Open-Label, Prospective, Randomized, 3-Arm, Controlled Trial

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Aim: High prevalence of metabolic disorders causes higher risk of cardiovascular diseases after kidney transplantation (KT), which remains the main burden impairing short-term and long-term survival. This open-label, prospective, randomized, 3-arm, controlled trial will evaluate the safety, tolerability and efficacy of metformin and empagliflozin in ameliorating metabolic profiles after KT.

Methods: After a screening assessment, eligible patients with an estimated glomerular filtration rate (eGFR) >45 mL/min/1.73m2 are randomly assigned to standard triple immunosuppression alone, standard immunosuppression plus metformin (500 mg twice daily), standard immunosuppression plus empagliflozin (25 mg once daily) from discharge. The primary endpoint is the differences in the visceral-to-subcutaneous fat area ratio over 12 months, evaluated by magnetic resonance imaging (MRI). Secondary outcomes include kidney graft function, glycometabolism, lipid metabolism, and inflammatory parameters. The trial will enroll 105 kidney transplant recipients, providing 90% power to detect the difference at 5% significance.

Keywords: metformin, empagliflozin, kidney transplantation, metabolic disorders, clinical trial

INTRODUCTION

Advances in patient selection, organ procurement and preservation, surgical technique, immunosuppression, and infection prevention have conferred significant improvement in rejection, infection, and subsequently decreased cause-specific graft failure rates after kidney transplantation (KT) (1). However, cardiovascular diseases (CVD) remain the main burden impairing both short-and long-term patient survival (2). Compared with the general population, conventional CVD risk factors, including obesity, liver and muscle insulin resistance, dyslipidemia, hypertension, and diabetes mellitus, are all highly prevalent in this population for long-term exposure to steroids and calcineurin inhibitors (3–6).

Previous studies demonstrated that adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) is a central regulator of multiple metabolic pathways and a key player in regulating cellular energy metabolism (7, 8). Activation of AMPK by pharmacological agents may hold a considerable potential to reverse the metabolic abnormalities in chronic

metabolic diseases (9, 10). Metformin, a widely used antidiabetic drug, acts as an AMPK activator by inhibiting complex I of the mitochondrial electron transport chain in many tissues, including adipose, skeletal muscle, and heart (11). A recent small clinical trial observed that metformin administration can safely ameliorate metabolic profiles in glucocorticoid-treated patients with inflammatory disease but without pre-existing diabetes (12). In addition, another antidiabetic drug sodium-glucose-cotransporter-2 (SGLT-2) inhibitors can improve metabolic parameters and cardiovascular risk in patients with or without diabetes in pre-clinical and clinical studies (13, 14, 14, 15). Another small clinical trial even reported that compared to metformin, significant improvement in anthropometric parameters and body composition, in overweight and obese women with polycystic ovary syndrome after treatment with empagliflozin (16). Hence, metformin and SGLT2 inhibitors may be used as potential adjuvant therapies to improve metabolic disorders after KT.

Although several preliminary clinical trials showed that metformin and SGLT-2 inhibitors can be used safely and improve glucose control after KT, but they are small-sample sized and only include patients with diabetes (17–19). We will conduct a prospective clinical trial with the aim of exploring their roles in improving metabolic profiles.

METHODS AND ANALYSES

Study Design

This study is designed as a 12-month, single-center, prospective, 3-arm, open-label, randomized clinical trial (**Figure 1**). This study has been registered at www.clinicaltrials.gov (NCT05013112) and is conducted in accordance with the Declaration of Helsinki and local regulations. Selected patients are recruited if they meet the following inclusion criteria: (1) living-donor kidney transplantation; (2) eGFR level $>$ 45 ml/min/1.73m2 at discharge; (3) 18$<$Age$<$65 years; (4) receiving standard triad immunosuppressive regimen. Our key exclusion criteria are as follows: (1) previous therapy with metformin or SGLT 2 over the previous 3 months; (2) pre-transplant diabetes; (3) alanine aminotransferase (ALT) or aspartate aminotransferase (AST) $>$ 2.5 or more of upper limit of normal; (4) Combined with HBV/HCV/HIV infection in the donor or recipient; (5) Malignancy history in the donor and recipient; (6) organ transplant history in the recipient. In addition, patients will exit from this clinical trial: (1) Serious deviation from clinical trial protocol; (2) Due to serious adverse reactions, the drug must be stopped in advance; (3) Withdrawal of informed consent; (4) Poor compliance and failure to use drugs as planned; (5) Lost to follow up.

Randomization and Masking

Eligible participants are randomly allocated to standard immunosuppression (placebo group), standard immunosuppression plus metformin (metformin group), or standard immunosuppression plus Empagliflozin (Empagliflozin group). The randomization sequence is created using a random number generation function and allocation to each group is done through block randomization in a 1:1:1 ratio. The budesonide is open label.

Procedures

Metformin is administered 500 mg twice daily orally from discharge based on the previous preliminary clinical trial (17). Empagliflozin is administered 25 mg once daily orally from discharge (18, 20). This will commence at randomization and continue for 12 months. The decision to continue with metformin beyond 12 months of follow-up is left to the discretion of the caring physician. In addition, during the follow-up, when kidney transplant recipients are diagnosed with PTDM, insulin therapy were adopted to reduce the glucose level at 6.1–6.7 mmol/L based on Chinese Management Guideline of post-transplantation diabetes mellitus after kidney transplantation (21).

Immunosuppressive regimen has been reported in our previous study (22). In brief, all participants will receive standardized triad immunosuppression, consisting of tacrolimus, mycophenolate mofetil/enteric-coated mycophenolate sodium, and steroids. Tacrolimus trough levels are measured by the enzyme multiplied immunoassay technique before breakfast and dose administration in the morning. Recommended tacrolimus trough levels are 5–10 ng/ml. MMF is started at 1000 mg one night before operation and maintained at 1000 mg twice daily with area under curve of mycophenolate mofetil maintained at 30–70 mg/h·L-1. If EC-MPS is selected, 720 mg is given the night before surgery and 720 mg bid thereafter. Methylprednisolone is injected intravenously at 500 mg during the operation and 200 mg per day 3 days after the operation. Then 60 mg prednisone is initiated, and gradually taper to 5–10 mg per day for maintenance.

Data Collection and Clinical Outcomes

Standard demographic, clinical and laboratory data (including medication details) will be collected prospectively from electronic medical records. Recipients are advised to receive routine follow-up weekly in months 0–3, every 2 weeks in month 4–6, monthly within 6–12 months, and every 3 months thereafter.

The general aim of this study is to determine the safety of metformin and empagliflozin in kidney transplant recipients and explore the role of metformin and empagliflozin treatment in improving metabolic profiles. Based on previous study, visceral-to-subcutaneous fat area ratio, evaluated by magnetic resonance imaging (MRI), is generally reported as a surrogate for metabolic risk and is markedly raised in patients with long-term exposure to steroids (23, 24). Hence, the primary outcome is the differences in the visceral-to-subcutaneous fat area ratio over 12 months among three groups. The visceral-to-subcutaneous fat area ratio will be measured at transplantation, and post-transplant 3, 6, 9, and 12 months. Kidney graft function will be evaluated when they receive routine follow-up. Secondary outcomes included glycometabolism (fasting plasma glucose, fasting insulin levels, 2-h post-prandial insulin levels, hemoglobin A1c, and insulin beta-cell function [indicated by the homeostasis model assessment of β cell function]), lipid metabolism (serum triglyceride, non-high-density lipoprotein, low-density

lipoprotein, total cholesterol), inflammatory parameters (C-reactive protein, interleukin-6, and tumor necrosis factor alpha) (**Table 1**). We will also examine cardiovascular disease and microvascular complications, including the following: major adverse cardiovascular events; all-cause mortality; atherosclerotic cardiovascular (ASCV) death; hospitalization or death from coronary artery disease, ischemic stroke, or heart failure; observable background diabetic retinopathy; and diabetic peripheral neuropathy.

Sample Size Estimation

Sample size calculation is based on the primary outcome. According to the previous study, the visceral-to-subcutaneous adipose tissue area ratio was 0.83 (SD = 0.48; $n = 58$) for kidney transplant recipients (25). The ratio is estimated to be half of that in the intervention group. The estimated sample sizes for 90% power at 5% significance is 28 patients in each group. Considering an estimated 20% dropout rate, we assure that the sample size exceeds the minimal number needed to ensure the validity of the mean, effect size and rationale of feasibility. Therefore, 105 individuals (35 each group) are expected to be enrolled.

Statistical Analysis

Two populations will be used in the analyses. The intention-to-treat (ITT) population will include all participants who have been randomly assigned, whereas the per-protocol (PP) population will include all participants who accomplish the entire intervention. The baseline characteristics of the study will be summarized as the means ± standard deviations for parametrically distributed data, geometric mean values (and 95% confidence intervals) for non-parametrically distributed data, and numbers (percentages) for categorical data.

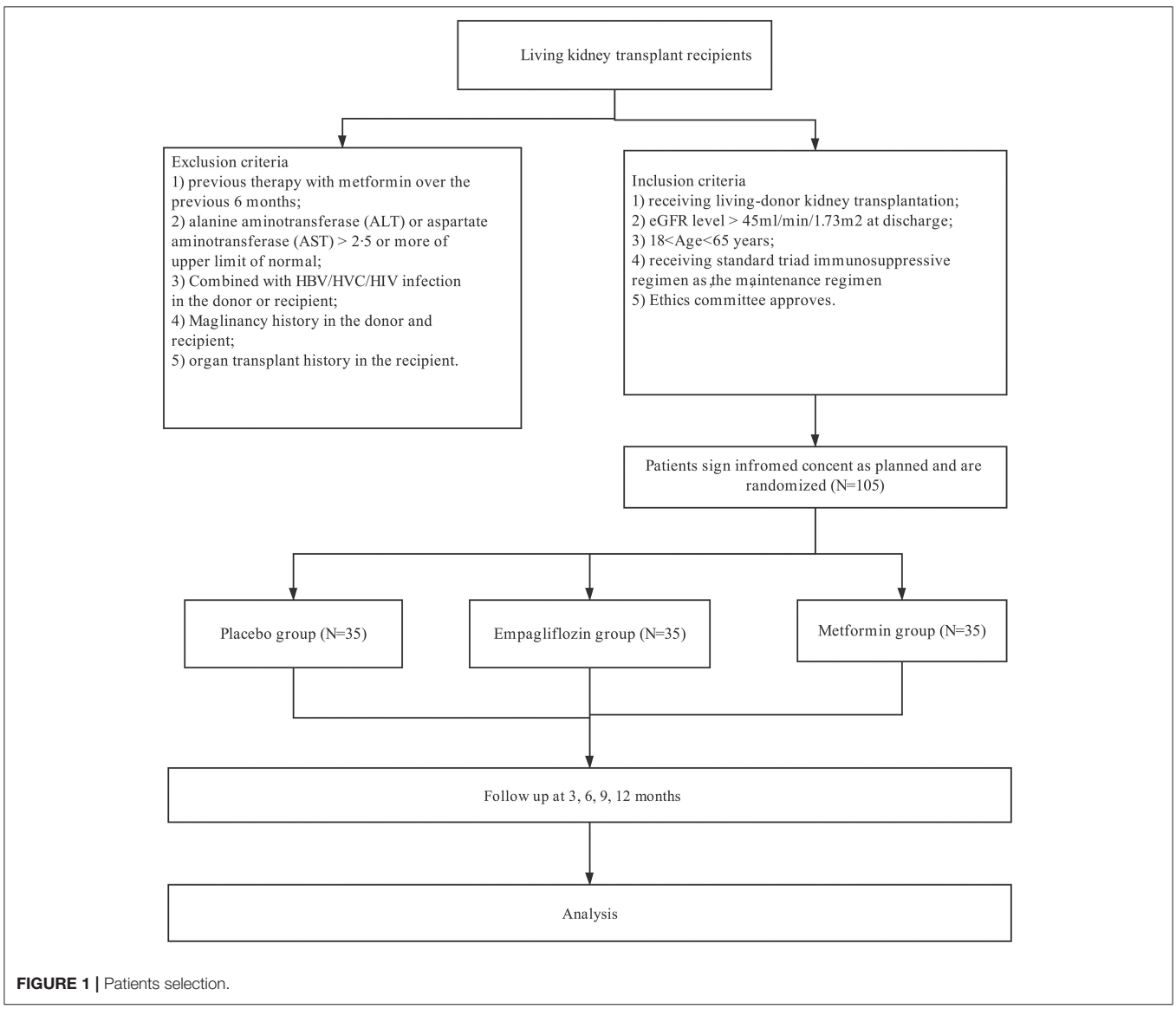

FIGURE 1 | Patients selection.

TABLE 1 | Routine follow-up.

Visit	V0	V1	V2	V3	V4
Time, days after kidney transplantation	−7day to 0 day	3 month	6 month	9 month	12 month
Participant-related information	×				
Informed consent	×				
History	×				
Inclusion/Exclusion	×				
Clinical examination	×	×	×	×	×
Efficacy and safety outcomes	×	×	×	×	×
Body weight	×	×	×	×	×
Height	×	×	×	×	×
Waist circumference	×	×	×	×	×
Visceral adipose tissue (VAT)	×	×	×	×	×
Subcutaneous adipose tissue (SAT)	×	×	×	×	×
VAT/SAT ratio	×	×	×	×	×
Glycometabolism	×	×	×	×	×
Fasting plasma glucose function	×	×	×	×	×
Fasting insulin levels	×	×	×	×	×
2-h postprandial insulin levels	×	×	×	×	×
Hemoglobin A1c	×	×	×	×	×
Insulin beta-cell function	×	×	×	×	×
Lipid metabolism	×	×	×	×	×
Serum triglyceride	×	×	×	×	×
Non-high-density lipoprotein	×	×	×	×	×
Low-density lipoprotein	×	×	×	×	×
Total cholesterol	×	×	×	×	×
Inflammatory parameters	×	×	×	×	×
C-reactive protein	×	×	×	×	×
Interleukin-6	×	×	×	×	×
Tumor necrosis factor alpha	×	×	×	×	×
Kidney graft function	×	×	×	×	×
Serum creatinine		×	×	×	×
Estimated glomerular filtration rate (eGFR)		×	×	×	×

Differences between participants who complete and withdraw from the trial will be analyzed by using a Student *t*-test or the Mann–Whitney test for continuous variables (e.g., age) and the chi-squared test for categorical variables (e.g., sex). For clinical outcomes, analysis of covariance will be used to examine differences among three groups, adjusting for potential confounding factors and effect modifiers (e.g., baseline age and sex). Based on the literature, some patients have higher risk of post-transplantation diabetes mellitus (PTDM), including those with family history of diabetes; or polycystic kidney disease; or age $\geq$60 years; or age 45–59 years plus (i) triglycerides $\geq$200 mg/dL; (ii) triglycerides 150–200 mg/dL and body mass index >27 kg/m2; (iii) triglycerides 150–200 mg/dL and high density lipoprotein (HDL) <40 mg/dL (men)/<40 mg/dL (women) Hence, subgroup analysis will be conducted based on the risk of PTDM, and was also conducted based on the components of the criteria for high-risk PTDM (26–28). Our statistical analysis will be performed by using R software, and the results will be considered significant at a *P*-value of <0.05.

DISCUSSION

This trial will provide more evidence on the safety and tolerability of metformin and SGLT2 agents in patients after renal transplantation. Most importantly, the study will also evaluate potential adjuvant therapies to ameliorating metabolic profiles in kidney recipients.

Successful developments in immunosuppression and clinical management make KT routines in adults with end-stage renal disease, but also unmask a greatly increased risk of premature metabolic disorders post-operatively. Generally accepted, long-term exposure to steroids can induce or worsen pre-existing insulin resistance, increase hepatic gluconeogenesis, and stimulate appetite and weight gain (5). In addition, calcineurin inhibitors can inhibit sterol 26–hydroxylase, diminish hepatic bile acid synthesis, reduce export of cholesterol from the liver, and inhibit β-cell growth and function (6). Compared with the general population, high prevalence of metabolic disorders increases the risk of post-transplant CVD, which makes CVD a main post-transplant burden. In Chadban's

study including 23210 first kidney-only transplant recipients from 1980 through 2018 from the Australia and New Zealand Dialysis and Transplant Registry, the authors assessed the trend of all-cause and cause-specific mortality at different periods post-transplant (29). Although morality declined over successive eras at all periods, CVD remained the most common cause of death despite of reduced account from 50% in 1985–1989 to 30% in 2015–2018. Further analysis showed that in the current era (2015–2018), the adjusted death rates due to CVD was 0.28, 0.50, 0.96 per 100 patient-years with a follow-up of 1–5, 5–10, and >10 years. However, less attention is focused on how to improve metabolic profiles in this population.

Previous studies demonstrated that therapeutics aimed at activating AMPK remain promising and beneficial for improving metabolic disorders in the context of obesity, diabetes, cancer, non-alcoholic fatty liver disease, cardiovascular diseases (7–10, 30–32). In Kulkarni's study, they studied participants >70 years ($N = 14$) in a randomized, double-blind, placebo-controlled, crossover trial in which they were treated with 6 weeks each of metformin and placebo (33). The authors observed that both metabolic and non-metabolic pathways were significantly influenced, including pyruvate metabolism, mitochondrial fatty acid oxidation, and collagen trimerization in adipose. Similar improvements of metabolic disorders were also reported in those patients diagnosed with cancer and treated by metformin in a well-designed clinical trial (34). Another recent clinical trial reported that metformin can improve metabolic profiles in Cushing's syndrome without diabetes and receiving systematic steroids ((≥20 mg/day for ≥4 weeks and remaining on ≥10 mg/day for the subsequent 12 weeks, or its cumulative dose-equivalent) (12). 53 patients were randomly assigned to receive either metformin ($n = 26$) or placebo ($n = 27$) for 12 weeks. Improvements in markers of carbohydrate, lipid, liver, and bone metabolism were observed in the metformin group compared with the placebo group. These studies demonstrated available benefits in metabolic profiles for patients without diabetes.

However, metformin was also associated with potential side effects, such as metformin-associated lactic acidosis (35). Metformin plasma concentrations are ∼2–4 folds higher in patients with type 2 diabetes and moderate to severe renal impairment (eGFR of 30 to <60 mL/min/1.73 m2 or <30 mL/min/1.73 m2, respectively) compared to healthy subjects (36). In a community-based cohort study, 75,413 patients with diabetes and time-dependent assessment of eGFR stage from January 2004 until January 2017 were included (37). The authors reported that there were 2335 hospitalizations with acidosis over a median follow-up of 5.7 years. Further analysis demonstrated that time-dependent metformin use was associated with incident acidosis only in patients with eGFR <30 mL/min/1.73m2 (HR: 2.07; 95% CI: 1.33–3.22), but not in patients with eGFR 45–59 mL/min/1.73m2 (HR: 1.16; 95% CI: 0.95–1.41) and eGFR 30 to 44 mL/min/1.73m2 (HR: 1.09; 95% CI: 0.83–1.44). This showed that metformin therapy may be safe in patients with eGFR 30–60 mL/min/1.73m2. For kidney transplant recipients, evidence of metformin is insufficient. A recent small clinical trial demonstrated that metformin can be administered safely in transplant recipients with stable kidney graft function but impaired glucose tolerance ($N = 19$) (17). Hence, considering potential benefits of metformin, it is time to reassess the safety and value of metformin in kidney transplant patients.

Another first-line anti-diabetic drug, SGLT2 had definite cardiovascular and renal function protection in patients with or without diabetes reported by many large clinical trials (13–15). In addition to improving glycometabolism, large clinical trials showed that SGLT-2 inhibitors can improve metabolic parameters in patients with or without diabetes (14, 38). Also, this action mechanism is insulin-independent; as such it does not increase the risk of hypoglycemia, making it attractive for use in normoglycemic individuals (39). Davies et al. demonstrated that assessed the effect of canagliflozin on the components of metabolic syndrome of metabolic syndrome in patients with T2DM and metabolic syndrome (38). The authors indicated that canagliflozin can significantly reduce HbAc1, fasting plasma glucose, body weight, body mass index, waist circumference, blood pressure, and triglycerides, and increased high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. Similar improvement of metabolic parameters was also seen in patients without diabetes. In the small clinical trial by Javed et al. the authors evaluated the effects of empagliflozin on metabolic parameters in polycystic ovary syndrome (16). Women with polycystic ovary syndrome were randomized to either empagliflozin 25 mg ($n = 19$) or metformin 1500 mg ($n = 20$) daily for 12 weeks with the main outcomes as the changes in anthropometric and body composition, and metabolic parameters. Despite of no statistical difference in metabolic parameters between two groups, there was a significant improvement in anthropometric parameters and body composition, in patients receiving empagliflozin.

However, SGLT2 agents are also restricted in kidney transplant recipients due to their mechanism of elimination and action by concentrate glucose in the urine. This can increase risk of urinary tract infections (40, 41), especially in patients with immunosuppression. Two recent small clinical trials have evaluated the safety and efficacy of SGLT2 in treating diabetes after kidney transplantation (18, 19). In the trial by Halden, 22 renal transplant recipients with diabetes were randomized to receive 10 mg empagliflozin with 20 receiving placebo as the control group once daily for 24 weeks. The authors shows that empagliflozin appeared safe and improved glycemic control in renal transplant recipients with NODAT compared with placebo. Also, a concomitant reduction in body weight was seen (443). In another preliminary prospective interventional trial including 14 kidney transplant recipients with NODAT and receiving insulin, the authors showed that empagliflozin can safely be used as add-on therapy when monitored closely (454). The latest clinical trial even showed that dapagliflozin can improve kidney function and reduce the risk of end-stage kidney disease and cardiovascular death in patients with stage 4 CKD and albuminuria (42). Hence, prospective studies are needed to explore the safety, tolerability and efficacy of metformin in improving metabolic profiles kidney transplant recipients with or without diabetes.

The limitation is that this study will explore the short-term impact of the intervention because the follow-up is set at 1 year. However, the development of metabolic diseases is a long and gradual process and can be influenced by other factors.

In summary, the AMPKT study will be the first dedicated clinical trial to explore the potential benefits and risks of metformin and SGLT2 inhibitors in kidney transplant recipients both with and without diabetes.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Hospital. Written informed consent was not provided because this is a protocol for a Clinical Trial.

AUTHOR CONTRIBUTIONS

SY, XW, TL, and TS were the initiators of this study. SY and MM wrote this protocol. SY, XW, TS, ZH, YF, and TL tool part in the study planning. SY and XW wrote the final manuscript. All authors have read and approved the final manuscript.

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Tocilizumab in the Treatment of Chronic Antibody-Mediated Rejection Post Kidney Transplantation: Clinical and Histological Monitoring

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Introduction: Chronic antibody-mediated rejection (cAMR) has very few effective therapeutic options. Interleukin-6 is an attractive target because it is involved in inflammation and humoral immunity. Therefore, the use of tocilizumab (anti-IL6 receptor, TCZ) is a potential valuable therapeutic option to treat cABMR in kidney-transplant (KT) recipients.

Materials and Methods: This single-center retrospective study included all KT recipients that received monthly TCZ infusions in the setting of cABMR, between August 2018 and July 2021. We assessed 12-month renal function and KT histology during follow-up.

Results: Forty patients were included. At 12-months, eGFR was not significantly different, 41.6 ± 17 vs. 43 ± 17 mL/min/1.73 m^2 ($p = 0.102$) in patients with functional graft. Six patients (15%) lost their graft: their condition was clinically more severe at the time of first TCZ infusion. Histological follow-up showed no statistical difference in the scores of glomerulitis, peritubular capillaritis, and interstitial fibrosis/tubular atrophy (IFTA). Chronic glomerulopathy score however, increased significantly over time; conversely arteritis and inflammation in IFTA ares improved in follow-up biopsies.

Conclusion: In our study, the addition of TCZ prevented clinical and histological worsening of cABMR in KT recipients, except for more severely ill patients. Randomized studies are needed to clarify the risk/benefit of TCZ in cABMR.

Keywords: kidney transplantation, tocilizumab, chronic antibody-mediated rejection, eGFR, kidney allograft biopsy

INTRODUCTION

Kidney transplantation remains the best therapeutic option regarding end-stage kidney disease for various reasons including improved survival and quality-of-life compared to those receiving dialysis therapy (1–4) However, the half-life of kidney transplants has not changed that much within the last decades at least in the United States as demonstrated by Lamb *et al.* This was particularly the case in low-risk populations like living-donor-recipients where half-life was 11.4 years in 1989 and 11.9 years in 2005. Finally, within this time-frame first-year attrition rates show dramatic improvements, whereas attrition rates beyond the first year show only small improvements (5). In 2012 Sellarés *et al.* have shown in a prospective cohort of 315 allograft recipients who underwent indication biopsies at 6 days to 32 years posttransplant that many actual failures after indication biopsies manifest phenotypic features of antibody-mediated (ABMR) or mixed rejection. They also underscore the major role of non-adherence (6). Indeed, non-adherence is a major risk factor for developing *de novo* donor-specific alloantibody (dnDSA) after kidney transplantation (7). dnDSA formation at post-transplantation is the leading cause of developing chronic antibody-mediated (cABMR) rejection (8, 9); they have a particularly worse impact upon kidney allograft survival especially when they bind complement, i.e., either C1q (9, 10) or C3d (11) components. Recently, Mayrdorfer *et al.* have reassessed the causes of kidney allograft failure in the modern immunosuppression era in a prospective cohort of 1,642 kidney transplant (KTx) recipients (12). They found that in 51.2% of patients with allograft failure, more than one cause was involved. The most frequent primary or secondary causes leading to graft failure were intercurrent medical events in 36.3% of graft failures followed by T cell-mediated rejection (TCMR) in 34% and ABMR in 30.7%. In 77.9%, a primary cause could be attributed to graft loss, of which ABMR was the most frequent etiology (21.5%).

At present, when cABMR is diagnosed we are left with very few therapeutical options. In a randomized controlled trial, Eskandary *et al.* reported, in cases of donor-specific alloantibody (DSA)-positive chronic ABMR, that bortezomib therapy was no more efficient than a placebo at changing the decline in slope of the estimated glomerular-filtration rate (eGFR) (13). Likewise, Moreso *et al.* reported, in a randomized controlled trial in the setting of cABMR with transplant glomerulopathy (TG) that the combination of polyclonal intravenous immunoglobulins (IVIG) and rituximab, as compared to a placebo, did not significantly modify the natural history of cABMR regarding transplant glomerulopathy (14).

Abbreviations: ah, arteriolar hyalinosis; aah, hyaline arteriolar thickening; cABMR, chronic antibody-mediated rejection; cg, chronic glomerulopathy score; cv, arterial intimal thickening; dnDSA, *de novo* Donor Specific Antibody; eGFR, estimated Glomerular Filtration Rate; ESKD, End-stage kidney disease; g, glomerulitis score; i, interstitial inflammation; IFTA, interstitial fibrosis and tubular atrophy; i-IFTA, inflammation in areas of interstitial fibrosis and tubular atrophy; KT, Kidney Transplantation; MFI, mean fluorescence intensity; mm, mesangial matrix expansion; ptc, peritubular capillaritis score; TCMR, T cell-mediated rejection; TCZ, Tocilizumab; t, tubulitis; v, intimal arteritis.

Indeed, in the setting of cABMR, IL-6 is an attractive target. IL-6 is a multifunctional pleiotropic cytokine that stimulates B- and T-cell functions (15, 16). Uncontrolled studies have suggested that blocking IL-6 receptor by tocilizumab (TCZ) might be valuable in patients presenting with cABMR (17) or with transplant glomerulopathy (18). Recently, Doberer *et al.* reported on a phase 2 randomized pilot trial to evaluate the safety (primary endpoint) and efficacy (secondary endpoint) analysis of the anti-IL-6 antibody clazakizumab in 20 KTx patients presenting with late cABMR (19). These data suggested a potentially beneficial effect of anti-IL-6 blockade on cABMR activity and progression.

In the present single-center study, we report on the long-term effects on tocilizumab monthly therapy in kidney transplant recipients presenting with cABMR and/or transplant glomerulopathy.

MATERIALS AND METHODS

Study Population

In this single-center retrospective study, we enrolled all KT recipients that received TCZ in the setting of chronic ABMR, between August 2018 and July 2021. All patients included had to meet the last Banff criteria for chronic active ABMR, category 2 of 2019 Banff classification (20).

All patients received intravenous TCZ at the dose of 8 mg/kg every month scheduled for 6 months. At that point, continuation or discontinuation of TCZ was reassessed every 6 months at the discretion of attending physicians.

We collected demographic data on donor and recipients in the hospital's medical electronic records. The research protocol was approved by the local ethical committee. All medical data were collected from our database [CNIL (French National committee for data protection) approval number 1987785v0].

Study Design

We assessed in this study the clinical, biological and kidney graft histological data at baseline, month +6 (M6) and month +12 (M12). Baseline was defined at the time of the kidney biopsy that resulted in TCZ introduction for each patient. Usually, follow-up kidney biopsies are performed every 6 months after starting TCZ therapy in order to assess whether TCZ is continued or not.

Data collected were serum creatinine level (μmol/L), eGFR (mL/min/1.73 m^2), proteinuria or albuminuria (g/L), presence of DSA and their mean fluorescence intensity (MFI), and histological Banff scores: chronic glomerulopathy score (cg), glomerulitis score (g) interstitial fibrosis and tubular atrophy (IFTA) inflammation in areas of interstitial fibrosis and tubular atrophy (i-IFTA), peritubular capillaritis score (ptc), interstitial inflammation (i), tubulitis (t), intimal arteritis (v), staining for C4d on endothelial cells of ptcs and medullary *vasa recta* (c4d), arterial intimal thickening (cv), mesangial matrix expansion (mm), arteriolar hyalinosis (ah), hyaline arteriolar thickening (aah), and total inflammation (ti).

After kidney transplantation anti-HLA antibodies and DSA are assessed on a yearly basis since 2007 and at each time we perform a for-cause kidney biopsy. Anti-HLA antibodies. and

DSA are assessed by a Luminex assay using the Immuncor® platform. eGFR was calculated using the Chronic Kidney Disease Epidemiology collaboration equation (CKD-EPI).

Immunosuppression

At the time of kidney transplantation, induction therapy consisted in anti-thymocyte globulin for all patients (Sanofi, Lyon, France). All patients received 1 g of mycophenolate mofetil (MMF) pre-operatively, followed by MMF 2 g/day up to POD 15, then tapered to 1 g/day. Prednisolone was given at the dose of 500 mg pre-operatively, tapered to 10 mg/day at day 30 post-kidney transplantation. If kidney allograft surveillance biopsy at 3 months post-transplant was normal prednisone was stopped. Tacrolimus was started at day 4 post-kidney transplantation and adjusted to achieve trough levels of 8–12 ng/mL the first month, and then 4–8 ng/mL.

Endpoint

The primary endpoint was to assess the evolution of renal function (eGFR) at M6 and M12 post-TCZ therapy in the setting of cABMR. Patients who have lost their graft within the first year after starting TCZ therapy were excluded from this analysis.

Secondary endpoints were the evolution of the Banff scores (according to the 2019 Banff classification) in kidney allograft biopsies post-TCZ therapy. We performed two analyses. First we merge all follow-up biopsies during the first year to realize paired comparison between baseline and First year biopsies post TCZ. Then, we assessed all biopsies available, whatever the time post-TCZ therapy and grouped them into 4 periods according to quartile time post-TCZ therapy: biopsies done <5 month, between ≥ 5–<9 months, between ≥ 9–<17 months and ≥ 17 months. The first index biopsy, i.e., the one allowing cABMR diagnosis was not included in follow-up analyses. Biopsies of patients that have lost their graft were included in the analyses. Finally, the incidence of allograft failure, i.e., graft loss was also assessed.

Statistical Analyses

Quantitative data are shown as means ± standard deviations (SD) or as medians with quartiles [Q1–Q3]. Qualitative data are shown as numbers and percentages. We decided to remove missing data for percentages calculation. The chi-squared test was used for categorical variables, especially the comparison of Banff scores between time periods. For the primary endpoint, eGFR values follow a normal distribution. Equality of the variance was also assessed and confirmed. We used a paired student *t*-test to compare eGFR at baseline vs. M6 and M12. For the Banff scores, we first did a global assessment of follow-up scores using a Kruskal-Wallis test. Then, a paired Wilcoxon test was used to compare Banff scores at baseline vs. follow-up, in a matched manner for each patient. A two-sided *p*-value of <0.05 was considered statistically significant. Statistical analyses were conducted using R statistical software.

TABLE 1 | Demographic characteristics of included KT recipients.

	Included KT recipients ($n = 44$)
Recipient age at KT, years Mean ± SD	43 ± 15
Gender (female), N (%)	16 (40)
Living donor, N (%)	7 (17,5)
ABO incompatible KT, N (%)	2 (5)
HLA incompatible KT, N (%)	12 (30)
Main nephropathies	
- Glomerular disease	11 (27,5)
- Polycystic disease	6 (15)
- Malformative uropathy	6 (15)
Transplantation rank	
- First	30 (75)
- Second	9 (22.5)
- Third	1 (2.5)

KT, Kidney Transplantation; HLA, Human Leukocyte antigen.

RESULTS

Study Population

Between August 2018 and July 2021, 40 patients received TCZ for cABMR post-kidney transplantation. The baseline characteristics of these patients are summarized in **Table 1**.

Immunosuppression at the time of cABMR consisted in tacrolimus for 34 patients (85%), MMF for 34 patients (85%), mTOR inhibitors for 6 patients (15.4 %), belatacept for 6 patients (15.4 %) and steroids for 25 patients (62.5%). DSAs were found in 22 patients (55%). Nineteen patients had class II DSAs whereas 7 patients had class I DSAs. For the 18 patients without detectable DSA, cABMR diagnostic was based on histological findings. All those 18 patients had a cg score >0 and a C4d staining in peritubular capillaritis and a [g+cpt] score ≥ 2.

Median time to cABMR diagnostic was 18.9 (6–55) months. TCZ was started after a median time of 34 days (23–155) after cABMR diagnosis. Median follow-up after cABMR diagnosis was 7 (4–13) months.

Immunosuppression

In some patient the diagnosis of cABMR had been established months/years before starting TCZ therapy. Therefore, some had received in the past either rituximab ($n = 16$; 40%), plasmapheresis ($n = 8$; 20%), high dose of steroids ($n = 21$; 52.5%) or antithymoglobulin ($n = 2$; 5%). For seven patients (17.5%) TCZ therapy was the first line therapy for cABMR.

Immunosuppressive regimen consisted in tacrolimus and mycophenolate acid for all patients at the time of rejection except for one patient that received belatacept and tacrolimus. Post-rejection, 18 patients (45%) were converted to a belatacept-based immunosuppression in association with tacrolimus (4 patients) or mycophenolate mofetil (14 patients). The other patients remained on tacrolimus and mycophenolate mofetil.

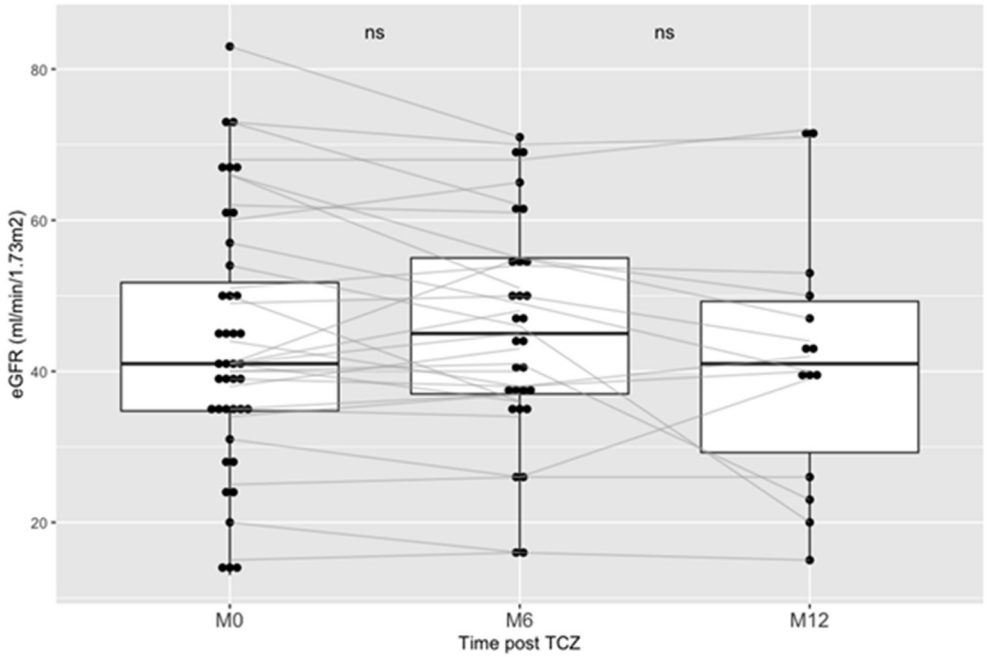

FIGURE 1 | Outcome of eGFR post Tocilizumab in kidney transplanted patients treated for chronic ABMR. Boxplots shows the eGFR (CKD-Epi) of patients at baseline of the introduction of TCZ, at Month-6 (M6) and at Month-12 (M12). TCZ stands for Tocilizumab. AMBR stands for antibody-mediated rejection.

Renal Function

Our primary endpoint was to assess the evolution of eGFR after TCZ treatment. At the time of cABMR diagnosis, mean eGFR was 43 ± 17 mL/min/1.73 m^2. At M6 post-TCZ therapy, mean eGFR was not significantly different, i.e., 45.3 ± 15 mL/min/1.73 m^2 ($p = 0.12$). Similarly, at M12 post-TCZ therapy, mean eGFR was not significantly different as compared to baseline, i.e., 41.6 ± 17 mL/min/1.73m^2 ($p = 0.102$). **Figure 1** shows the evolution of eGFR between baseline and M12 for each patient. We then assessed the eGFR slopes between M0 and M6 and between M0 and M12 using a linear model regression: we did not find a significant difference in eGFR ($p = 0.56$ between M0-M6 and $p = 0.77$ between M0-M12).

However, when we included in the analyses an eGFR = 5 ml/min/1.73 m^2 for the 5 patients who lost their graft within the 12 months post-TCZ, eGFR was 40 ± 19 ml/min.1.73m^2 at M6 post-TCZ ($p = 0.01$ as compared to baseline) and eGFR was 32 ± 21 ml/min.1.73m^2 at M12 post-TCZ ($p = 0.02$ as compared to baseline).

Regarding proteinuria, mean proteinuria at baseline was 1.0 ± 0.9 g/L. At M6 and M12 post-TCZ therapy mean proteinuria were 0.8 ± 1.1 g/l. and 0.9 ± 1.1 g/L, statistically not different from baseline, respectively $p = 0.95$ and $p = 0.28$.

Six patients (15%) lost their graft after an average time of 2.5 [2.5 – 6.5] months post-cABMR diagnostic. These patients had a significantly lower eGFR at the time of cABMR diagnostic (first biopsy) as compared to other patients: 24.5 ± 16 vs. 46.3 ± 15 ml/min/1.73 m^2 ($p = 0.006$). Similarly, their proteinuria level was significantly higher at the time of cABMR diagnostic, i.e., 1.8 ± 1 vs. 0.8 ± 0.9 g/L ($p = 0.022$). Finally, they had at baseline a more severe histological presentation, i.e., with ct = 3, ci = 3 and v = 2 than in those who did not lose their graft ($p = 0.007$, $p = 0.002$ and $p = 0.001$ respectively).

Histologic Evolution

From the 44 patients, 38 (70%) have had a follow-up kidney biopsy after TCZ introduction and 28 (63.6%) had at least 2 kidney biopsies post-TCZ. Average times of kidney biopsies were: 3.1 (1–5) months, 4.2 [1.2 – 10] months, 4.2 [1.9 – 6.7] months, 6.8 [3.5 – 9.4] months for the second, third, fourth and fifth biopsy post-TCZ respectively. Median time between follow-up biopsies was 173.5 (105–224) days.

At baseline, half of biopsies had a glomerulitis score (g) ≥2 and a peritubular capillaritis score (ptc) ≥2. Twenty five percent had a chronic glomerulopathy score (cg) ≥ 2. Eighteen percent of patients had an IFTA score ≥2 at baseline.

We assessed the significant modification of Banff score between biopsies post-TCZ therapy.

First, we compared the Banff scores between baseline and first-year follow-up post-TCZ treatment in 20 paired patients (**Table 2**). There were no statistical differences in the acute and chronic histologic scores of those patients in the follow-up biopsies as compared to diagnostic biopsies.

The **Table 3** shows the global Banff scores of all patients according to biopsies performed during quartile times post-TCZ therapy: <5 month, ≥5–<9 months, ≥9–< 17 months and ≥17 months. We then compared the Banff scores between baseline and all period of follow-up post-TCZ treatment in all patients.

TABLE 2 | Histological Banff scores during the first year post-TCZ treatment for cAMR.

Banff Scores	Baseline ($N = 20$)	5–12 months ($N = 20$)	p value*
Acute scores: *N* (%)**			
t score			0.595
0	12 (60.0%)	13 (65.0%)	
1	7 (35.0%)	7 (35.0%)	
2	1 (5.0%)	0 (0.0%)	
i score			0.220
0	18 (90.0%)	19 (95.0%)	
1	0 (0.0%)	1 (5.0%)	
2	2 (10.0%)	0 (0.0%)	
v scores			0.311
0	18 (94.7%)	19 (100.0%)	
1	1 (5.3%)	0 (0.0%)	
N-Miss	1	1	
g scores			0.968
0	4 (20.0%)	4 (21.1%)	
1	6 (30.0%)	5 (26.3%)	
2	10 (50.0%)	10 (52.6%)	
N-Miss	0	1	
ptc scores			0.361
0	3 (15.0%)	6 (30.0%)	
1	3 (15.0%)	1 (5.0%)	
2	14 (70.0%)	13 (65.0%)	
Chronic scores: *N* (%)**			
cg			0.683
0	16 (80.0%)	13 (68.4%)	
1	1 (5.0%)	2 (10.5%)	
2	3 (15.0%)	4 (21.1%)	
N-Miss	0	1	
cv			0.833
0	7 (36.8%)	5 (27.8%)	
1	4 (21.1%)	4 (22.2%)	
2	8 (42.1%)	9 (50.0%)	
N-Miss	1	2	
IFTA			0.512
0	5 (31.2%)	9 (47.4%)	
1	7 (43.8%)	5 (26.3%)	
2	4 (25.0%)	5 (26.3%)	
N-Miss	4	1	
iIFTA			0.161
0	5 (25.0%)	10 (52.6%)	
1	6 (30.0%)	5 (26.3%)	
2	9 (45.0%)	4 (21.1%)	
N-Miss	0	1	

**p-value compares patient paired Banff scores of biopsies between baseline and 5–12 months post-TCZ therapy.*

***Missing values are removed from the percentage calculation.*

TABLE 3 | Histological Banff scores comparison post TCZ treatment for cAMR.

Banff scores	Baseline $N = 40$	<5 months $N = 12$	5–9 months $N = 14$	9–17 months $N = 14$	≥17 months $N = 16$
Acute scores: *N* (%)**					
i score					
0	36 (92)	9 (75)	12 (92)	14(100)	14 (87)
1	1 (3)	2 (17)	1 (8)	0	1 (6)
≥2	2 (5)	1 (8)	0	0	1 (6)
t score					
0	26 (65)	5 (42)	7 (54)	11 (79)	9 (56)
1	11 (27)	5 (42)	6 (46)	2 (14)	6 (37)
≥2	3 (7)	2 (17)	0	1 (7)	1 (6)
v score					
0	34 (92)	7 (64)	12 (100)	14(100)	14 (100)
1	3(8)	4 (36)	0	0	0
g score					
0	8 (20)	3 (27)	4 (33)	2 (15)	1 (8)
1	10 (25)	3 (27)	2 (17)	3 (23)	1 (8)
≥2	22 (55)	5 (45)	6 (50)	8 (61)	11 (85)
ptc score					
0	11 (27)	4 (33)	4 (31)	5 (36)	2 (12)
1	7 (17)	3 (25)	1 (8)	2 (14)	5 (31)
≥2	22 (55)	5 (42)	8 (61)	7 (50)	9 (56)
Chronic scores: N (%)**					
IFTA score					
0	11 (33)	4 (40)	6 (50)	6 (43)	2 (13)
1	16 (48)	4 (40)	2 (17)	7 (50)	9 (60)
≥2	6 (18)	2 (20)	4 (33)	1 (7)	4 (27)
i-IFTA score					
0	11 (29)	2 (18)	6 (46)	6 (46)	4 (25)
1	12 (32)	7 (64)	3 (23)	6 (46)	8 (50)
≥2	15 (39)	2 (18)	4 (31)	1 (8)	4 (25)
cg score					
0	27 (67)	10 (91)	8 (61)	9 (75)	5 (31)
1	3 (7)	1 (9)	2 (15)	2 (17)	3 (19)
≥2	10 (25)	0	1 (8)	1 (8)	8 (50)
cv score					
0	12 (33)	4 (36)	4 (33)	2 (15)	0
1	6 (17)	4 (36)	3 (25)	4 (31)	4 (27)
≥2	8 (50)	3 (27)	5 (42)	7 (54)	11 (73)

***Missing values are removed from the percentage calculation.*

There was no statistical difference in the follow-up histologic scores except for the intimal arteritis score (v) = 1 that was present in 36 % in biopsies performed <5 months but was not found in later biopsies ($p = 0.001$). Also, the number of biopsies with a cg score ≥2 significantly increased over time, i.e., 0% in biopsies <5 months and 50% in biopsies >12 months ($p = 0.037$). There was no statistical difference during the follow-up biopsies for the IFTA, i-IFTA, g, ptc, i, t, and cv scores suggesting a relative stability of the histological lesions.

Evolution of biopsy scores for each patient are shown using alleviate figures for IFTA (**Supplementary Figure 1**), for cg (**Supplementary Figure 2**) for g (**Supplementary Figure 3**) and ptc scores (**Supplementary Figure 4**). We assessed more

precisely the outcome by using a paired Wilcoxon test to compare the evolution of Banff scores between baseline and each period of follow-up for each patient:

For the cg score, the average cg score was higher in >17 months biopsies as compared to baseline, i.e., 2.4 ± 0.8 vs. 1.5 ± 0.9, but not statistically significant ($p = 0.053$). The arterial intimal thickening (cv) score was also higher in follow-up biopsies as compared to baseline, i.e., 1.7 ± 0.4 vs. 1.2 ± 0.9 respectively, also without reaching significance ($p = 0.056$).

However, the iIFTA score seemed to decrease with time, i.e., in 9–12 months biopsies, iIFTA score was 0.6 ± 0.6 vs. 1.1 ± 0.8 at baseline ($p = 0.053$).

There was no statistical difference between baseline and follow-up biopsies (<5 months, 5–9 months, 9–17 months and >17 months) for the IFTA, the glomerulitis (g) the peritubular capillaritis (ptc), the interstitial inflammation (i), the tubulitis (t) and the intimal arteritis (v) scores (data not shown).

Then, we assessed the evolution of patients with an history of circulating DSA as compared to DSA(−) patients. We compared g, cg, IFTA, iIFTA, and ptc scores in each follow-up biopsy post-TCZ therapy (second biopsy, third biopsy, fourth biopsy, fifth biopsy and sixth biopsy post-TCZ). There were no statistical differences in the Banff score between DSA(+) and DSA(−) patients except for the ptc score after the third biopsy in which a score ≥2 was present in 6 patients (66%) in DSA(+) group as compared to 1 patient (16%) in the DSA(−) group ($p = 0.03$). Four DSA (−) patients have lost their graft at the end of follow-up (22%). In the DSA(+) group, 2 patients (9%) have lost their graft. The difference did not reach significance ($p = 0.476$).

DISCUSSION

In this study, we assessed the clinical and histological outcomes of kidney transplant recipients experiencing chronic active ABMR treated with IV tocilizumab. We have shown that at 6 months post-TCZ and 12 months post-TCZ, renal function and proteinuria remained stable in patients with a functional graft. However, eGFR was significantly lower at 12 months if we included the value of 5 ml/min/1.73 m^2 for patients who have lost their kidney graft within the first year. We have also assessed the evolution of Banff scores in follow-up kidney allograft biopsies and showed that the parameters of microvascular inflammation (g and ptc) and the chronicity markers (cg, IFTA, i-IFTA) also remained stable during the follow-up. To summarize, although a control group is missing, we assume that TCZ therapy may have allowed preventing clinical and histological worsenings in the setting of cABMR in our cohort.

IL-6 has pleiotropic effects upon the inflammatory response. Sites of action are as numerous as the possible therapeutic fields of tocilizumab: impact on hematopoiesis and on keratocyte proliferation, differentiation of osteoclasts (21). Regarding the humoral response, IL-6 plays a role in the differentiation of the mature B-cell into a cell capable of secreting antibodies (22). It has been showed that TCZ significantly and non-specifically reduce the IgG synthesis in highly sensitized kidney transplant recipients treated for cABMR (23). However, in highly sensitized kidney transplant candidates, tocilizumab as a monotherapy limited B cell maturation but however, it had almost no effect on anti-HLA alloantibodies (24). Chandran et al. conducted a randomized controlled clinical trial of clinically stable kidney transplant recipients on calcineurin inhibitor, mycophenolate mofetil, and prednisone, with subclinical graft inflammation noted on surveillance biopsies during the first-year post-transplant: they were randomized to receive either TCZ (8 mg/kg every 4 weeks, six infusions) or placebo. They showed an increase of circulating Treg as compared to controls, and tocilizumab-treated subjects were more likely to show improved Banff ti-score (62.5% vs. 21.4%, $p = 0.03$) (25).

Two recent studies addressed the use of IV tocilizumab for treating cABMR. Lavacca et al. included fifteen cABMR patients for which the first-line therapy was tocilizumab (18). They were followed for a median time of 20.7 months. They found that despite the majority of patients experienced advanced transplant glomerulopathy (TG) at diagnosis (60% with cg3), glomerular filtration rate and proteinuria stabilized during the follow-up, with a significant reduction in donor-specific antibodies. In addition, protocol biopsies after 6 months demonstrated significant amelioration of microvascular inflammation and no TG, C4d deposition, or IF/TA progression. Finally, gene-expression and immunofluorescence analysis showed upregulation of three genes (TJP-1, AKR1C3, and CASK) involved in podocyte, mesangial, and tubular restoration. The second single-center retrospective study reported on nine ABMR kidney transplant patients resistant to apheresis, rituximab, and intravenous immunoglobulins that were treated with monthly IV tocilizumab (26). They were compared with a historical cohort of 37 patients with similar clinical, immunological, and histological characteristics. They found that 1-year graft survival and the decline in renal function did not differ between patients who received tocilizumab and those who did not. In addition, histological follow-up showed that despite a decrease in inflammation and tubulitis scores after tocilizumab, the course of antibody-mediated lesions and chronic glomerulopathy were similar in both groups (26).

In our study, the addition of TCZ may have prevented clinical and histological worsening of cABMR in kidney transplant recipients, except for more severely ill patients. Indeed, 6 patients lost their graft. When we assessed the clinical characteristics of these patients at baseline, we showed that they had a significant more severe presentation with a lower eGFR (24 ml/min/1.73 m^2), higher level of proteinuria (1.8 g/L) and worse histological presentation (ci, ct and v scores). We assume that for these patients TCZ treatment was initiated too late.

Most of our patients had follow-up kidney biopsies within the first-year post-TCZ treatment. We did not observe significant worsening in the Banff scores over time. However, during TCZ therapy most of our patients (34/40) were clinically stable without rejection. Although not reaching the level of statistical significance we observed an improvement of some histological parameters such as peritubular capillaritis.

Recently, Sethi et al. assessed infections occurring among 148 kidney recipients treated with tocilizumab 8 mg/kg IV monthly ($n = 83$) or IVIG/rituximab ($n = 65$) for donor-specific

antibodies and antibody-mediated rejection through 1 year after treatment cessation (27). There were 106 infections observed over 190.1 person-years, yielding an incidence rate of 558 infections/1,000 patient-years; however a lower incidence rate of infections was observed among tocilizumab-treated compared with IVIG/rituximab-treated patients (463 infections/1,000 patient-years vs. 730 infections/1,000 patient-years; $P = 0.02$). Twenty-five of 49 infections (51%) in the IVIG/rituximab group required hospitalization compared with 31 of 57 (54%; $P = 0.85$) in the tocilizumab group. Finally, there were no infection-related deaths in either group. On multivariable Poisson regression, there was a lower incidence rate of infections associated with tocilizumab compared with IVIG/rituximab. These findings are very reassuring at using in the long run tocilizumab therapy in kidney transplant recipients.

Our study has several limitations inherent to the retrospective and uncontrolled design. The number of patients in the present study is limited, although no other published study on the subject has included more patients. Large randomized clinical trials are needed to clarify the benefit of TCZ for treating cABMR in solid-organ transplant patients because the safety profile of TCZ therapy in kidney transplant recipients is reassuring (27).

Recent data highlight the potential role of TCZ in controlling the humoral and inflammatory response and its potential benefit in cABMR treatment. The available studies and our study seem to show that it is possible to stabilize the decline in renal function and histological rejection lesions. Indeed, randomized studies are needed in this area, as cABMR suffers from the lack of effective treatments. Blocking the IL-6 pathway by either anti-IL-6 receptor or by anti-IL-6 monoclonal antibodies seems to a relevant avenue.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of CNIL (French National Committee for Data Protection) approval number 1987785v0. N° BRIF: BB-0033-00069. Informed consent was obtained from all subjects involved in the study.

AUTHOR CONTRIBUTIONS

JN, PM, TJ, and LR designed the study. JN, PM, BJ, and LR recruited the patients. DG reviewed the kidney biopsies. RL, FI, LM, and JN collected the data. JN and LR wrote the manuscript. PM and TJ edited the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.790547/full#supplementary-material

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REVIEW
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Ex-vivo Kidney Machine Perfusion: Therapeutic Potential

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Kidney transplantation remains the gold standard treatment for patients suffering from end-stage kidney disease. To meet the constantly growing organ demands grafts donated after circulatory death (DCD) or retrieved from extended criteria donors (ECD) are increasingly utilized. Not surprisingly, usage of those organs is challenging due to their susceptibility to ischemia-reperfusion injury, high immunogenicity, and demanding immune regulation after implantation. Lately, a lot of effort has been put into improvement of kidney preservation strategies. After demonstrating a definite advantage over static cold storage in reduction of delayed graft function rates in randomized-controlled clinical trials, hypothermic machine perfusion has already found its place in clinical practice of kidney transplantation. Nevertheless, an active investigation of perfusion variables, such as temperature (normothermic or subnormothermic), oxygen supply and perfusate composition, is already bringing evidence that *ex-vivo* machine perfusion has a potential not only to maintain kidney viability, but also serve as a platform for organ conditioning, targeted treatment and even improve its quality. Many different therapies, including pharmacological agents, gene therapy, mesenchymal stromal cells, or nanoparticles (NPs), have been successfully delivered directly to the kidney during *ex-vivo* machine perfusion in experimental models, making a big step toward achievement of two main goals in transplant surgery: minimization of graft ischemia-reperfusion injury and reduction of immunogenicity (or even reaching tolerance). In this comprehensive review current state of evidence regarding *ex-vivo* kidney machine perfusion and its capacity in kidney graft treatment is presented. Moreover, challenges in application of these novel techniques in clinical practice are discussed.

Keywords: kidney transplantation, *ex-vivo* machine perfusion, *ex-vivo* therapy, graft preservation, kidney preconditioning, ischemia-reperfusion injury, graft rejection, organ regeneration

INTRODUCTION

Kidney transplantation (Tx) remains the gold-standard treatment for end-stage kidney disease. Due to advanced kidney replacement therapies, improved care, and increased survival of such patients, the number of people on the waiting list for a deceased donor kidney is stably high or growing. In 2020, in the EuroTransplant region, <3,000 deceased donor kidneys and <1,000 living

donor kidneys were transplanted while ∼11,000 patients were on the active kidney waiting list (1). To meet growing organ demands, utilization of grafts donated after circulatory death (DCD), or retrieved from extended criteria donors (ECD) is unavoidable. The main challenge preventing the expansion of the donor pool is the susceptibility of ECD kidneys for ischemia-reperfusion injury (IRI). Moreover, DCD and ECD grafts are more immunogenic, which leads to difficulties in immune regulation after implantation and a higher risk of acute and chronic rejection (2–4).

The development of organ preservation techniques has the potential to overcome these challenges. Therefore, it has been the main focus of research in the solid organ Tx field for at least 30 years (5, 6). Kidney graft preservation relied on static cold storage (SCS) for a long time (7–9) but increasing donor age and comorbidities urgently require more reliable preservation techniques. Since publication of the largest randomized clinical trial (RCT) comparing hypothermic dynamic kidney machine perfusion (HMP) and SCS (10), HMP took root in routine clinical practice (11–14). HMP improves at least short-term outcomes of all types of kidney grafts, especially of DCD and ECD organs, by achieving the first goal of organ preservation—maintain organ quality until implantation. However, to increase the number of transplantable organs, the main focus now is on the improvement of graft quality, graft conditioning, and repair (6). Extensive research on different *ex-vivo* dynamic preservation temperatures [normothermic (NMP) and subnormothermic (SNMP)], perfusate composition, oxygenation, and perfusion duration revealed that machine perfusion could be used as a tool to treat kidneys prior to implantation by creating nearly-physiological conditions (15). Moreover, machine perfusion seems to be a perfect platform for the various biological and pharmacological agents' delivery directly to the kidney graft. *Ex-vivo* treatment is attractive because it would allow avoiding toxicity caused by systemic recipient treatment and logistic difficulties of the donor therapy (16–18). In addition, some novel promising agents just failed to reach the graft when applied systemically (19–21). This issue could be solved by targeted therapy delivery during *ex-vivo* machine perfusion. Moreover, perfusion parameters and perfusate markers would allow constant evaluation of the kidney state during treatment (22). Multiple experimental and clinical works investigated various *ex-vivo* kidney perfusion therapies for three main goals: (i) to reduce ischemia-reperfusion injury, (ii) reduce the immunogenicity of the kidney graft and (iii) promote healing and regeneration of already injured organ.

In this review, we discuss current evidence of kidney *ex-vivo* perfusion techniques and graft treatment during *ex-vivo* perfusion. Of note, in the overview of *ex-vivo* therapies, we did not include usual perfusate components, such as oxygen, nutrition supplement, heparin, vasodilators, and antibiotics, as this is out of our scope. We aim to outline the newest experimental and clinical studies of kidney *ex-vivo* cell therapy, gene therapy, application of nanotechnologies, delivery of different gasses, biological, and pharmacological agents for kidney treatment and pre-conditioning.

OVERVIEW OF KIDNEY *EX-VIVO* MACHINE PERFUSION PROTOCOLS

Ex-vivo organ perfusion techniques are usually classified according to the perfusate temperature. Below, we briefly present the concepts and the current protocols of hypothermic and (sub)normothermic kidney machine perfusion. The main differences of perfusion techniques are summarized in **Table 1**.

Hypothermic Machine Perfusion

During HMP, cold (4–10°C) acellular preservation solution is pumped through the kidney vasculature (5). Several kidney HMP devices are currently commercially available, including LifePort® (Organ Recovery Systems; Itasca, Illinois), Kidney Assist® (OrganAssist; Groningen, Netherlands), Waters RM3® kidney perfusion system (Rochester, Minnesota) and Waves IGL® (Lyon, France). In the current *ex-vivo* perfusion machines, roller or centrifugal pumps are used to generate pressure-controlled pulsatile flow, avoiding perfusion-related graft injury. For kidney HMP, low pressure (25–30 mmHg) is preferable to prevent kidney edema and endothelial damage (5, 23). The perfusion solutions used are qualitatively different from SCS solutions. The only clinically proven fluid for kidney HMP is Kidney Perfusion Solution-1 (KPS-1®), which has the same composition as Belzer MPS® UW (Machine Perfusion Solution University of Wisconsin) (14). Unlike the conventional UW solution and other SCS solutions, KPS-1 has an extracellular-like Na+/K+ balance. Moreover, it contains other impermeants (gluconate and mannitol instead of lactobionate and raffinose) and glucose. A variant of commonly used HTK (histidine-tryptophan-ketoglutarate, Custodiol®) solution Custodiol-N® supplemented with dextran 40 was used for kidney HMP in several porcine models (24). Moreover, a novel Custodiol-MP® solution has been recently developed exclusively for aerobic machine perfusion (25). Despite promising results of experimental models, further studies are necessary to prove the superiority of Custodiol-N® or Custodiol-MP® over KPS-1® in terms of kidney graft function and transplant outcomes (24, 25).

MP was widely used at the beginning of the solid organ Tx era when the first kidney transplant programs were developed. This method was considered the only safe and reliable way to preserve grafts, especially for long periods of organ storage. These machines were large and difficult to transport (26, 27). Later, Collins et al. presented a method to preserve and transport kidneys on ice in a preservation solution (7). More elaborated preservation solutions granted safe, easy, and much cheaper SCS with satisfactory Tx outcomes decreasing HMP's popularity, inaugurating the era of SCS (7–9). SCS served well as a preservation method of healthy kidney grafts. However, with the growing demand for organs and the aging donor population, the usage of kidneys from ECD lately became unavoidable. The limited applicability of SCS for higher-risk organs encouraged extensive research on alternative and more sophisticated preservation methods. The HMP landmark study in 2009 by Moers et al. brought the machine perfusion back to clinics. This EuroTransplant study, which included 672 kidneys retrieved in 60 European Tx centers, revealed a significantly

TABLE 1 | Comparison of different kidney machine perfusion techniques.

	HMP	NMP	SNMP
Temperature	4–10°C	35–39°C	20–32°C
Need of oxygen	+/–	+	+
Perfusate	Without oxygen carrier	With oxygen carrier	With/without oxygen carrier
Cost	Moderate	Expensive	Moderate/expensive
Personnel	Single operator	Team	Single operator/team
Device malfunction	Low risk for graft loss	High risk for graft loss	High risk for graft loss
Graft evaluation	Limited capability	Extensive capability	Moderate capability
Current evidence	Clinically proved	Clinical studies ongoing	Experimental stage
Commercially available devices	LifePort®, Waters RM3®, kidney assist®, waves IGL®	Kidney assist®	Kidney assist®
Therapies/interventions tested	• Cell therapy (MSC) • Gene therapy (siRNA) • Biological therapy (etanercept) • Thrombolytics, fibrinolytics, anticoagulants (tPA, CHC, thrombalexin) • Gases (CORM-401) • Others (metformin, doxycycline, propofol)	• Cell therapy (MSC, MAPC) • Gene therapy (siRNA) • Biological therapy (αCD47ab) • Nanotechnologies (nanoparticles) • Thrombolytics, fibrinolytics, anticoagulants (tPA + plas-minogen) • Gases (CORM-3, Argon, CORM-401) • Cytosorb hemadsorbtion • Others (EPO, cyclic helix B peptide, metformin, SUL-121)	Cell therapy (MSC) Gene therapy (shRNA) Nanotechnologies (NB-LVF4) Gases AP39 (H_2S donor)

HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion; SNMP, subnormothermic machine perfusion; MSC, mesenchymal stromal cells; MAPC, multipotent adult progenitor cells; siRNA, small interfering ribonucleic acid; shRNA, short hairpin ribonucleic acid; NB-LVF4, nano-barrier membrane; tPA, tissue plasminogen activator; CHC, corline heparin conjugate; CORM, carbon monoxide releasing molecule; EPO, erythropoietin.

improved graft survival at 1- and 3-years as well as reduced DGF rates in HMP kidneys compared to SCS (10). Even though this study included mainly donation after brain-death (DBD) grafts, Zhong et al. also found significant benefits in 1- and 3-years graft survival of HMP in the DCD kidneys cohort (28). An independently powered extension of Jochmans et al. initial RCT showed reduced delayed graft function (DGF) rates in DCD kidneys that underwent HMP (29). Similarly, another RCT reported that HMP significantly diminished DGF rates and improved 1-year death-censored graft survival in ECD kidneys in comparison with SCS (30). The superiority of kidney HMP vs. SCS was also confirmed by many systematic reviews and meta-analyses: reduction of DGF rates was observed in both DCD (31, 32) and ECD kidneys (33), and across all donor types (11–14, 34). Similarly, meta-analyses reported improved ECD kidney graft survival at 1-year (33) and among all donor types at 3-years (13). A Cochrane Review, including 16 studies published during the previous 10-years including 2,266 patients, revealed that HMP significantly reduced DGF rates in both DBD and DCD kidney Tx. However, the number needed to treat to prevent one episode of DGF was lower for DCD kidneys (14). Several systematic reviews and meta-analyses demonstrated significant reduction rates in kidney primary non-function (PNF) following HMP compared to SCS (12, 35).Nevertheless, the HMP effect on short- and long-term kidney graft function remains inconclusive (14).

In depth, knowledge about the exact mechanism, how pulsatile hypothermic perfusion improves kidney outcome, is scarce. However, several experimental studies brought some clarity into the field. Similar to SCS, during HMP, the rate of metabolism is reduced to ∼10% of physiological temperature. Although HMP does not prevent cold-related depletion of adenosine triphosphate (ATP) and accumulation of metabolic products in the graft (5), the dynamic manner of preservation gives its beneficial effects. Firstly, HMP allows the elimination of debris, toxic metabolites, and free radicals produced during hypothermia (36, 37). Hemodynamic stimulation of the graft vasculature helps prevent endothelial damage, leading to anti-inflammatory effects and may even reduce the graft's immunogenicity. Pulsatile flow generates vascular shear stress, which influences endothelial gene expression and function (5, 38). Moreover, HMP enhances endothelial nitric oxide (NO) synthase (eNOS) phosphorylation, preventing vasospasm and promoting NO-depended vasodilatation at reperfusion. Good microcirculation at the time of implantation vastly increases the chances of immediate graft function (39). On the other hand, even though cellular metabolism during HMP is reduced, it is not entirely ceased. Therefore, the problem of oxygen deficiency remains: a decrease in ATP levels results in inhibition of Na+/K+ pumps and leads to acidosis. Mitochondrial dysfunction and ROS production due to oxygen deficiency promote graft lesions during reperfusion (40). In a porcine DCD model, Kaminski et al. reported a rapid decrease in perfusate oxygen pressure from 150 mmHg at 10 min of perfusion to 6.8 mmHg at 200 min and kidney cortical oxygen pressure dropped from 10.2 to 0 within the first 60 min of perfusion (41). While healthy kidney grafts endure anoxia at low temperature for some time, such conditions may be harmful to ECD or DCD kidneys significantly diminishing the optimal preservation time. Not surprisingly, despite reported superiority of HMP when compared to SCS, RCT and meta-analyses reveal a lack of evidence that HMP

improves long-term graft function or long-term survival of DCD kidneys (14, 29). Moreover, even with HMP, the kidney graft outcome depends on cold ischemia time (CIT). Therefore, preservation time cannot be extended (30, 42). HMP itself does not have a conditioning effect and does not improve kidney quality but rather helps maintaining kidney quality as it was at the time of retrieval.

These limitations of HMP led to a growing interest in oxygenated HMP ($HMPO_2$). During HMP higher levels of ATP are produced than during SCS, and oxygen is further able to support ATP synthesis (43). Rodent models revealed that oxygenation in HMP reduced nuclear injury, tubular damage, macrophage activation, increased kidney function and even suppressed T-cell response after implantation (37, 44). In pre-clinical porcine models, $HMPO_2$ improved early kidney graft function and reduced fibrosis (43, 45–48). The COPE-COMPARE study (49, 50) included 106 paired kidneys retrieved from DCD donors aged ≥50 years and compared $HMPO_2$ to standard HMP. It demonstrated that $HMPO_2$ is safe and feasible resulting in significantly less severe complications after Tx in the $HMPO_2$ group. $HMPO_2$-perfused grafts did not show significant improvement in estimated glomerular filtration rate (eGFR) at 12 months post-transplant when comparing functioning kidneys from both groups. However, sensitivity analysis, considering all-cause graft failure, showed significantly improved 1-year kidney graft function in the $HMPO_2$ group. Interestingly, $HMPO_2$-preserved kidneys had a significant relative risk reduction of acute rejection compared to HMP. No statistically significant difference was seen in terms of DGF and PNF (49). Another clinical trial (COPE-POMP) randomized ECD kidneys to end-$HMPO_2$ after SCS vs. SCS alone with graft survival at 12 months post-transplant as a primary endpoint. The minimum $HMPO_2$ time was 120 min before implantation. Both groups had comparable 1-year graft survival. Moreover, no significant differences in terms of DGF, PNF, eGFR, and acute rejection between the groups were observed. The overall graft survival rate was high in this study leading to the assumption that the analysis might be underpowered (50). All in all, it seems that oxygen is the key factor when considering HMP not only as a preservation technique but also as an organ conditioning tool. However, additional clinical studies are necessary to confirm this hypothesis.

Normothermic Machine Perfusion

Despite the benefits of HMP, the detrimental effect of the cold remains, and the kidney graft outcome is still very dependent on CIT. Even after adoption of HMP into clinical practice, the utilization of marginal kidneys, especially DCD, remains limited. The emerging technology of NMP that reduces or even eliminates CIT and harm caused by low temperatures has a great potential in expanding the donor pool (5, 6). During NMP, the organ is perfused with oxygenated blood or other oxygen carrier containing perfusate, nutrients, and medications at body temperature (35–37°C) (5, 6, 15). The preclinical trials by Hosgood and Nicholson found that the optimal arterial pressure for kidney NMP is the lower end of the physiological range for kidney autoregulation [mean arterial pressure (MAP) ∼95 mmHg] (51). The main difference between HMP and NMP perfusion solutions is that NMP requires an oxygen carrier. Leukocytes and thrombocytes depleted autologous whole blood has been used for kidney NMP in experimental studies (52–54). Elimination of white cells and platelets allows restoration of organ function without induction of inflammatory reactions and thrombosis, occurring during normal *in-vivo* reperfusion. The remaining plasma contains albumin and globulins, which maintain stable osmotic pressure and electrolytes that regulate pH. However, it can be logistically demanding to harvest autologous blood and prepare perfusate on the spot in clinical situations. Moreover, the remaining fibrinogen may promote micro thrombus formation (15). Therefore, red blood cells (RBC)-based solution is used much more commonly. RBCs efficiently carry oxygen, as well as, their flow reduces shear stress in kidney vessels maintaining normal endothelial function (15) but the increased risk of hemolysis due to RBC contact with artificial surfaces in the perfusion circuit should not be under estimated. Longer-banked RBCs may not be suitable because of higher levels of non-transferrin-bound iron and time-dependent metabolic alterations (15, 55–57). As an alternative, artificial oxygen carriers, including polymerized bovine hemoglobin-based oxygen carriers (HBOC) and pyridoxylated bovine hemoglobin, as well as manufactured, acellular oxygen-carrying media, including Lifor, Aqix RS-I, STEEN solution, have been proposed. The properties of different oxygen carriers are discussed in detail elsewhere (15). In addition, in most of the protocols NMP perfusates contain heparin (if it is blood- or RBC-based perfusate), vasodilators, mannitol, corticosteroids, antibiotics, nutrient preparations with glucose, amino acids and insulin (5, 6, 15).

Such graft preservation in almost physiological conditions enables normal cellular metabolism and recovery of ATP production (58). Some experimental works revealed that 1 h NMP promotes IL-6, IL-8 (59), and heat shock proteins (HSP) expression in kidney grafts (59, 60). Moreover, induction of protective stress responses, down-regulation of cell death, and enhanced proliferation have been observed (61). It has been demonstrated that long periods of NMP can support *de novo* protein synthesis and promote recovery of cytoskeletal integrity in ischemically-damaged kidneys (62). Interestingly, the release of inflammatory cytokines and chemokines into the perfusate and recirculation during NMP has been observed by several investigators (63–65). Transcriptional profiling of NMP perfused kidneys revealed that not only oxidative phosphorylation genes but also many immune and pro-inflammatory pathway genes are significantly up-regulated after 4 h of NMP. Most likely, circulating cytokines promote inflammatory gene expression; therefore, their removal could be beneficial (discussed below) (64, 65). On the other hand, NMP initiates donor-derived T- and B- lymphocytes, natural killer (NK)-cells, macrophages, and granulocytes diapedesis and removal (63).

Current evidence shows, that NMP itself has a therapeutic effect on kidney grafts, though the full mechanism remains to be revealed. In addition, NMP should be an ideal platform for the delivery of various *ex-vivo* therapies as pharmacokinetics and pharmacodynamics of drugs should not be altered by low

temperature (5, 6). Another advantage is the possibility of kidney evaluation in physiological conditions before implantation, which could both alleviate decision-making in graft utilization and allow graft assessment during *ex-vivo* therapy (22). Hosgood et al. established a kidney quality score based on renal blood flow (RBF), urine output (UO) during NMP, and macroscopic graft appearance (66). Extensive research is ongoing to find an ideal perfusate or urine biomarker (or a combination of biomarkers) for graft quality and function assessment (22).

So far, most of the experimental and clinical experience has been acquired with short-term pre-implantation kidney NMP. One of the pioneering groups, established the NMP technology using modified pediatric cardiopulmonary bypass equipment and demonstrated its feasibility and superiority over SCS in multiple animal experiments (52, 58, 59). In 2013 they published the first pilot clinical series, in which 18 ECD kidneys underwent 1 h pre-implantation NMP revealing a significantly lower incidence of DGF when compared to the consecutive historical control group (ECD kidneys implanted after SCS) (67). Later they reported successful implantation of a pair of previously considered non-transplantable human kidneys after 1 h NMP at the end of SCS (68). Currently, the same group is conducting the first RCT (ISRCTN15821205), comparing pre-implantation NMP vs. SCS for DCD kidneys. Three hundred thirty-eight patients have been recruited, and results of this trial are expected in 2021 (69). On the other hand, just a few studies have directly compared NMP with HMP or $HMPO_2$ so far. Several experimental models demonstrated the superiority of a short period of NMP after HMP (52) or prolonged SCS (58) vs. HMP-only. Contrary to those findings, in the recent study by Vallant et al. porcine kidneys after 4 h of end-ischemic HMP had higher urine output, oxygen consumption, perfusate flow rates, and lower number of apoptotic cells than paired grafts that underwent 4 h of end-ischemic NMP (70). This is in consistence with the results of Darius et al. (46) and Blum et al. (71) who also did not find a beneficial effect of kidney NMP compared to HMP or $HMPO_2$ in their experiments. One of the reasons for this inconsistency with previously mentioned results could be different protocols and compositions of preservation solutions. Importantly, another RCT has recently started in the Netherlands, aiming to compare 2 h NMP after HMP vs. HMP-only for DCD or ECD kidneys (NCT04882254).

Currently, Kidney Assist® (OrganAssist; Groningen, Netherlands) is the only commercially available device for kidney NMP. As this machine is not portable, kidney NMP remains a static method and can be used only in combination with SCS or HMP, which are the only commercially available options for organ transportation. Moreover, the optimal duration of NMP is still to be determined. Even though clinically, short-term pre-implantation kidney NMP has been demonstrated as sustainable, the maintenance of NMP for long periods is challenging. One of the main issues is the toxic products unavoidably deriving from the cells' activity that need to be eliminated (70). Moreover, the NMP procedure itself is expensive and requires a lot of human resources; not surprisingly, its cost-effectiveness remains questionable. The risk of the device malfunction, which would lead to organ loss, should also be taken into account. Nevertheless, there is growing evidence demonstrating the benefits of long-term NMP. Recently, a Dutch group registered a new clinical trial PROPER (NCT04693325), aiming to prolong NMP up to 6 h. One of the leading groups in kidney graft preservation at the University of Toronto explored prolonged NMP up to 16 h in porcine experiments. They revealed that long-term normothermic preservation significantly reduces tubular injury and improves kidney function, compared to SCS, HMP or end-ischemia short-term NMP (72–78). The most recent works already demonstrated the safety and feasibility of 24 h kidney NMP, though these grafts were not implanted (79–82). Importantly, Weissenbacher et al. in porcine and non-transplantable human kidneys experiments revealed, that one key factor allowing prolongation of NMP is urine recirculation (80–82). Perfusate homeostasis and stable kidney arterial flow could be achieved only by urine recirculation (80, 82). Moreover, proteomics analysis showed decreased damage-associated molecular patterns, angiotensinogen levels, and enhanced levels of enzymes involved in kidney metabolism, perfused with urine recirculation (81). Pool et al. recently demonstrated that perfusate composition also significantly impact kidney injury and perfusion parameters during prolonged NMP (83). Basile et al. observed stable perfusion parameters and no evidence of damage during 72 h of NMP, revealing that even several days of perfusion could be feasible (79). However, further investigation of principal perfusate components is necessary to establish optimal NMP conditions, allowing long-term NMP. In the studies by Weissenbacher et al. a pre-clinical automatic portable NMP machine was used, confirming that it is only a matter of time until NMP becomes feasible even for organ transportation (80–82).

Although some challenges remain to be overcome, the results of the previously mentioned studies suggest that long periods of NMP may be necessary for re-conditioning of the more severely damaged kidneys. Moreover, the ability to extend *ex-vivo* preservation time could allow applying regenerative techniques and other treatments regimen. It is most likely, that different NMP protocols and perfusate components may be necessary considering different donation situations and kidney quality. Finally, kidney Tx surgery could become an elective daytime procedure.

Subnormothermic Machine Perfusion

A less investigated but also promising alternative for kidney graft preservation is SNMP. Organ perfusion at 20–32°C aims to avoid cold-induced graft injury but does not increase metabolism to a level requiring oxygen carriers for adequate oxygenation (5). Currently, this technique is still at an experimental stage. One study showed that 1 h end-ischemia kidney perfusion at 37°C preserves tubular and kidney functions better than the same duration perfusion at 32°C, which raised a concern that lower than physiological temperatures may reduce RBC oxygen-carrying capacity (84). However, a study by Hoyer et al. revealed that SNMP is feasible and beneficial even without oxygen carrier: 7 h perfusion of porcine DCD kidneys at 21°C with Custodiol-N, supplemented with dextran 40, resulted in a better-preserved organ structure when compared to oxygenated HMP

or SCS. Moreover, a 2-fold increase in creatinine clearance (CrCl) compared to oxygenated HMP, and a 10-fold increase in CrCl, compared to SCS was demonstrated (85). Another group demonstrated that 4 h porcine kidney perfusion with autologous blood at 22°C significantly reduced IRI-related structural injury, hemorrhage and clotting, as well as increased UO and RBF in comparison to kidneys, perfused at 15 and 37°C. Moreover, SNMP diminished the expression of Toll-like receptor signaling molecules [high mobility group box 1 (HMGB1), myeloid differentiation factor 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)] and reduced the levels of the kidney injury marker neutrophil gelatinase lipocalin (NGAL) and IL-6 (86). In another study, the same authors showed the feasibility of a hemoglobin-based oxygen carrier (HBOC-201) for 22°C kidney SNMP (87). STEEN solution has also been used for prolonged (24 h) SNMP at 21°C and demonstrated the advantage over 37°C blood-based solutions in terms of vascular resistance during perfusion (88). Brasile et al. used 32°C for kidney perfusion with EMS medium (89).

Moreover, SNMP has been successfully used for delivery of pharmacological agents (90). For example, the cytoprotective property of the H_2S donor AP29 in University of Wisconsin (UW) solution seemed more pronounced at 21 °C than in hypothermic or normothermic conditions, suggesting that SNMP might be an optimal platform for certain agents delivery (90, 91).

Lastly, a controlled oxygenated rewarming strategy has been proposed as a safer gradual transition from cold to warm before reperfusion, avoiding a sudden heat shock (92–94). According to the protocol of Minor et al. (93) and von Horn and Minor (94) following SCS the kidney is perfused *ex-vivo* starting at 8°C and 30 mmHg and gradually elevating temperature and pressure up to 35°C and 75 mmHg during the first 90 min of 2 h perfusion. Pre-clinical studies demonstrated improved mitochondrial recovery (94) and kidney function following auto-Tx (95) after controlled oxygenated rewarming compared to SCS alone (93). The same group recently demonstrated that 2 h of controlled-oxygenated rewarming after 6 h SCS improved kidney function of ischemically damaged porcine kidneys at a similar level as 8 h NMP (96). One successful Tx of a human ECD kidney after gradual oxygenated rewarming has been reported (93). Despite promising pre-clinical results, further investigation is necessary to prove the applicability of these techniques and establish optimal protocols in terms of timing and perfusate composition.

KIDNEY GRAFT THERAPY DURING *EX-VIVO* MACHINE PERFUSION

Many different pharmacological and biological therapies, nanotechnologies, and hemadsorbtion techniques have been applied for kidney grafts during *ex-vivo* machine perfusion. Experimental and clinical studies investigating kidney *ex-vivo* machine perfusion therapies are summarized in **Table 2** and discussed in detail below.

Cell Therapy

One of the emerging strategies in *ex-vivo* machine perfusion therapeutics is cell therapy. Mesenchymal stromal cells (MSC) are multipotent cells mainly derived from bone marrow and adipose tissue, but also present in the umbilical cord, placenta, peripheral blood, and other tissues. Due to their stem-cell-like properties, after the engraftment into organs, they can differentiate into various functional cells and interfere with detrimental pathophysiological processes (124). International Society for Cellular Therapy established criteria defining MSC, including plastic-adherence when maintained in standard culture conditions, expression of cluster of differentiation (CD)73, CD90, and CD105 surface markers, lack of expression of endothelial and blood cell markers CD45, CD34, CD14 or CD11b, CD79α or CD19, and human leukocyte antigen (HLA)-DR, and capacity to differentiate into osteoblasts, chondroblasts, and adipocytes *in vitro* (125). Low immunogenicity (the lack of HLA class II expression, low HLA class I and costimulatory molecules expression), immunomodulatory and regenerative properties, as well as unelaborate growth *in vitro* (126) make MSC an attractive therapeutic agent in the field of solid organ Tx (124, 127). Like stem cells, they are able to differentiate into specified cells or deliver organelles to injured cells and consequently restore the function of the damaged organ (128). However, the secretion of growth factors, cytokines, and extracellular vesicles (EV) containing lipids, proteins, mRNAs, miRNAs, non-coding RNAs, and sometimes genomic DNA as well as the modulation of the graft's microenvironment are probably even more crucial mechanisms of action (129–132). Specifically, MSCs may not directly replace damaged kidney epithelial cells, but rather MSCs secretome may promote kidney regeneration from residual mature kidney cells, which become capable, dedifferentiate, and replicate (133). Nevertheless, it has been revealed that not only paracrine activity but also direct cell-to-cell interactions between MSC and host cells play a crucial role in acquiring regenerative, anti-inflammatory, and pro-tolerogenic effects in the target organ (127).

Several clinical kidney Tx trials already showed that systemic recipient treatment with autologous or allogenic MSC modulates the immune response and allows reducing doses of immunosuppressive drugs (134–136).Those studies focused on systemic immune modulation but not on the reparative or regenerative capacities of MSC therapy. Despite promising results, including safety and feasibility, it has become clear that intravenous infusion of MSC might not be ideal for several reasons. Firstly, it has been observed that the majority of intravenously delivered MSC are trapped in the microcapillary system of the lungs and liver hence not reaching the target organ (19–21). Another major problem is the short life span of intravenously infused MSC, meaning that multiple infusions may be necessary (19, 21). On the other hand, intra-arterial delivery of MSC directly to the kidney graft is feasible, efficient, prolongs MSC survival and cell-to-cell contact *in situ*, as well as, off-target migration of infused cells is minimal (137, 138), supporting the idea that cell therapy could be successfully applied for organ preconditioning and repair *ex-vivo* prior to implantation.

TABLE 2 | Summary of kidney *ex-vivo* perfusion therapies.

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
Cell therapy					
Gregorini et al. (97)	5×10^6 MSC EV derived from 5×10^6 MSC	Rat kidneys; 20 min WI → 4 h HMP MSC $n = 5$; EV $n = 5$; No additives $n = 5$	HMP 4 °C; perfusate: UW (Belzer) solution	Assessment and sampling during HMP	• ↓ histological kidney damage score (MSC, EV); • ↑ gene pathways for molecular transport, respiratory electron transport and the citric acid cycle (MSC); • ↑ Idh2, Ndufs8, Pdhb (MSC, EV); ↑ Calb1, Slc16a1, Atp6v0d2 gene expression (EV); • ↓ LDH, lactate, MDA, glucose; ↑ pyruvate in perfusate (MSC, EV).
Brasile et al. (79)	1×10^8 MSC	Non-transplantable human kidneys; 29.4 ± 7.4 h SCS → 24 h SNMP $N = 5$ kidney pairs	SNMP 32°C; perfusate: exsanguinous metabolic support solution	Assessment and sampling during SNMP	• No MSC migration out of the vasculature into the kidney parenchyma; • ↑ ATP; • ↓ pro-inflammatory cytokines (eotaxin, G-CSF, IL-6, IP-10, MIP-1a, MIP-1B, RANTES, TNF-α, MCP-3, Flt-3L, GM-CSF, fractalkine, MDC, IL-1B); • ↑ growth factors (EGF, FGF-2, TGF-α); • ↑ restoration of cytoskeletal integrity (ZO-1 protein location in the cell); • ↑ DNA synthesis (PCNA); • ↑ mitosis
Pool et al. (98)	1×10^5 human A-MSC 1×10^6 human A-MSC 1×10^7 human A-MSC BM-MSC (fluorescent-labeled) Added after 1 h of NMP	Porcine kidneys; 30 min WI → 3.5–5 h SCS → 7 h NMP. $N = 3$ per group of different concentrations; Labeling and detection: $n = 10$	NMP 37°C; RBC-based perfusate with WME*	Assessment and sampling during NMP	• ND in macroscopic changes and haemodynamics; • MSC (when infused $\geq 1 \times 10^6$) identified only in the lumen of glomerular capillaries. MSC still intact after tissue engraftment; • Minority of glomeruli positive for fluorescent pre-labeled BM-MSC; • ↓ number of MSC in perfusate during NMP (with or without kidney)
Pool et al. (99)	1×10^7 human BM-MSC 1×10^7 human A-MSC Added after 1h of NMP	Porcine kidneys; 20 min WI → 2–3 h oxygenated HMP → 7 h NMP BM-MSC $n = 5$; A-MSC $n = 5$; No additives $n = 5$	NMP: 37°C; RBC-based perfusate*	Assessment and sampling during NMP.	• ↓ NAG in perfusate (A-MSC vs. control); • ↓ LDH (BM-MSC); • ↓ NGAL (BM-MSC and A-MSC); • ↑ HGF (BM-MSC and A-MSC); • ↑ Endothelin-1 (BM-MSC vs. A-MSC); • ↑ IL-6 and IL-8 (BM-MSC and A-MSC); • ND in histology scores, arterial flow rate, cumulative diuresis, kidney function
Lohman et al. (100)	1×10^6 porcine A-MSC 1×10^6 human A-MSC	Porcine kidneys; 75 min WI → 14 h oxygenated HMP → 2 h NMP Porcine A-MSC $n = 7$; Human A-MSC $n = 7$; Vehicle group $n = 7$; No additives $n = 7$	NMP*	AutoTx after contralateral nephrectomy, follow-up 14 days	• MSC retention in kidney cortex; • No effects on perfusion haemodynamics, no adverse effects after Tx; • ND in plasma crea, GFR, NGAL, kidney damage assessed by histology
Thompson et al. (101)	50×10^6 MAPC Added after 1 h of NMP	Non-transplantable human kidneys MAPC $n = 5$; Control (10 ml bolus of crystaloid) $n = 5$	NMP 36.5 °C; RBC-based perfusate*	Assessment and sampling during NMP	↑ UO; ↓ NGAL, ND in KIM-1 in urine; Restored RBF within the kidney medulla 4 h after MAPC cell infusion (US MicroFlow imaging and CEUS); ↓ IL-1β, ↑ IL-10; ↑ IDO activity (Kyn:Trp ratio); ND in oxygenation, biochemical parameters, IRR, RBF.

(Continued)

TABLE 2 | Continued

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
2. Gene therapy					
Yuzefovych et al. (102)	Lentiviral vectors encoding shRNA targeting β2-microglobulin (shβ2m) and the class II transactivator (shCIITA) (1.5×10^{11} particles)—silencing rat MHC I and MHC II gene expression	Rat kidneys; 10 min SCS → 2 h SNMP (gradual rewarming and increasing flow speed) Treatment group: (shβ2m and shCIITA-encoding vector) $n = 7$; Control group: (vector with non-sense shRNA) $n = 5$	SNMP 31–32 °C; perfusate: WME with 5% BSA*	Assessment and sampling during SNMP; AlloTx, follow-up 6 weeks	• ↓ β2m and CIITA levels; • ND in perfusate LDH levels, histology findings • ↓ IL-17 or IFN-γ, IL-12 and MCP-1 levels during entire perfusion time; ↓ IL-6 at the beginning, but ↑ at 2 h; ↑ IL-10, MIP-1a, MIP-2, IP-10, TNF-α, and EGF at 2 h • ND in perfusate RANTES levels
Yang et al. (103)	Naked caspase-3 siRNA. 3 mcg/ml into the renal artery before SCS and 0.15 μg/ml into autologous blood (used for perfusion)	Porcine kidneys; 10 min WI → 24 h SCS → 3 h NMP ("reperfusion") siRNA $n = 3$; no additives $n = 3$	NMP ("reperfusion") 38 °C, perfusate: heparinized autologous whole blood*	Assessment and sampling during NMP ("reperfusion")	• ↓ caspase-3 precursor and active subunit; ↓ active caspase-3 positive cells (40% reduction); • ↓ apoptotic cells were; • Marginally ↓ MPO positive cells; • Marginally ↑ RBF; • ↑ oxygen consumption; • Neutralized perfusate pH
Moser et al. (104)	100 μM doxycycline 50 nM MMP-2 inhibiting siRNA	Rat kidneys; 10 min WI → 60 min flushing with 4°C KPS-1 → 22 or 5 h HMP 4–7 rats per group: Treatment group 1 (22 h HMP, doxycycline); Treatment group 2 (22 h HMP, MMP-2 siRNA); Control group 1 (22 h HMP); Control group 2 (5 h HMP)	HMP; 4°C, perfusate: KPS-1	Assessment and sampling during HMP	• ↓ MMP-2 in the perfusate (siRNA); • ↓ LDH and cytochrome c oxidase in the perfusate (doxycycline); • ↓ protein release into the perfusate (doxycycline); • ↓ NGAL (doxycycline and MMP-2 siRNA); • Protection of mitochondrial membrane (doxycycline and MMP-2 siRNA)
3. Biological therapy					
Diuwe et al. (105)	Etanercept 1.5 mg into the perfusion fluid after the 1st h of HMP	Human kidneys; SCS → HMP. Treatment group $n = 47$; Control group $n = 47$	HMP 4°C; perfusate KPS-1	Tx, follow-up from 12 to 46 months	• ND in patient survival at 12 and 24 months, patient survival with functioning graft at 12 and 24 months; PNF, DGF, immediate function, acute rejection, serum crea levels on day 7 or at months 1, 3, 6, 12, and 24 after Tx; • 2.3-fold ↑ risk in the recipient's death, 2.6-fold ↑ risk in graft loss, 2.5-fold ↑ risk in occurrence of the composite endpoint (occurrence of any event)
Hameed et al. (106)	αCD47ab: 100 μg immediatly after organ flush at the time of retrieval → 200 μg αCD47Ab into the kidney arterial line, immediately prior NMP start	Porcine kidneys. 6 h SCS → 1 h NMP	NMP; 37°C; RBC-based perfusate*	Assessment and sampling during NMP	• Addition of αCD47Ab to the cold flush → no binding; • Infusion into the arterial line at the start of NMP → widespread binding along the glomerulus and kidney tubular epithelium (detectable at the end of NMP); • ↑ RBF, ↓ IRR, ND in oxygen consumption, UO, CrCl, FENa; • ND in expression of IL-6, TNF-α, IL-1β, and IL-18; • ↓ kidney tubular debris after NMP, ND in other histological parameters; • ↓ increase in oxidative stress during NMP, ND in cell death.

(Continued)

TABLE 2 | Continued

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
4. Nanotechnologies					
Brasile et al. (107)	NB-LVF4 100 μg/min 66 μg/g of kidney weight through arterial line	Canine kidneys. Treatment group 1 (only NMP) $n = 5$; Treatment group 2 (NMP and autoTx) $n = 4$; Treatment group 3 (NMP and alloTx) $n = 4$; Control group (NMP and alloTx) $n = 4$	SNMP 32 °C; acellular perfusate*	Assessment after NMP, autoTx, and alloTx after both-sides nephrectomy	• Coverage in 90% of the vascular luminal surface (small and large vessels); • No occlusion of vessels, stable perfusion pressures and vascular flow rates; • Normal serum chemistries, electrolyte profiles and hematology, stable crea levels after autoTx; • Delayed onset of rejection (day 30 vs. day 6 in control group)
Tietjen et al. (108)	antiCD31-nanoparticles (NP) 50 mg/ml	Non-transplantable human kidneys; NMP 8.5 h (3 last kidneys after initial experiments) Imaging group $n = 1$; Concentration determination group $n = 3$; Treatment group $n = 3$; Control group (control nanoparticles) $n = 1$	NMP 36 °C; RBC-based perfusate*	Assessment during NMP.	• CD31-NP accumulated 5 to 10-fold more than control-NP; • ↑ accumulation in glomeruli compared with surrounding interstitial microvasculature
DiRito et al. (109)	ICAM-2-NP with 10 μg/ml plasminogen + 100 μg/kg of kidney weight tPA	Non-transplantable human kidneys SCS➔ 1 h NMP ICAM-2-NP alone; ICAM-2-NP with fibrinolysis; Control-NP-alone; Control-NP with fibrinolysis	NMP 36 °C; RBC-based perfusate*	Assessment and sampling during NMP	• In the absence of tPA + plasminogen, ICAM-2-NP and Control-NP accumulated at identical levels at the site of vascular obstructions; • Together with fibrinolytic therapy ↓ retention of the non-targeted Control-NP and ∼3-fold ↑ retention of ICAM2-NPs in the glomeruli and ∼20-fold ↑ in the microvessels
5. Thrombolytics, fibrinolytics, anticoagulants					
Nghiem et al. (110)	tPA 200 mg	Human kidneys with 50% thrombosed glomeruli detected by wedge biopsy. SCS ∼ 35.5 h ➔ HMP 7–24 h; $n = 14$	HMP ∼4.1°C, perfusate: HTK solution (Custodiol)	Assessment and sampling during HMP (wedge biopsies before and after HMP), Tx	• ↓ in thrombosed glomeruli from 50 to 23%; • Perfusion parameters (IRR, flow rate) improved during 7–24 h of HMP
Woodside et al. (111)	tPA 50 mg	Human DCD kidneys; SCS ➔ HMP ≥1 h; tPA $n = 19$; no additives $n = 19$; *24 kidneys implanted to recipients (12 in each group)	HMP 4–6°C; perfusate: IGL Pulsatile Perfusion Solution*	Assessment during HMP, implantation	• ND in RBF and IRR during HMP; • Eradicated microthrombi (only 1 tPA group kidney had significant microtrombi after HMP); • ND in DGF, patient survival, death-censored graft survival, crea, GFR, kidney discard rates; • 1 patient in tPA group required reoperation for bleeding from unknown source
DiRito et al. (109)	10 μg/ml plasminogen + 100 μg/kg of kidney weight tPA	Non-transplantable human kidneys; SCS➔ 1 h NMP Plasminogen + tPA $n = 9$; Only tPA $n = 3$; Only plasminogen $n = 3$	NMP 36 °C; RBC-based perfusate*	Assessment and sampling during NMP	• completely cleared microvascular obstructions in all tPA +plasminogen kidneys, remaining obstructions in tPA only group, only minor reduction of obstructions in plasminogen goup; • ↑ vascular resistance in the tPA only, plasminogen only groups at ∼30–60 min; stable resistance in tPA + plasminogen group • ↑ urine production, ↓ NGAL, IL-6, ICAM-1 in tPA+ plasminogen group

(Continued)

TABLE 2 | Continued

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
Sedigh et al. (112)	Corline heparin conjugate (CHC) 50 mg	Porcine kidneys (DBD model); HMP 20 h Treatment group $n = 5$; Control group (unfractionated heparin) $n = 5$	HMP; 5.0 ± 0.47°C; perfusate: KPS-1	Assessment and sampling during HMP	• CHC successfully binds to the ischemic vessel walls of kidney arteries and tissue; • ND in vascular resistance, histological changes (histological changes were minimal in all samples)
Sedigh et al. (113)	CHC 50 mg	Porcine kidneys (DBD model); HMP 20 h. Treatment group $n = 6$; Control group $n = 6$	HMP; 1–4°C, perfusate KPS-1	Assessment during HMP; *Ex-vivo* normothermic reperfusion for 3 h	• CHC distributed in the glomerular vasculature; • ↑ rate of crea decline, ↑ UO, ↓ serum lactate levels; • ND in HMP parameters, ↓ IRR and ↓ MAP (needed to reach the same RBF) during NMP; • ↓ urinary NGAL levels; • ↓ tubular injury, ND in glomerular and vascular morphology • ND in hemostatic dynamics
Hamaoui et al. (114)	Thrombalexin. 2.1/4.2 μM	Porcine kidneys; 15 min WI ➔ 5 h SCS ➔ 4 h stabilization HMP ➔ 1.5 h HMP treatment Treatment group $n = 19$; Control group (unmodified perfusate or anticoagulantly inert "tail only" proteins) $n = 19$ Non-transplantable human kidneys: 78h SCS ➔ 4h HMP; $n = 2$	HMP; perfusate: UW solution	Normothermic hemoreperfusion (NHRP) for 6 h	• Thrombalexin binding and adherence to kidney microvasculature after pre-treatment, continued adherence after 6 h of NHRP; • ↑ RBF and perfusion flow index during NHRP; • ND in functional capillary density; 44% larger D capillaries, 50% faster RBC velocity, 3.5× greater capillary blood flows and perfusion indices; • Marginally ↓ lactate and d-dimers during NHRP; • ND in histology, fibrin(ogen) deposition in interstitial tissues and peritubular capillaries; • Human kidneys: ↑ RBF and perfusion indices, ↓ lactate, d-dimer, fibrinogen levels
Gases					
6. Hosgood et al. (115)	Nitric oxid donor sodium nitroprusside (SNP); 25 mg/250 ml 5% glucose administered for 5 min before and during the first hour of NMP at 25 ml/h; Water soluble carbon monoxide releasing molecule (CORM-3); 0.0146 g/60 ml saline infused into the arterial arm for 5 min before and during the first hour of NMP at 50 ml/h	Porcine kidneys; 10 min WI ➔ 16 h SCS ➔ 2 h NMP SNP $n = 6$; CORM-3 $n = 6$; iCORM-3 (inactive) $n = 6$; Control (no intervention) $n = 6$	NMP 39°C	Assessment during NMP; *Ex-vivo* reperfusion with non-leucocyte-depleted autologous blood	During NMP: • ↑ RBF, ↑ IRR fall (SNP and CORM-3 vs. controls); During reperfusion: • ↑ RBF (CORM-3 vs. control), ↓ IRR (SNP vs. controls); • ↑ crea fall (SNP and CORM-3 vs. iCORM-3); • ↓ UO (SNP and iCORM-3 vs. CORM-3 and control); • ↓ oxygen consumption at 3 h (SNP and control vs. CORM-3); • ↑ tubular dilatation and vacuolation, number of condensed tubular nuclei (SNP vs. CORM-3)
Smith et al. (116)	Argon (70% Ar, 25% O_2, 5% CO_2)	Porcine kidneys; 15 min WI ➔ 17 h SCS ➔ 1 h NMP ➔ 30 min SCS Treatment group $n = 6$; Nitrogen control (70% N2, 25% O_2, 5% CO_2) $n = 6$; Oxygen control (95% O_2, 5% CO_2,) $n = 6$	NMP 38°C; perfusate: leukocyte depleted blood	Assessment and sampling during NMP; 3 h warm *ex-vivo* reperfusion with autologous blood	• Numerically ↑ RBF during NMP in argon and nitrogen groups; • ND in oxygen consumption, CrCl, total UO; • ND in urinary cytokines (IL-6, IL-8, TNF-α), urinary HIF-1α; • ND in HIF-1α tubular cytoplasmic and nuclear staining; • ND in morphology

(Continued)

TABLE 2 | Continued

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
Bhattacharjee et al. (117)	CORM-401 200 μM (in Plasmalyte solution) after HMP delivered through pulsatile action at 37 °C, over the period of 20 min	Porcine kidneys; 60 min WI → 4 h HMP → 10 h NMP Treatment group: $n = 5$; Control group (iCORM-401): $n = 5$	HMP 4 °C; perfusate: UW solution; NMP 37 °C; perfusate: isogeneic oxygenated blood	Assessment and sampling during NMP (reperfusion)	• ↓ ATN score, intrarenal hemorrhage, apoptosis; • ↓ KIM-1 and NGAL in urine; • ↑ RBF, total UO; • ↓ urinary protein, ↑ CrCl; • ↓ gene expression of TLR2 and 6, MyD88, NF-κB and HMGB1
Juriasingani et al. (90)	20 nM AP39 (H_2S donor)	Porcine kidneys; 30 min WI → 4 h SNMP Treatment group (SNTAP) $n = 7$; Control group 1 (SNMP without treatment) $n = 6$; Control group 2 (SCS) $n = 7$	SNMP 31°C; perfusate: UW solution and non-stressed autologous blood	Assessment during SNMP; 4 h *ex-vivo* reperfusion at 37°C with stressed autologous blood	• ↑ UO during preservation and reperfusion; • ND in ATN score; ↓ apoptosis; • 214 genes differentially expressed in SNTAP vs. SCS groups (↓ pro-apoptotic (BCL10), heat shock response (HSPD1 and HSPA1A) genes, regulators of those pathways (BAG3, DDIT3); ↑ proliferation (MAPK7) and oxidative stress response (NRROS) genes; • 614 genes differentially expressed in the SNTAP group vs. SNMP group, including ↓ genes associated with the HIF-1α-mediated hypoxia response pathway (EGR1, PCK1, PDK3, RGCC); ↓ proinflammatory (IL6, HMGB2) and pro-cell death (HOXD8, HOXD10) genes; ↑ genes mediating TGF-β pathway (SMAD3 and NRROS) and HIF-1α degradation (AJUBA); ↑ proliferation (MAPK7) and oxidative stress response (NRROS) genes
7. Other pharmacological agents					
Yang et al. (118)	Erythropoietin (EPO) 5,000 U/l	Porcine kidneys; 10 min WI → 16 h SCS → 2 h NMP. Treatment group $n = 3$; Control group (no additives) $n = 3$	NMP 38°C; perfusate heparinized leucocyte-depleted autologous blood*	Assessment and sampling during NMP	• ↓ apoptotic cells in tubular areas; • ↓ macrophages (ML1P+); ↑ neutrophils (MPO+) in tubular lumens, but ↓ in interstitial areas; • ↑ apoptosis of neutrophils; • ↑ caspase-3 activity, expression of 17 kD active caspase-3; • ↓ IL-1β active protein, ↑ its precursor; • ↓ tubular dilation and cytoplasmic vacuolisation in tubular epithelium; • ↑ UOt; • ↓ WBC in hemoperfusate, ND in Hgb level; • ND in RBF, oxygen consumption
Yang et al. (119)	Cyclic helix B peptide (CHBP) 10.56 nmol/l in flushing solution and perfusate	Porcine kidneys; 20 min WI → 18 h SCS → 3 h NMP (reperfusion) Treatment group $n = 5$; Control group (no additives) $n = 5$	NMP 37°C, perfusate: whole heparinized autologous blood*	Assessment and sampling during NMP (reperfusion)	• ↑ RBF, oxygen consumption, UO at 2 h; • ↓ serum potassium; • ND in serum crea level, CrCl, serum pH, ALT, AST, LDH; • ↓ tubular epithelial vacuolation, tubular dilatation, interstitial expansion; • ↓ apoptotic cells in tubular areas, but ↑ in interstitium and tubular lumens; • ↓ MPO positive cells, expression of caspase-3; • ND in HSP70 expression

(Continued)

TABLE 2 | Continued

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
Huijink et al. (120)	Rats: Metformin 300 mg/kg for donor 12 and 2 h before kidney retrieval; 30 or 300 mg/l added to NMP perfusate Pigs: Metformin 4 mg/l to HMP perfusate and 20 mg/ml increasing dose to NMP perfusate	Rat kidneys; 15 min WI → 24 h SCS → 90 min NMP Group 1: no metformin $n = 5$; Group 2: metformin for donor $n = 5$; Group 3: 30 mg/l metformin in NMP; Group 4: metformin for donor and 30 mg/l in NMP; Group 5: 300 mg/l metformin in NMP; Group 6: metformin for donor and 300 mg/l in NMP; Porcine kidneys; 30 min WI → 3 h HMP → 4 h NMP. Group 1: no metformin $n = 6$; Group 2: metformin in HMP and NMP $n = 7$	Rats: NMP 37°C, perfusate: WME; Pigs: HMP°C, perfusate: UW Machine Perfusion Solution; NMP 37°C, perfusate: leucocyte depleted, autologous, blood	Assessment and sampling during perfusion	• ND in perfusion parameters, CrCl (rats, pigs); • ↑ total UO in metformin-preconditioned rat kidneys; ↓ total UO in metformin-perfused rat and porcine kidneys; • ↓ protein excretion in metformin-preconditioned rat kidneys vs. control; ND between other groups; • ↓ LDH in metformin-preconditioned and/or metformin-perfused (30 mg/l) rat kidneys. ND in porcine kidneys; • ↓ tubular necrosis, proximal tubular cell vacuolation (rats); ND in porcine kidneys histology • ↓ EDN-1 (rats, 300 mg/l); ↓ eNOS (rats, 30 mg/l), ↓ VCAM-1 (rats, 30 mg/l), ↓ IL-6 (rats, 30 mg/l) • ↑HSP70 (pigs)
Moser et al. (121)	100 μM doxycycline	Rat kidneys; 10 min WI → 60 min flushing with 4°C KPS-1 → 22 h or 5 h HMP Treatment group (22 h HMP) $n = 4$; Control group 1 (22 h HMP) $n = 4$; Control group 2 (5 h HMP) $n = 4$	HMP 4 °C, perfusate: 20 ml KPS-1	Assessment and sampling during HMP	• ↓ LDH, NGAL and total protein increase at 22 h HMP; • ↓ cells separation and extracellular space enlarging, caused by HMP; • ↓ mitochondria damage and formation of dense bodies; • ↑ levels of triosephosphate isomerase (TPI), phosphoglycerate mutase (PGM), dihydropteridine reductase-2, pyridine nucleotide-disulfide oxidoreductase, phosphotriesterase- related protein, aminoacylase-1A,N(G),N(G) dimethylarginine dimethylaminohydrolase, and phosphoglycerate kinase 1
Nakladal et al. (122).	50 μM SUL-121, SUL-150 [*(R)*-enantiomer of SUL-121] and SUL-151 [*(S)*-enantiomer of SUL-121]	Porcine kidneys; 24 h SCS → NMP	NMP 37°C; perfusate: oxygenated medium (RPMI 1640)	Assessment during NMP	• ↓ intrarenal pressure (profound effect with SUL-121 and SUL-150, only a minor effect with SUL-151); • 35% ↑ in RBF from baseline with fixed pressure (80 mmHg) (SUL-150)
Snoeijs et al. (123)	Water-soluble (cyclodextrin-complexed) propofol: 40 μmol/100 mg kidney weight during flush-out and 32 μmol/100 mg kidney weight at 1 h after the start and 1 h before the end of HMP	Porcine kidneys; 45 min WI → 22 h HMP; Treatment $n = 6$; Control $n = 6$	HMP 4 °C; perfusate: UW solution	AutoTx, 10 days follow-up	• ↓ increase in MDA conc. after reperfusion; • ↓ increase in IRR at the beginning of reperfusion; • ↓ crea concentration after 10 days; • ND in histological changes; • ND the extent of neutrophil infiltration and peritubular capillary inflammation; • No adverse events

(Continued)

TABLE 2 | Continued

References	Treatment, dose	Model	Perfusion parameters	Assessment of outcomes	Main findings
8. Hemadsorbtion					
Hosgood et al. (64)	Cytosorb hemadsorption	Porcine kidneys; 22 h SCS → 6 h NMP; Treatment group $n = 5$; Control group $n = 5$	NMP 37.4 °C; whole blood-based perfusate*	Assessment and sampling during NMP	• ↓ RBF at 30 min; ↑ mean RBF, oxygen consumption at 1 h (sign.) and 3 h (numerically) but not at 6 h • ND in UO, CrCl, FENa; • ↓ urinary NGAL • ↑ IL-1β, IL-1α, IL-1RA, TNFα, IL-10 in the control group throughout perfusion, whereas levels remained low in the treatment group; • ↓ IL-6 at 3 and 6 h. Numerically ↓ IL-8 at 6 h; ↓ CRP at 1, 3, and 6 h • ↓ thromboxane B2, PGE, prostacyclin
Ferdinand et al. (65)	Cytosorb hemadsorption	Non-transplantable human kidneys; 4 h NMP Treatment group $n = 5$; Control group $n = 5$	NMP 37.4°C; whole blood-based perfusate*	Assessment and sampling during NMP. Indirect comparison with human kidneys transplanted after 1 h of NMP	• ND in RBF, UO, oxygen consumption, acid-base homeostasis during NMP; • 46 genes sign. ↑ and 181 ↓ in the treatment group after 4 h of NMP; • ↓ transcriptional response included NLRP3 inflammasome activation-associated genes and neutrophil recruiting chemokines; • ↓ "TNFα signaling *via* NFκB" pathway, ↑ OXPHOS and fatty acid metabolism pathways (this gene signature was associated with lower incidence of prolonged DGF in transplanted kidneys)

↑ increase; ↓ decrease; ND, no difference; MSC, mesenchymal stromal cells (A-adipose-derived, BM-bone marrow-derived); EV, extracellular vesicles; WI, warm ischemia; HMP, hypothermic machine perfusion; SNMP, subnormothermic perfusion; NMP, normothermic perfusion; UW, University of Wisconsin; ATP, adenosine triphosphate; crea, creatinine; G-CSF, granulocyte colony stimulating factor; IL, interleukin; IP-10, interferon gamma-induced protein-10; MIP, macrophage inflammatory protein; RANTES, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted; TNF, tumor necrosis factor; MCP, monocyte chemotactic protein; Flt-3L, Fms Related Receptor Tyrosine Kinase 3 Ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; MDC, macrophage-derived chemokine; EGF, epidermal growth factor; FGF, fibroblast growth factor; TGF, tumor growth factor; ZO, zonula occludens; DNA, deoxyribonucleic acid; PCNA, proliferating cell nuclear antigen; WME, William's E Medium; RBC, red blood cell; NAG, N-acetyl-β-d-glycosaminidase; LDH, lactate dehydrogenase; NGAL, neutrophil gelatinase-associated lipocalin; HGF, hepatocyte growth factor; Tx, transplantation; GFR, glomerular filtration rate; MAPC, multipotent adult progenitor cells; KIM, kidney injury molecule; RBF, renal blood flow; US, ultrasound; CEUS, contrast-enhanced ultrasound; IDO, indoleamine 2.3-dioxygenase; kyn, kynurenine; trp, tryptophan; (sh)RNA, (short hairpin) ribonuclear acid; MHC, major histocompatibility complex; SCS, static cold storage; BSA, bovine serum albumin; IFN, interferon; siRNA, small interfering ribonucleic acid; MPO, myeloperoxidase; MMP, matrix metalloproteinase; KPS, kidney perfusion solution; PNF, primary non-function; DGF, delayed graft function; CD, cluster of differentiation; ab, antibody; IRR, intrarenal resistance; UO, urine output; CrCl, creatinine clearance; FENa, fractional excretion of sodium; NB-LVF4, nano-barrier membrane; ICAM, intracellular adhesion molecule; tPA, tissue plasminogen activator; DCD, donated after circulatory death; DBD, donated after brain death; MAP, mean arterial pressure; HTK, histidine tryptophan ketoglutarate; AST, aspartate aminotransferase; HIF, hypoxia inducible factor; ATN, acute tubular necrosis; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; HMGB, high mobility group; WBC, white blood cells; Hgb, hemoglobine; ALT, alanine aminotransferase; HSP, heat shock protein; EDN, eosinophil-derived neurotoxin; eNOS, endothelial nitric oxide synthase; VCAM, vascular cell adhesion molecule; CRP, C reactive protein; PGE, prostaglandin E; OXPHPOS, oxidative phosphorylation.

**Perfusate includes more components, such as heparin, nutrition agents, antibiotics, vasodilators, etc.*

Five experimental studies in which MSCs were delivered into the kidney *via ex-vivo* machine perfusion have been published (79, 97–100). Gregorini et al. perfused rat kidneys, harvested after 20 min of warm ischemia, for 4 h with hypothermic UW solution containing MSC or extracellular vesicles derived from MSC. Both treatments significantly reduced histological lesions such as bleb formation, tubular necrosis, tubular lumen obstruction, and overall diminished global kidney damage score after HMP. Moreover, gene sets and individual genes responsible for molecular transport, citric acid cycle, respiratory electron transport, and antioxidant activity were significantly up-regulated. Correspondingly, perfusate biochemical analysis showed significantly lower levels of ischemia markers lactate and lactate dehydrogenase (LDH), oxidative stress marker malondialdehyde (MDA), increased pyruvate, and decreased glucose levels, demonstrating more active kidney metabolism during HMP. Importantly the positive effect was even more considerable in the EV group, compared to MSC-perfused kidneys. It suggests that prompt and direct delivery of free soluble EV mediators could be beneficial during short-term *ex-vivo* perfusion (97). Brasile et al. used 24 h NMP (so-called exsanguinous metabolic support) to deliver MSC to non-transplantable human kidneys focussing on the regenerative potentiality of this therapy. Infusion of 10^8 MSC (higher doses led to higher MAP, lower vascular flow, and diminished oxygen consumption) resulted in significantly increased ATP concentration in both kidney cortex and medulla, reduced synthesis of pro-inflammatory cytokines, and increased synthesis of growth factors. Moreover, up-regulated DNA synthesis and increased incidence of mitosis associated with MSC therapy

were observed. Interestingly, 24 h NMP alone normalized the cytoskeletal integrity of injured kidneys, and MSC therapy further improved this restoration by 4.81%. During 24 h of perfusion, MSC remained in the vascular compartment of the kidney and did not migrate to parenchyma, as proved by histological evaluation and perfusate investigation (79). Similarly, in the study by Pool et al. human MSC stayed in glomerular capillaries of porcine DCD kidneys and remained undamaged throughout 7 h of NMP. Nevertheless, MSC accumulated only in the minority of glomeruli rather than distributed uniformly, even in well-perfused kidneys. Moreover, in this study, a gradual decrease of MSC counts in the perfusate was observed in experiments with kidney as well as without kidney connected to the perfusion machine. That suggests that MSC might be susceptible to perfusion conditions, such as flow and pressure, suggesting to be a limitation of long-term machine perfusion (98). In contrast, Brasile et al. could recover more than 95% of initially infused MSC from the perfusate at the end of 24 h perfusion (79). However, it should be taken into account that Pool et al. used human-derived MSC in porcine kidneys, so there could be a possibility of a xeno-effect, even for leukocyte-depleted perfusate lacking some immune components (98). In a later study the same group demonstrated that porcine DCD kidneys, 7 h perfused with MSC in 37°C, presented with lower levels of kidney injury markers (NGAL and LDH) and increased levels of hepatocyte growth factor (HGF), as well as immunomodulatory cytokines IL-6 and IL-8 in the perfusate. No differences in kidney function or diuresis were observed, which could also be explained by the relatively short perfusion time. Importantly, no apparent differences between adipose and bone marrow-derived MSC were found (99), even though several studies suggested that MSC of different sources may differ in activity and treatment effects (139, 140). The main drawback of the before-mentioned experiments is the lack of data from kidney assessment after reperfusion *in vivo*. Recently, Lohman et al. published their results of porcine DCD kidney auto-Tx after 14 h oxygenated HMP and subsequent 4 h NMP with human or porcine adipose-derived MSC. After 14 days of follow-up, no beneficial effects on kidney function or kidney injury markers were observed. Nevertheless, the treatment neither negatively affected perfusion parameters nor caused adverse events after auto-Tx, thus encouraging further machine perfusion studies with subsequent Tx *in vivo* to investigate MSC impact on ischemia-reperfusion injury, organ regeneration capacity, and immune modulation (100).

Another type of cells, potentially useful for kidney treatment during *ex-vivo* machine perfusion, are multipotent adult progenitor cells (MAPC). Genetically MAPCs are similar to MSCs, reside in the bone marrow, and even have comparable function and mechanism of action. Particular growth and expansion characteristics lead to phenotypically different features of those two cells' populations (141). MAPC immunomodulatory capacity has already been demonstrated in a couple of Tx studies. Treatment with allogenic MAPC in a rat heterotopic heart transplant model allowed to withdraw pharmacological immunosuppressive therapy and successfully achieve long-term survival (142). MAPCs have also been successfully used in a human liver Tx case, which resulted in a pro-tolerogenic profile of the recipient's leucocytes and reduced immunogenicity (143). Thompson et al., in their very recent study, used *ex-vivo* NMP to deliver MAPC to non-transplantable human kidneys. MAPC treatment resulted in higher UO, lower NGAL concentration in perfusate, but not other kidney injury biomarkers kidney injury molecule-1 (KIM-1) and flavine mononucleotide (FMN). MAPC was also associated with the changes in cytokine profile—decreased IL-1β and increased IL-10 levels, as well as up-regulated indoleamine-2,3-dioxygenase activity, which is known for its role in pro-tolerant mechanisms and suppression of inflammatory processes (101).

Although the results of machine perfusion cell-therapy studies are promising, several questions remain to be answered. The fate of *ex-vivo* delivered MSC or MAPC is still not determined. It is known that their lifespan in the target organ is limited; however, there is evidence that once immunomodulatory processes have been promoted, these beneficial effects are maintained even after inactivation or death of MSC (144, 145). Therefore, it is necessary to investigate if supportive cell therapy would be beneficial after implantation and at which time points it would be the most efficient. Additionally, it is crucial to thoroughly check into possible immunogenicity of allogenic MSC and MAPC in the machine perfusion setting, as extraction and preparation of autologous cells in case of Tx is usually logistically demanding (146). The perfect duration and conditions of machine perfusion also need to be determined. As discussed previously, several hours of *ex-vivo* cell therapy could be sufficient to promote immunomodulatory and anti-inflammatory processes; however, apparently a much longer time is needed for kidney regeneration and repair (79, 99, 101). Recent study revealed that machine perfusion conditions also affect MSC viability, metabolism, and function, which may not allow reaching the maximum effect of cell therapy (147). Therefore, further work on machine perfusion prolongation, *ex-vivo* organ viability maintenance, and optimal conditions for therapeutic cells is needed.

Gene Therapy

Ex-vivo machine perfusion is a promising platform for organ-specific gene therapy or even genetic engineering. One of the emerging approaches is posttranscriptional gene silencing with small interfering double-stranded RNA (siRNA), which induces degradation of homologous mRNA transcripts and blocks the desired gene expression (148, 149). They can be transported by viral vectors or just injected as a synthetic "naked" form. The main advantage of siRNA, as a tool for gene therapy, is its simple delivery—they are small and do not need to cross the nucleus membrane to become active. siRNA, silencing the expression of RelB, Caspase 3, IKKb, Fas or complement genes, delivery by simple intravenous, or hydrodynamic injection or *via* renal artery, has been already successfully applied in multiple rodent ischemia-reperfusion models (148–152).

However, even though gene therapy techniques, including siRNA, have been developing for the past 20 years, they remain in the experimental stage, not proceeding to clinics. The main

problem is low efficiency and insufficient organ specificity of non-viral or viral gene therapy *in vivo* (153). Short lasting effects due to rapid degradation and excretion of the agent, delivered *via* the systemic route, is challenging too. Therefore, *ex-vivo* machine perfusion of the graft could help overcome those drawbacks by specific and efficient application of the gene therapy hence avoiding off-target effects (154).

Yang et al. published the first *ex-vivo* kidney perfusion study with siRNA—naked synthetic caspase-3 siRNA was infused directly into the renal arteries of ischemic porcine kidneys prior to 24 h of SCS followed by addition to autologous blood perfusate used for 3 h NMP. As expected, this treatment significantly reduced caspase-3 precursor and active subunit expression in perfused kidneys. Moreover, treated grafts had 40% lower number of apoptotic cells, shown by histology and immunohistochemistry (IHC). The caspase-3 siRNA group also demonstrated marginally improved RBF, significantly increased oxygen consumption, and improved perfusate pH regulation at 3 h of perfusion. However, no difference in CrCl and UO was observed (103). In another study, siRNA inhibiting matrix metalloproteinase-2 (MMP-2) gene expression was added to HMP perfusate. Ischemic rat kidneys were perfused for 22 h, which diminished MMP-2 and NGAL levels in perfusate to a level similar to that observed at 5 h of perfusion without treatment. Moreover, protection of mitochondrial membranes was observed (104). It suggests that gene therapy could help prolong organ preservation time until implantation and protect from additional injury. Recently, Yuzefovych et al. (102) adopted a similar technique to reduce the immunogenicity of the rat kidney allografts by silencing rat MHC I and MHC II expression. Rat kidneys underwent 2 h of SNMP with short hairpin RNA (shRNA), designed to target rat β2-microglobulin (β2m) and rat class II transactivator genes (CIITA) and carried by a lentiviral vector. Kidneys were implanted, and recipients were followed up for 6 weeks. As a result, transcript levels of β2m and CIITA were reduced by 71 and 70%, respectively. As the vector contained the sequence for nanoluciferase, the bioluminescence activity in plasma and urine was detectable during the whole 6 weeks after Tx, confirming a stable transferred gene expression. Moreover, a cytokine shift toward a pro-tolerogenic milieu was detected in perfusate during perfusion [increased secretion of IL-10, macrophage inflammatory protein (MIP)-1a, MIP-2, interferon γ-induced protein (IP)-10, epidermal growth factor (EGF), and decreased IL-12, IL-17, monocyte chemoattractant protein (MCP-1), interferon γ (IFN-γ)]. Importantly, no vector-related damage of the kidney allograft, as confirmed by LDH levels and histological investigation became evident. Even more, as no off-target distribution of the vector was observed, this diminishes the burden of risks associated with a lentiviral vector, such as tumorigenesis and other systemic side effects (102, 155).

Undoubtedly, these results are a step forward toward the goal to make a kidney graft immunologically invisible and reduce or even eliminate the need for systemic immune suppression for the recipient. Nevertheless, the long-term immunological state of the recipient, the incidence of acute and chronic rejection, the need for pharmacological immune suppression, graft function, and other clinical questions were not investigated in this study and remain to be answered.

Biological Therapy

The idea to use *ex-vivo* kidney machine perfusion to modulate the biological response of the graft before Tx by administering biological agents into perfusion solution has recently emerged. Inhibition of pro-inflammatory molecules, or their precursors' secretion and action by using specific monoclonal antibodies, blocking target sites, or even changing gene expression at the organ level before implantation gives a rationale that IRI, immunogenicity, and, therefore, need for systemic therapies could be reduced (105, 106). In a clinical study by Diuwe et al. (105) the tumor necrosis factor (TNF)-α inhibitor Etanercept was added to HMP of human kidneys, which were subsequently implanted. Although no negative impact on perfusion parameters was observed, no difference in patient survival at 12 and 24 months, PNF, DGF, an immediate graft function, acute rejection, or serum creatinine levels could be found. Unexpectedly, the proportional hazard Cox model showed that etanercept caused a 2.3-fold increase in the risk of recipient's death and a 2.6-fold increased risk of graft loss. It should be taken into account that all recipients still received a standard immunosuppression regimen of three drugs: corticosteroid, tacrolimus, and mycophenolic acid ester or sodium salt, therefore this study was not able to determine if the need or at least dosage of systemic immune suppression could be reduced by local biological therapy during machine perfusion. Moreover, it is not clear if, in the hypothermic environment, the highest effectiveness of the TNF-α inhibitor could be reached. It is more likely that in higher temperatures, the bioavailability and activity of the drug may be different. Therefore, adoption of (S)NMP into clinical practice would allow more efficient testing of drugs and determining optimal conditions for *ex*-vivo application of biological agents (105). Recently, an experimental study using αCD47Ab, an inhibitor of thrombospondin mediated IRI signaling, was conducted. One dose of αCD47Ab was infused *via* the renal artery immediately following cold perfusion of porcine kidneys at the time of retrieval, while another dose was added to NMP *via* the arterial line. Interestingly, the addition of αCD47Ab into the cold solution did not result in antibody binding to the kidney structures, whereas after 1 h of NMP containing the agent, αCD47Ab was detectable widely spread along the glomerulus and kidney tubular epithelium. Moreover, increased RBF and lower intrarenal resistance (IRR) during NMP could be observed in treated kidneys. However, oxygen consumption, UO, CrCl, and fractional sodium excretion (FENa) did not differ from control organs. Histologically, a significant increase in tubular dilatation and vacuolation in 1 h of NMP was detected in all kidneys whereas αCD47Ab-treated organs had reduced kidney tubular debris. NMP-induced oxidative stress was reduced in treated kidneys but the extent of cell death remained similar in comparison to control kidneys. Although no reperfusion was performed and the beneficial effects of this treatment on IRI remain questionable, the finding of successful delivery and binding of the antibody to graft structures during only 1 h of NMP encourages further investigation of

machine perfusion as a platform for organ-targeted biological therapy (106).

Nanotechnologies

Ex-vivo machine perfusion is an attractive platform to apply novel nanotechnology in the solid organ Tx field. The main limitation of its use *in vivo* is some difficulties with systemic administration. NPs are not able to escape from the bloodstream and reach extravascular targets (156, 157). Moreover, they tend to be trapped in liver and spleen phagocytes (158) or be absorbed by serum proteins and form "protein corona "which also disturbs specific targeting (159). Therefore, delivery of NPs directly to the graft in leukocyte-depleted, serum-free perfusate is promising.

So far, endothelial cells of kidney graft vasculature have been chosen as the main target for nanomedicine. Firstly, because the endothelium is the primary point, where ischemia-reperfusion or immune response-caused graft injury starts and secondly because it is directly accessible to the perfusate (107, 108). Brasile et al. used SNMP of 32 °C as a platform to deliver a receptor-mediated bioengineered nano-barrier membrane (NB-LVF4), made of laminin, vitrogen, fibronectin, and type IV collagen, to canine kidney grafts, to "immunocloak" the vasculature and reduce the antigenicity of the endothelium. The main idea was to provide a physical nano-barrier between recipient's immune cells and graft endothelium without interfering with the transport of nutrients and oxygen and thus making the vascular surface non-immunogenic and non-thrombogenic. Three hours of perfusion with the agent allowed a coverage of 90% of small and large kidney vessels without any occlusion or disturbances in perfusion parameters. Autotransplanted kidneys revealed a good function, proving that NB-LVF4 local treatment *ex-vivo* did not additionally damage the kidney. AlloTx experiments demonstrated a significant delay in the onset of rejection in treated kidneys in the absence of any systemic immunosuppression (107).

NPs are also attractive due to their ability to release the specific agents gradually. It has been found that pharmacological agents encapsulated within NPs can be incorporated into the endothelium, thereafter slowly releasing drugs by hydrolysis (160). This slow-release is especially interesting in handling the host's immune response against allografts, as it only evolves within several weeks after implantation. The work by Tietjen et al. focused on the improvement of NPs targeting kidney graft endothelium during NMP. Anti-human CD31 antibodies conjugated NPs were used to facilitate the binding and internalization of NPs by endothelial cells by delivery to non-transplantable human kidney grafts *via* NMP for 8.5 h. CD31-conjugated NPs accumulation was 5 to 10-fold higher than for non-conjugated NPs with no extravascular accumulation being observed. However, this increase was much less pronounced than observed *in vitro* where CD31-NPs accumulation was 80-fold higher than for control-NPs. Interestingly, non-specific NPs accumulated within the interstitial microvessels, where RBC-enriched vascular plugs were found. The extent of non-specific binding accordingly correlated to worse kidney perfusion. Nevertheless, despite the limitation of specific targeting of NPs in poor-perfused kidneys, the feature of non-specific accumulation of NPs could serve as a diagnostic/prognostic tool indicating vascular obstruction (108). The same group recently pre-treated non-transplantable human kidneys with tissue plasminogen activator (tPA) and plasminogen *via* NMP aiming ameliorate cold storage-caused microvascular obstructions to be able to deliver intercellular adhesion molecule (ICAM)-2-NPs. They observed a 3-fold increased retention of ICAM-2-targeted NPs in the glomeruli as well as ∼20-fold increase in the microvessels meaning that more effective delivery of NPs and graft modification could be achieved by application of thrombolytics as an essential first step during NMP (109).

Thrombolytics, Fibrinolytics, Anticoagulants

Formation of microthrombi is one of the most common problems in DCD kidneys or grafts retrieved from donors with disseminated intravascular coagulation, which often accompanies head injuries or multiorgan failure. Microvascular thrombosis can lead to poor graft perfusion and subsequent DGF or even PNF. Therefore, the discard rate of such kidneys remains high. Moreover, interactions among endothelial damage, inflammation, and activation of the coagulation system are well-known resulting in microthrombi, subsequent microcirculatory failure, and the "no-reflow" phenomenon as usual manifestations of IRI and acute antibody-mediated rejection (161, 162). As conventional systemic anticoagulation after kidney graft implantation does not eradicate pre-existing thrombi, does not guarantee the prevention of thrombosis, and carries its risk of bleeding (163, 164), the idea of anticoagulants or thrombolytics introduction directly to the graft has gained interest (165, 166). Several attempts of local thrombolysis or kidney pre-treatment with anticoagulants using *ex-vivo* machine perfusion have been published. Nghiem et al., used 14 human kidneys with biopsy-proven 50% thrombosed glomeruli that underwent 12–16 h of HMP with 200 mg tPA and were subsequently implanted. Reduction in glomerular thrombosis from 50 to 23% was observed at the end of perfusion, and 10 out of 14 recipients had immediate graft function after implantation (110). In the later RCT, after HMP with tPA, microthrombi were eradicated in all but one DCD kidney. No significant difference in kidney function, recipient survival, and death-censored graft survival could be observed which could also be due to the small study population (111). Despite promising results, it should be considered that tPA activity is reduced at lower temperatures suggesting that HMP, used in both studies, might not be optimal (167). DiRito et al. recently revealed that microvascular plugging in the kidney graft is induced by prolonged cold storage, which promotes fibrinogen production in proximal tubular cells and accumulation within the tubular epithelium. Upon restoration of physiological temperatures during NMP or implantation, rapid fibrinogen secretion to urine and microvasculature as well as RBC aggregation causes microvascular obstructions, occurring within 15 min after normothermic temperature restoration impairing adequate graft perfusion. Interestingly, those cold-induced obstructions seem to be different from traditional microthrombi. The investigators delivered tPA with

plasminogen at the beginning of 1 h NMP of non-transplantable human kidneys and managed to clear microvascular obstructions completely. It subsequentially resulted in more stable perfusion parameters, decreased levels of NGAL, IL-6, and ICAM-1, and increased urine production. On the other hand, differently from the above-mentioned studies, neither tPA nor plasminogen alone had such an effect (109).

Another kidney *ex-vivo* perfusion study used thrombalexin, an endothelial localizing, cell membrane binding synthetic thrombin inhibitor. Nineteen ischemically damaged porcine kidneys and 2 non-transplantable human kidneys underwent 1.5 h of HMP with thrombalexin and were subsequentially hemoperfused for 6 h at physiologic temperature to mimic reperfusion. Perfect thrombalexin binding and adherence to graft microvasculature were confirmed after HMP remaining stable during 6 h of reperfusion. In comparison to the control group, treated kidneys demonstrated superior blood flow, 44% larger D capillaries, 50% faster RBC velocity, 3.5 times improved capillary blood flows and perfusion indices in orthogonal polarization spectral imaging, as well as lower d-dimer levels, confirming the anticoagulant activity of thrombalexin. Accordingly, cortical lactate levels were lower in treated kidneys, showing reduced ischemic damage and giving evidence that HMP could be successfully used to deliver cytotopic anticoagulant to the graft thus improving macro-and microvascular perfusion (114).

Sedigh et al. used HMP to deliver Corline heparin conjugate (CHC), a macromolecular heparin, consisting of >20 heparin molecules, to porcine DBD kidney grafts. CHC not only inhibits coagulation, platelet adhesion, and complement activity but also, differently from conventional heparin, irreversibly binds to collagen structures and therefore may be able to restore damaged endothelial glycocalyx and also locally express functional heparin (112, 113). In their first study, the group confirmed the safety and feasibility of CHC pre-treatment during HMP. It successfully bound to vessel walls of the ischemic kidneys, did not cause excess histological damage, or changed perfusion parameters (112). The later work on 3 h *ex-vivo* reperfusion showed that pre-coating vessels with CHC during HMP reduces preservation injury and improves organ function at least in the acute period. Treated kidneys had higher OU, faster decline in creatinine levels, lower urine NGAL levels, and less tubular damage in histological specimens (113).

Although none of those studies investigated the fate of delivered anticoagulant agents in longer periods after reperfusion and the actual need for systemic anticoagulation therapy afterwards, cytotopic delivery, and coating graft with anticoagulants during machine perfusion seems to be promising and provides a field for further studies before implementing this strategy into clinical practice.

Gases

Several investigators used *ex-vivo* machine perfusion to deliver various gases (other than pure oxygen or 95% oxygen and 5% carbon dioxide mix) for kidney graft treatment. Multiple animal models revealed that such gaseous molecules, like carbon monoxide (CO) or NO, have cytoprotective, anti-inflammatory, antiapoptotic, or vasoregulatory effects and may be beneficial in the reduction of IRI or immune responses in the Tx setting (168–170). However, despite the long history of experiments, systemic donor or recipient treatment with gases never set foot in clinical practice due to side effects, difficulties to deliver gases in a safe and controlled manner, and logistic issues, which all might be solved by *ex-vivo* organ-specific treatment.

Hosgood et al. after 10 min of WI and 16 h of SCS, applied 2 h of NMP with NO donor sodium nitroprusside (SNP) or CO-releasing molecule (CORM-3) infused during the first hour of perfusion to porcine kidneys. Despite the short-acting time of these agents, the treatment not only increased RBF at the time of preservation but also improved hemodynamic parameters during 3 h of *ex-vivo* reperfusion. However, in this study, the renoprotective effects of CORM-3 were more apparent than for SNP in terms of RBF, oxygen consumption, UO, and CrCl. Moreover, the histological evaluation showed an increase in ischemic structural changes, such as tubular dilatation, vacuolation, and the number of condensed tubular nuclei in SNP-treated kidneys (115). In the later work, Bhattacharjee et al. used the fourth generation of CO releasing molecules CORM-401, which is more potent and allows more controlled CO release than previously synthesized molecules (117, 171). It has been demonstrated that at 37°C, CORM-401 releases 15 times more CO than at 5°C during the same period, suggesting that NMP could be superior to HMP in case of gas delivery. Another important finding of the study was that 200 μM of CORM-401 in plasmaLyte solution, delivered *via* the renal artery, over 10 h results only in a minimal and non-toxic level of carboxyhemoglobin (COHb) hence considering it safe. CORM-401 was delivered to kidneys originating from a porcine DCD model in a pulsatile manner at 37°C for 20 min followed by immediate reperfusion with autologous blood. This treatment increased RBF and total OU during reperfusion, reduced graft injury (lower histological acute tubular necrosis score, less necrosis, intrarenal hemorrhage, and apoptosis, diminished KIM-1 and NGAL levels in urine), urinary protein levels, and increased CrCl to compare with control kidneys. Interestingly, this treatment had an anti-inflammatory effect by significant downregulation of toll-like receptor (TLR)-2, 4, and 6, as well as MyD88, NF-κB, and HMGB1 genes expression (117).

A very recent study investigated the third gasotransmitter's (H_2S) donor AP39 use during SNMP (21°C) (90). Its beneficial cytoprotective effects have already been demonstrated by supplementing preservation solutions of SCS in murine kidney IRI and Tx models (172, 173). Juriasingani et al. stored porcine DCD kidneys for 24 h in UW supplemented with AP39 at 21°C. Surprisingly, this strategy preserved kidneys better than SCS in UW without additives, giving rationale that delivery of H_2S at higher temperatures could be even more beneficial (91). Indeed, SNMP with AP39 increased UO both during preservation and 4 h of normothermic reperfusion with stressed autologous blood. It also lowered the extent of apoptosis after reperfusion. RNA sequencing detected 214 genes, differentially expressed in the treatment group compared to the SCS group, including downregulated pro-apoptotic, heat shock response genes including regulators of those pathways as well as increased proliferation and oxidative stress response genes.

When compared to SNMP without additives, the treatment group differentially expressed 614 genes with reduced expression of genes associated with the hypoxia inducible factor (HIF)-1α-mediated hypoxia response pathway, pro-inflammatory, and cell death-attenuating genes. On the contrary, the genes mediating proliferation, oxidative stress response, transforming growth factor (TGF)-β pathway, and HIF-1α degradation were upregulated (90).

Among other gases, 70% of argon has also been investigated as potential gasotransmitter for ischemically damaged porcine kidneys during 1 h of NMP. However, subsequent 3 h of *ex-vivo* reperfusion showed no measurable beneficial effect in graft histology, functional parameters, or inflammatory markers (116).

Nevertheless, current evidence suggests that *ex-vivo* delivery of gases, especially using gas-releasing molecules, is likely feasible and safe. Development of machine perfusion strategies and increasing experience of S(NMP) perfusion may be especially beneficial in targeted kidney graft treatment with gas not only due to more efficient gas release from gas-donor molecules to compare with hypothermic conditions but also due to easier control of such treatment *via* perfusion parameters. The efficacy still needs to be determined in auto- or alloTx models and subsequent clinical trials.

Other Pharmacological Agents

EPO

Several studies investigated erythropoietin's (EPO) role in cytoprotection and preconditioning of kidney graft in the course of *ex-vivo* machine perfusion. Endogenous EPO is mainly produced by kidney cortical fibroblasts and not only participates in erythropoiesis but also acts locally through autocrine and paracrine axis *via* receptors in kidney tubular epithelium, endothelial cells, and mesangium. As a protective agent, it coordinates the response to injury of kidney cells by modulating pathways of apoptosis, necrosis, and inflammation (174). Nevertheless, to achieve a therapeutic effect, high doses of EPO are required. It vastly increases the risk of complications, such as hypertension and thrombosis, when administered systemically (17, 18). EPO contributes to tissue remodeling when added to NMP perfusate of ischemically damaged porcine kidneys. Two hours of NMP with EPO increased caspase-3 activity and the number of neutrophils, free cells, and cellular debris in tubular lumen but reduced the number of apoptotic cells and macrophages in tubulointerstitial areas compared to NMP alone. Reduced activity of pro-inflammatory cytokine IL-1β was also observed. Moreover, tubular apoptosis, dilatation, and cytoplasmic vacuolization in the tubular epithelium were significantly diminished, and UO increased in EPO-treated kidneys. The augmentation of apoptotic cells in tubular lumen but not in interstitial areas shows that EPO likely alleviates the clearance of dead inflammatory material, which could be beneficial for tissue conditioning before implantation and confrontation with the host's immune system (118). In another porcine study, cyclic helix B peptide, derived from the 3-dimensional structure of EPO using the cyclization method, was investigated. Such a structure improves cytoprotection but avoids induction of erythropoiesis and its related negative effects. Therefore, such peptide would be potent for systemic use. In this work, *ex-vivo* machine with whole blood as perfusate was used to simulate reperfusion and cyclic helix B peptide effect during the kidney reperfusion phase. Similar to the previous work, such treatment significantly decreased the number of apoptotic cells in the tubular areas, but increased in the lumen, again confirming EPO contribution in apoptotic cells clearance. Downregulation of caspase-3 expression (both precursor and active subunits) and upregulation of heat shock protein (HSP)70 in the treatment group was observed. Furthermore, cyclic helix B peptide improved the hemodynamic parameters RBF and oxygen consumption and increased UO. Even though drug administration during reperfusion is outside of the subject of this review, the results of this study suggest that cyclic helix B peptide could be used as an alternative to conventional EPO during the preservation phase for improved organ conditioning and protection (119).

Metformin

Huijink et al. investigated the potential beneficial effect of metformin on kidney graft injury by the donor pre-treating and/or adding it to NMP solution in rats and pigs experiments (120). Metformin is not only known as an antihyperglycemic agent but also has some pleiotropic effects, due to its ability to inhibit the complex 1 of the mitochondrial respiratory chain, coordinate cellular energy state, inhibit apoptosis, and regulate endothelial function *via* NO production (175, 176). Organo-protective action of metformin has also been demonstrated in an ischemia-reperfusion setting (177, 178). Nevertheless, the results of the Huijink et al. study were inconclusive as the beneficial effect on kidney integrity was observed mainly in rodent experiments when metformin was used for preconditioning (donor treatment). However, perfusate supplementation during NMP was not as effective. Nevertheless, downregulation of eosinophil-derived neurotoxin (EDN)-1, eNOS, vascular cell adhesion molecule (VCAM)-1, IL-6 genes in rat kidneys and upregulation of HSP-70 in porcine kidneys was observed. Moreover, metformin-perfused kidneys revealed lower kidney injury in histological specimens, encouraging further investigation of metformin-treatment using an *ex-vivo* perfusion platform (120).

Doxycycline

Another kidney graft pharmacological treatment strategy is inhibition of matrix metalloproteinases, which play a role in the pathogenesis of ischemia-reperfusion (179), as well as acute and chronic immune injury (180, 181). Activation of MMPs leads to acute tubular injury, necrosis, apoptosis, tubular atrophy, fibrosis, and damage of the basal membrane in the kidney (179, 182). Moser et al. observed increased levels of MMP-9 and MMP-2 during HMP both in human and rat kidneys, along with elevated levels of total protein, NGAL, and LDH in perfusate, indicating preservation injury. Twenty-two hours of rat kidney HMP resulted not only in higher levels of MMPs and kidney injury markers but also in structural impairment of mitochondrial integrity compare to kidneys perfused for only 5 h. Upon supplementing HMP perfusate

with doxycycline, which is not only an antibiotic but also a clinically approved MMP inhibitor, 22 h HMP levels of MMP-2 and MMP-9, LDH, cytochrome c oxidase, NGAL, and total protein dropped to the levels of 5 h perfusion without treatment. Moreover, the doxycycline effect on mitochondrial membrane protection was demonstrated by electronic microscopy (104). Extensive proteomic analysis showed a significant increase in 8 proteins in doxycycline perfused rat kidneys, including glycolysis enzymes triosephosphate isomerase (TPI), phosphoglycerate kinase 1 (PK-1), phosphoglycerate mutase (PGM), urea cycle enzyme aminoacyclase-1A, NO synthesis regulator N(G),N(G), dimethylarginine dimethylaminohydrolase, as well as, other enzymes, such as dihydropteridine reductase-2, pyridine nucleotide-disulfide oxidoreductase and phosphotriesterase-related protein. Interestingly, TPI, PK-1, and N(G),N(G), dimethylarginine dimethylaminohydrolase levels were reduced by HMP, while treatment with doxycycline allowed correction of this reduction, again proving mitochondrial preservation (121).

SUL Compounds

Another class of novel agents that protect cells from hypothermia-associated damage by preserving mitochondrial structure and function and reactive oxygen species (ROS) scavenging are 6-chromanol derivates (SUL compounds) (183, 184). Nakladal et al. added SUL-121 and its enantiomers SUL-150 and SUL-151 into the NMP solution of porcine kidneys after 24 h of SCS. An apparent and immediate increase in RBF and decrease in intrarenal pressure, mainly through SUL-150 enantiomer, was observed. *In vitro* experiments with isolated intraarterial arteries using various agonists showed that the beneficial vascular effect of SUL-121/SUL-150 is mediated by specific competitive inhibition of α1-adrenoreceptors on the vascular smooth muscle. This study did not investigate SUL-121 impact on ROS production and mitochondrial function when it is administered specifically during machine perfusion. Therefore, although *ex-vivo* kidney graft treatment with 6-chromanol derivates after prolonged cold ischemia seems feasible and promising, further investigation is necessary (122).

Propofol

Besides the anesthetic properties, the well-known agent propofol has common structural elements like α-tocopherol and acts as antioxidant by preventing lipid peroxidation in cell membranes (185, 186). Snoeijs et al. investigated renoprotective features of propofol by delivering it to ischemically damaged porcine kidney grafts *via* 22 h HMP. To make it water-soluble, they prepared a cyclodextrin inclusion complex. The treatment prevented lipid peroxidation, reduced the increase in renovascular resistance at reperfusion after autologous kidney implantation. Moreover, treatment with propofol slightly improved kidney function in the early period after Tx. However, leucocyte infiltration in kidneys was not diminished by propofol treatment. The main advantage of HMP delivery was that it allowed high tissue concentrations of the agent without any adverse effects after graft implantation. Propofol concentrations were undetectable in recipients' plasma in the early reperfusion periods (123).

Hemadsorbtion

It has been recently found that, despite leukocytes- and complement-free perfusate, kidney tubular epithelial cells and circulating cells locally produce cytokines and chemokines, hence up-regulating inflammatory pathways within the graft already during *ex-vivo* machine perfusion (59, 65). As it is well-known that pro-inflammatory mediators aggravate the severity of IRI, the removal of cytokines and chemokines from the kidney using machine perfusion seems to be a logical strategy of organ preparation before implantation. This question was analyzed in two recent experimental studies (64, 65). Cytosorb hemadsorbtion filter, which is currently widely used in intensive care to treat severe inflammatory states, such as systemic inflammatory response syndrome and sepsis (187, 188), was connected to the perfusion machine and used to remove cytokines from perfusate during kidney NMP (64, 65). In the first study on porcine kidneys, a significant increase of IL-1β, IL-1α, IL-1RA, TNFα, IL-10 was observed after 6 h of NMP in control kidneys, whereas levels of these cytokines remained low in the hemadsorbtion group. Decreased levels of IL-6, IL-8, c reactive protein (CRP), and thromboxane B2 were also observed in treated kidneys. No effect on UO, CrCl, and FENa was observed, but kidney injury marker NGAL levels in urine were significantly lower in the Cytosorb group (probably due to direct filtering of the NGAL molecule). Interestingly, even though overall blood flow throughout 6 h of NMP was increased in the treatment group a rapid decrease in RBF was observed at 30 min of perfusion, following a decline of vasodilators prostaglandin E (PGE) and prostacyclin. Cytosorb advantage is the ability to filter a wide range of mediators according to their size (10–50 kDa). However, it is not specific and also removes anti-inflammatory agents, such as IL-10 or IL-1RA, and vasodilators PGE and prostacyclin. This problem could be partly solved by adding vasodilators to the perfusate when hemadsorbtion is used (64). Ferdinand et al., used the same technique to perfuse non-transplantable human kidney pairs, focused on changes in graft gene expression after cytokine removal. They showed that NMP has a double effect on gene regulation. It promotes oxidative phosphorylation pathway genes, which are crucial for energy generation. However, at the same time, several pro-inflammatory and immune pathway genes are induced. Using the samples from the clinical study of kidney NMP, the investigators found that this "gene signature" is associated with prolonged DGF after implantation. In non-transplantable human kidneys, NMP with Cytosorb hemadsorbtion not only diminished levels of cytokines but also significantly reduced inflammatory gene expression and up-regulated genes of the oxidative phosphorylation pathway. These surprising results suggest that locally produced and during NMP recirculating cytokines likely play a role in kidney gene regulation and its shift toward a pro-inflammatory state, culminating in aggravated graft injury after implantation. Hemadsorption during *ex-vivo* kidney perfusion seems to be a promising approach to cease this inflammation exacerbating loop however, more experimental studies followed by kidney implantation and clinical studies are necessary to strengthen this hypothesis (65).

CHALLENGES AND CONSIDERATIONS

In the last decade, significant progress has been made in the kidney preservation field. Many *ex-vivo* machine perfusion therapeutic strategies have been developed and proved feasible in multiple experimental and a few clinical studies. Nevertheless, the kidney machine perfusion therapeutics is still in its infancy therefore, several challenges remain and need to be considered.

Currently, among other *ex-vivo* kidney perfusion strategies, only HMP is fully established and routinely used in clinical practice. Therefore, it could be the fastest and easiest way to implement kidney machine perfusion therapeutics into clinical practice. Indeed, several studies demonstrated a successful delivery of MSC and EV (97), siRNA (104), fibrinolytics and anticoagulants (110–114), doxycycline (104, 121), and propofol (123). However, the pharmacokinetics and pharmacodynamics of different agents at 4°C is not well-known (105). The possibility that low temperature might reduce the effectiveness of the therapy (167) or even enhance the accumulation of the agent, which can be damaging after reperfusion, should be considered. Moreover, the kidney outcomes after HMP still depend on CIT (30, 42). Therefore, it could be used only for several hours of therapy, which might not be sufficient for tissue regeneration strategies.

NMP, which ideally provides a physiological environment, is a more attractive platform for kidney *ex-vivo* therapeutics. Not surprisingly, most of the experimental studies used physiological temperature for kidney graft therapy. Long-term warm perfusion is crucial to enhance kidney tissue repair and regeneration (62, 79). SNMP was also found to be effective, especially for gas delivery (90). However, none of these strategies have been adopted in clinical practice yet. The results of the first clinical trial of short-term end-ischemic NMP should answer some questions and probably even open the window of opportunities for kidney perfusion therapeutics (69).

Nevertheless, the optimal protocol, especially for long-term perfusion, still needs to be established including, optimal timing, perfusion settings, oxygen partial pressure or perfusate composition. Moreover, the discussion regarding kidney evaluation parameters during perfusion, which is crucial to observe the graft state during therapy, is still ongoing The rationale for the use of perfusion readings, such as pressure, flow and resistance, is based on the kidney structure, which is rich in capillary network with filtration function. The release of vasoconstrictors from capillaries following the ischemic and inflammatory insults, determines accumulation of erythrocytes and microthrombosis, leading to a diminished flow and increased resistance in the graft. The hypoxia directly activates single-layer endothelium cells, favoring a pro-coagulant and pro-inflammatory phenotype of the kidney vasculature, with consequent disruption of the blood flow, increased leukocyte infiltration, and a further decline in kidney function. On the basis of kidney structure and physiology, perfusion readings are considered as a valuable tool to assess kidney viability before implantation. On the other hand, the relative predictive value of perfusion parameters is low and they cannot be considered as the only criteria to determine, whether to transplant the kidney. In general, there is likely no universal perfusate marker or universal perfusion parameter. However, the best combination of different parameters, sensitive and specific kidney quality score, is an objective of the current research (22). The optimal timing and duration of the different treatments during kidney perfusion also requires further investigation. Due to the short action or life-span of many agents, it might be necessary to continue some therapies systemically after implantation.

The main limitation of the aforementioned studies is that the results mostly reveal the expression of different molecular markers, and thorough investigation of the treatment impact on kidney function after implantation, clinical alloimmune response and transplant outcomes is lacking. Most of the experiments only evaluated the preservation stage or used short *ex-vivo* reperfusion models, which might not reflect the real conditions after implantation. Therefore, more animal models of kidney implantation and longer follow-up periods are necessary before kidney perfusion therapeutics clinical studies. Lastly, implementation of novel gene and cell therapies might be challenging due to ethical regulation, logistics (e.g., MSC retrieval and growth), and high cost. On the other hand, the application of those therapies *ex-vivo via* the machine perfusion platform is more ethically acceptable and less risky than systemic recipient or donor treatment. Therefore, machine perfusion might facilitate the adoption of novel techniques and therapies into clinical practice.

CONCLUSIONS

The rapid development of kidney *ex-vivo* machine perfusion techniques and strategies started a new era in kidney Tx. Many (pre-) clinical studies on *ex-vivo* kidney perfusion using various modalities have already demonstrated its safety and feasibility. Further, IRI and graft immunogenicity may be reduced, and both regeneration and repair may be promoted. Kidney perfusion therapeutics offers an opportunity to overcome the challenges faced with ECD or DCD kidneys, increase the pool of transplantable organs and improve outcomes after implantation. Nevertheless, further extensive research is necessary to transfer these techniques from the experimental to clinical stage.

To date criteria for kidney machine perfusion to improve graft survival/function are still pending. Clinical trials are warranted to define organs that need *ex-vivo* machine perfusion before transplantation for optimal graft function. These data would allow a cost-effective use of machine perfusion.

AUTHOR CONTRIBUTIONS

PSt was responsible for the study concept design and critical revision of the drafted manuscript. RZ, PM, PSc, BL, and KS were responsible for the literature review, interpretation, and drafting of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Clinical Relevance of Absolute BK Polyoma Viral Load Kinetics in Patients With Biopsy Proven BK Polyomavirus Associated Nephropathy

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Introduction: The absolute BK viral load is an important diagnostic surrogate for BK polyomavirus associated nephropathy (PyVAN) after renal transplant (KTX) and serial assessment of BK viremia is recommended. However, there is no data indicating which particular viral load change, i.e., absolute vs. relative viral load changes (copies/ml; percentage of the preceding viremia) is associated with worse renal graft outcomes.

Materials and Methods: In this retrospective study of 91 biopsy proven PyVAN, we analyzed the interplay of exposure time, absolute and relative viral load kinetics, baseline risk, and treatment strategies as risk factors for graft loss after 2 years using a multivariable Poisson-model.

Results: We compared two major treatment strategies: standardized immunosuppression (IS) reduction ($n = 53$) and leflunomide ($n = 30$). The median viral load at the index biopsy was 2.15E+04 copies/ml (interquartile range [IQR] 1.70E+03–1.77E+05) and median peak viremia was 3.6E+04 copies/ml (IQR 2.7E+03–3.3E+05). Treatment strategies and IS-levels were not related to graft loss. After correction for baseline viral load and estimated glomerular filtration rate (eGFR), absolute viral load decrease/unit remained an independent risk factor for graft loss [incidence rate ratios [IRR] = 0.77, (95% *CI* 0.61–0.96), $p = 0.02$].

Conclusion: This study provides evidence for the prognostic importance of absolute BK viremia kinetics as a dynamic parameter indicating short-term graft survival independently of other established risk factors.

Keywords: polyomavirus nephropathy, viral load, viral kinetic, graft survival, renal transplantation

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INTRODUCTION

The viral reactivation of BK Virus in an immunocompromised patient may induce BK polyoma virus associated nephropathy (PyVAN) as a serious complication following renal transplantation. PyVAN has a prevalence of 1–10% (1–3). The hallmarks of the diagnosis are the quantitative detection of BKPyV-DNAemia in blood and urine *via* PCR (4, 5), as well as distinct histological and immunohistochemical findings in the renal biopsy as a gold standard for organ invasive infection (6). The histomorphological phenotype of PyVAN is characterized by tubulointerstitial nephritis including the detection of virus-infected tubular epithelial cells by immunohistochemical staining using BK large T-antigen raised against SV40 (7). A more recent diagnostic option is gene expression analysis from biopsy to distinguish PyVAN from T-cell-mediated rejection (TCMR) (8). Untreated PyVAN can lead to progressive graft damage and presents clinically in the form of an asymptomatic deterioration of graft function causing graft failure in up to 10–30% of the patients (3, 9–11).

The absolute viral load is an important diagnostic surrogate for "presumptive PyVAN" (BKPyV load of 10E4 in blood, without biopsy confirmation). While serial assessment of BKPyV viremia after kidney transplantation (KTX) is recommended (12), none of the previously published studies could address serial assessment of viral load kinetics as a risk factor for worse outcomes, mostly because of limited sample-size (13–15). Additionally, complete viral clearance is considered as a treatment success, with however, limited suitability for treatment guidance: the median time to reach complete viral clearance is up to 9 months with a high proportion of patients never achieving this goal (range 25–76%) (16–18).

This underlines the necessity of further solid virological parameters indicating response during treatment. Continuous assessment of BKPyV viremia may be promising, however, there is no data indicating which particular viral load change, i.e., absolute viral load decrease (in copies/ml) vs. relative viral load changes (as percentage of the preceding viremia) is associated with worse renal graft outcomes.

The risk of graft loss and deterioration of graft function may be further influenced by distinct treatment strategies. Currently, the optimal treatment strategy of PyVAN is unknown. The main recommended pillar of treatment remains the reduction of the immunosuppression (IS) (12, 19–21). Reduction of IS includes reduction of calcineurin inhibitors [CNI, Tacrolimus and Cyclosporin A (CyA)] and the reduction or discontinuation of mycophenolate mofetil (MMF) (21). The increased probability of graft rejection associated with decreased immunosuppression necessitates the careful monitoring of renal function and a low biopsy indication threshold (22). Several treatments with antiviral agents, such as leflunomide (a disease modifying drug with immunoregulatory features used to treat different types of rheumatic conditions) (23, 24), cidofovir (25, 26), fluoroquinolones (27), and immunoglobulin therapy (intravenous immunoglobulins [IVIG]) (28, 29) with variable results were attempted.

In this large single-center study of 91 patients with biopsy proven PyVAN, we aimed to analyze the interplay of baseline risk, BK viral load dynamics (absolute and relative changes), and treatment strategies on graft survival after 2 years. By analyzing the kinetics of BK viremia after diagnosis, we aimed at identifying patients under higher risk of graft loss in relation to absolute viral load and relative viral load changes. Serial measurements of BK viral load, graft function, and IS level enabled detailed assessment of graft loss risk using time dependent multivariable models.

Abbreviations: AZA, Azathioprine; BKPyV, BK polyomavirus; CNI, calcineurin inhibitor; CyA, Cyclosporin A; HLA, human leukocyte antigen; IS, immunosuppression; KTX, kidney transplantation; IVIG, intravenous immunoglobulins; MMF, mycophenolate mofetil; PCR, polymerase chain reaction; PyVAN, polyomavirus associated nephropathy; SV40, simian virus 40; TCMR, T-cell-mediated rejection; TX, transplantation.

MATERIALS AND METHODS

Study Design

In this retrospective single-center cohort study, all renal transplant recipients, transplanted between 2001 and 2018 at the Medical University of Vienna with biopsy proven PyVAN were considered eligible for study ($N = 111$; 3.6% of all Tx, $N = 3{,}039$). Following criteria were applied for study inclusion: (a) all patients with biopsy proven PyVAN between 2001 and 2018 supported by compatible histopathological findings and immunohistochemical staining of SV40; (b) two or more positive BK virus- PCR findings during the post-transplantation (TX) period (serial assessment of BK Virus *via* PCR was introduced at 2001); and (c) a follow-up of at least 2 years after index biopsy. This study aimed to assess the association of absolute and relative BK viral load changes over time with transplant survival in the 24 months after the diagnosis of PyVAN and the potential difference in the relation to baseline risk and treatment strategies. The primary outcome was death censored graft-loss, defined as initiation of any renal replacement therapy. The flowchart of the study is shown in **Figure 1**.

Parameters and Clinical Findings

This study was approved from the Medical University of Vienna Institutional Review Board (Nr:1291/2020), Vienna, Austria, assuring adherence to the declarations of Helsinki and Istanbul.

Data were assessed retrospectively using electronic and archived medical records. We included following variables: (a) baseline demographic and transplant-associated data (age, gender, number of previous transplants, and donor type [living vs. deceased]), underlying renal disease, number of human leukocyte antigen (HLA) mismatches (0–6), and cold ischemia time (hours), (b) immunosuppression regimen before and after diagnosis of the PyVAN and the trough level of calcineurin inhibitor (Tacrolimus, CyA), (c) viral load in plasma (copies/ml measured by PCR) at months 0, 1, 3, 6, 9, 12, and 24 after PyVAN diagnosis, (d) graft function at diagnosis of PyVAN (estimated glomerular filtration rate measured by the CKD-EPI equation [eGFR CKD-EPI] in ml/min/1.73 m^2 (30)) as well as at months 1, 3, 6, 9, 12, and 24 after PyVAN diagnosis, (e) date of graft loss, and (f) BANFF single lesions at the time of PyVAN diagnosis and rejection diagnosis. The eGFR-slopes were used to visualize graft function on a longitudinal scale over the follow-up period.

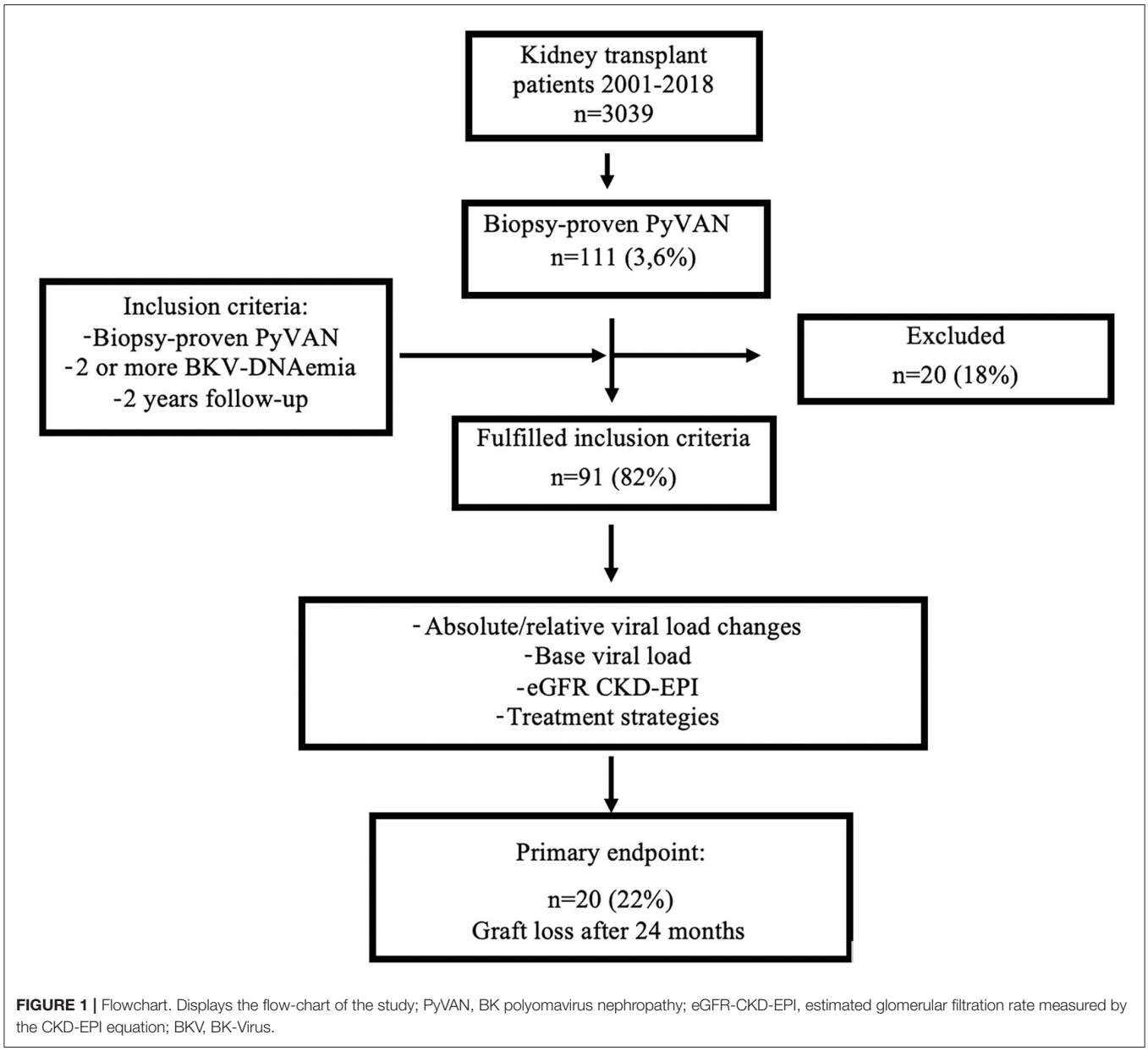

FIGURE 1 | Flowchart. Displays the flow-chart of the study; PyVAN, BK polyomavirus nephropathy; eGFR-CKD-EPI, estimated glomerular filtration rate measured by the CKD-EPI equation; BKV, BK-Virus.

Treatment of PyVAN and Degree of Immunosuppression

Standard of care for the treatment of PyVAN consisted of two major strategies: (a) a standardized reduction of CNI and/or MMF or (b) switching from MMF or Azathioprine to Leflunomide. After PyVAN diagnosis, the dose of MMF/Azathioprine was either reduced by half or discontinued. In a second step, the dose of the administered CNI was reduced (Tacrolimus levels were targeted to <6 ng/ml, CyA <150 ng/ml). Leflunomide was administered by a daily dose of 20–40 mg/day. Cidofovir or IVIG were used as rescue treatments in rare cases. After transplantation, administration of corticosteroids (or equivalent dose of dexamethasone) was standardized according to our centers protocol with 250 mg on day 1, 125 mg on day 2, 50 mg for days 3–7, 25 mg for days 8–15, 10 mg for days 16–30, and 5 mg further on.

To analyze the effects of the levels of Tacrolimus and CyA at each time-point (months 1, 3, 6, 9, 12, and 24 after the biopsy), we scaled the level of CNI in three categories (low, medium, and high level of CNI exposure), according to the trough levels. Categories were defined as following: low (ng/ml): Tacrolimus 3–5, CyA <40; medium (ng/ml): Tacrolimus 5–7, CyA 40–80; and high (ng/ml): Tacrolimus > 7, CyA > 80.

Biopsy

Biopsies were performed upon unexplainable graft dysfunction and/or proteinuria (as a standard procedure of post-TX

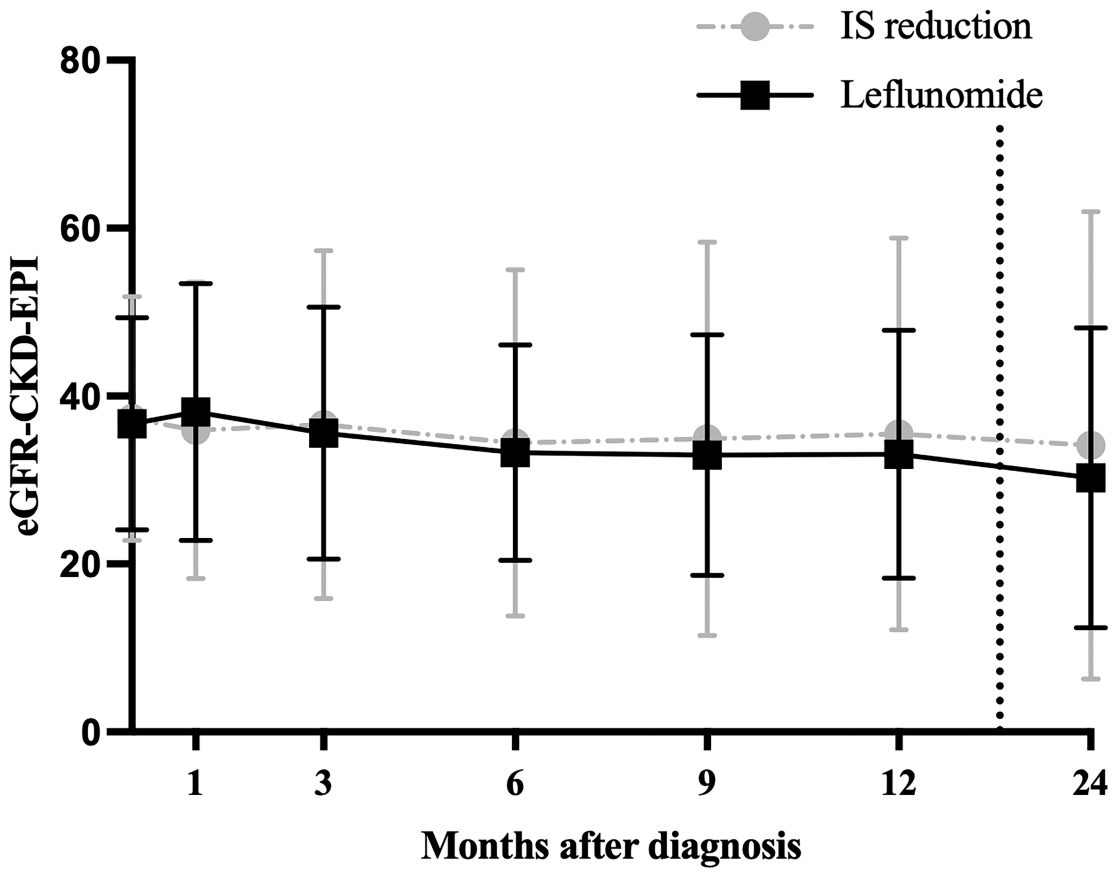

FIGURE 2 | Graft function in the 24 months after biopsy; shows the eGFR in ml/min/1.73 m^2 measured by the CKD-EPI equation between two major therapy groups. IS: immunosuppression reduction vs. Leflunomide. Dotted line between months 12 and 24 was used for visualization purposes.

care). None of the included cases originated from protocol biopsies which have been introduced at our center in June 2017. BK viremia without impairment of graft function was not considered as an indication for biopsy. Histopathologic findings were evaluated on formalin-fixed paraffin-embedded sections applying standard methodology. The biopsies were examined for histological signs of viral infection, such as intranuclear inclusions, cellular atypia, tubular epithelial cell degeneration, with rounding, detachment, and cell-apoptosis, and immunohistochemical staining for SV40 large T-antigen (2, 17). Diagnosis and single lesions were scored according to Clasification of Rejection (BANFF) criteria at the time of diagnosis (31). All rejections were treated according to a center-specific protocol with either pulse of steroids or thymoglobulin as described previously (17).

Dynamics of BK Viremia

Screening for BK infection was performed by testing for BK viremia every 3 months during the first year after TX. Serum viral load was recorded at diagnosis (PyVAN biopsy), at months 1, 3, 6, 9, 12, and 24 after the biopsy as well as months 1, 2, and 3 before the biopsy. Real time PCR was performed by DNA isolation from 200 μl of plasma using the automatic extractor NucliSens EasyMag (bioMérieux, Marcy l'Etoile, France) and eluted to a final installment of 70 μl. BK-polymavirus was quantified using Taqman PCR in real-time with primers and samples inside the small capsid protein VP3 (32). Complete BK viral load clearance was defined as a reduction of viral load under the detection level (<70 copies/ml) (33).

Statistical Analysis

The research was conducted using a pre-designed model of data collection and all information were inserted into Excel and consequently transferred to the SPSS and Stata analysis system (SPSS: An IBM Company, IBM Corporation, Armonk, NY, USA; Stata 16 Stata Corp, College Station, TX, USA). We present continuous data as mean ± SD, categorized data as absolute count with the relative frequency. To test the null hypothesis of no difference, we used a *t*-test with normally distributed continuous data, Mann–Whitney *U*-test for analysis with no normal distribution. For categorized data, we used the Fisher's exact test. Generally, a two-sided $p < 0.05$ was considered statistically significant. Values from clinical and demographic data were randomly missing only in rare cases and were not included in the statistical analyses.

TABLE 1 | Demographic and clinical characteristics of the study population.

	Treatment group					*P*-value*
	All patients (*N* = 91)	**IS reduction* (*N* = 53)**	**Leflunomide* (*N* = 30)**	**IVIG (*N* = 5)**	**Cidofovir (*N* = 3)**	
Male sex, *N* (%)	63 (69)	39 (74)	21 (70)	1 (20)	2 (67)	0.80
Deceased donor, *N* (%)	74 (81)	43 (81)	23 (77)	0 (0)	3 (100)	0.78
ABO incompatible TX *N* (%)	5 (5.5)	3 (5.7)	2 (6.7)	0 (0)	0 (0)	>0.99
Age at transplantation (years), mean ± SD	51 ± 15	53 ± 16	50 ± 15	58 ± 9	43 ± 7	0.39
Cold ischemia time (h), mean ± SD	12 ± 8	11 ± 7	14 ± 10	11 ± 8	13 ± 6	0.17
HLA Mismatch, median (IQR)	3 (2–4)	3 (2–4)	3 (1–4)	4 (2)	2 (2–4)	0.48
Sensitized, *N* (%)	15 (16)	10 (19)	5 (17)	0 (0)	0 (0)	>0.99
Donor age in years, median (IQR)	55 (46–67)	57 (48–66)	52.5 (43–71)	54 (31–76)	46 (27–46)	0.98
eGFR three months after Tx, median (IQR), ml/min/1.73 m^2	42.5 (32.9–54.5)	48.4 (35.8–56.7)	40.6 (32.6–50.1)	28.0 (17.7–41.9)	58.9 (41.5–58.9)	0.09
Maintenance IS						
Tacrolimus, *N* (%)	77 (85)	44 (83)	27 (90)	5 (100)	1 (33)	>0.99
CyA, *N* (%)	6 (7)	4 (8)	1 (3)	0 (0)	1 (33)	0.60
Belatacept, *N* (%)	1 (1)	1 (2)	0 (0)	0 (0)	0 (0)	>0.99
Sirolimus, *N* (%)	4 (4)	1 (2)	2 (7)	0 (0)	1 (33)	0.29
Induction IS, *N* (%)	60 (66)	39 (74)	20 (61)	1 (20)	0 (0)	0.36
IL-2 Antibodies, *N* (%)	40 (44)	27 (51)	13 (39)	0 (0)	0 (0)	ND
CD20 Antibodies, *N* (%)	4 (4)	2 (4)	2 (7)	0 (0)	0 (0)	ND
Apheresis, *N* (%)	15 (16)	10 (19)	5 (17)	0 (0)	0 (0)	ND
Depleting antibodies, *N* (%)	12 (13)	6 (11)	3 (10)	2 (40)	1 (33)	ND

*TX, Transplantation; HLA, human leukocyte antigen, sensitized: latest CDC PRA > 40% and/or donor specific antibody levels MFI > 1,000 (34); eGFR, estimated glomerular filtration rate; IS, immunosuppression; CyA, Cyclosporin A; d, days; IL, interleukin; ND, not done; SD, standard deviation; IQR, Interquartile range, *p was calculated for comparison of the IS-reduction and Leflunomide group; intravenous immunoglobulins (IVIG) and cidofovir group were not compared due to low sample size; t-test for normally distributed continuous data, Mann–Whitney U-test for analysis without normal distribution, Fischer's exact test for categorized data.*

Poisson-Regression

To assess the effects of the viral load changes on the graft loss as the outcome of interest, we used the Poisson-regression model. Poisson regression, a form of generalized linear regression, is well-suited to model event rates if the exposure time (offset) for each observed individual is known and matters. The exponentiated regression coefficient equals the incidence rate ratio (*IRR*), which quantifies the effect of a covariable on the event rate. Event rates for graft loss are presented as rate per 100 patient months. We therefore used multivariable Poisson models to estimate *IRR*s with 95% *CI*s of several covariables. As the main exposure covariable, we used changes in viral load (differences between time intervals of the naturally log-transformed plasma viral loads). Other covariables were chosen based on clinical considerations in the counterfactual framework, meaning that they needed to be common causes for changes in viral load and graft loss (baseline eGFR, baseline viral load, levels of the immunosuppression over time, and PyVAN therapy). Individuals may have had several periods of different exposure-levels (viral load), resulting in a panel-design. We included exposure times (offset) for each observation period, which were allowed to differ between individuals and observation periods in our models. Using the patient as the panel identifier, we allowed for the panel design by using random effect models or cluster robust estimators if random effect models could not be computed. We used the Wald-test to test the null hypothesis of $IRR = 1$, meaning no effect of a covariable on graft loss rate.

RESULTS

Study Cohort

Of 111 biopsied patients with PyVAN, 20 patients did not fulfill the inclusion criteria. In total 91 patients were included in the final analysis with a mean follow-up after diagnosis of 646 ± 193 days. Most patients were men ($N = 63$; 69.3%) and received their first kidney transplant ($N = 75$; 82%). Deceased donor was the most common type of transplantation ($N = 74$; 81%). Mean recipient age was 51 ± 15 years. As shown in **Table 1**, the majority of patients received Tacrolimus based IS (87%).

Induction IS was administered in 60 patients, most frequently Basiliximab (34/57%). Depleting antibodies were administered in 12 (14%) patients. Median HLA-mismatch was 3 (IQR 2–4), and

TABLE 2 | Polyomavirus associated nephropathy (PyVAN)-associated data of the study population.

	Treatment group					*P*-value*
	All patients (*N* = 91)	**IS reduction* (*N* = 53)**	**Leflunomide* (*N* = 30)**	**IVIG (*N* = 5)**	**Cidofovir (*N* = 3)**	
Time to first positive PCR in serum (d) median (IQR)	113 (81–215)	113 (85–243)	106 (77–178)	92 (18–143)	119 (181–277)	0.84
Days until PyVAN, median (IQR)	181 (125–317)	175 (122–347)	185.5 (130.2–334)	343 (109–353)	192 (122–222)	0.60
eGFR at PyVAN diagnosis, mean ± SD	36 ± 14	37 ± 15	37 ± 13	20 ± 16	38.0 ± 14	0.67
BK viremia at PyVAN diagnosis, median (IQR); copies/ml	2.1E+04 (1.7E+03–1.8E+05)	9.6E+03 (1.6E+03–9.2E+04)	8.7E+04 (1.6E+03–3.2E+05)	9.3E+05 (2.8E+04–1.9E+08)	1.0E+04 (4.6E+02–3.4E+04)	0.14
Max. BKV load, median (IQR); copies/ml	3.6E+04 (2.7E+03–3.3E+05)	2.4E+04 (1.9E+03–1.9E+05)	8.70E+04 (5.10E+03–6.0E+05)	9.3E+05 (2.0E+05–7.6E+07)	1.0E+04 (4.6E+02–3.4E+04)	0.43
Rejection at the time of PyVAN diagnosis *N* (%)	29 (32)	14 (27)	9 (30)	3 (60)	3 (100)	0.61
ABMR *N* (%)	2 (2)	2 (4)	0 (0)	0	0 (0)	ND
TCMR *N* (%)	27 (29)	12 (23)	9 (30)	3 (60)	3 (100)	0.46
Rejection before PyVAN diagnosis *N* (%)	30 (33)	16 (30)	8 (27)	1 (20)	3 (100)	>0.99
Rejection after PyVAN diagnosis *N* (%)	17 (18.7)	8 (15)	7 (23)	0	2 (33)	0.71

*PCR, polymerase chain reaction; PyVAN, BK polyomavirus nephropathy; BKV, BK-Virus; eGFR, estimated glomerular filtration rate according to the CKD-EPI equation; ABMR, antibody mediated rejection; TCMR, T-cell mediated rejection; ND, not done; SD, standard deviation; IQR, interquartile range, *p refers to a comparison of patients in the IS reduction and patients in the Leflunomide group; t-test for normally distributed continuous data, Mann–Whitney U-test for analysis without normal distribution, Fischer's exact test for categorized data.*

median donor age was 55 (IQR 46–66.5) years. Graft function measured by the CKD-EPI formula 3 months after TX was 43 (IQR 33–55) ml/min/1.73 m^2.

Treatment Groups

Since treatment strategies may further influence outcomes, patients were divided into four groups, according to the PyVAN treatment strategy. In this study, 53 (58%) patients underwent standardized reduction of immunosuppression (details as shown in methods section), 30 (33%) patients were switched from MMF/Azathioprine to Leflunomide. Furthermore, five (6%) patients received IVIG and three (3%) patients Cidofovir as rescue medications. **Table 1** displays the demographic- and baseline clinical parameters of all 91 study participants. Baseline characteristics did not differ significantly between two major treatment groups (IS-reduction vs. Leflunomide). Due to the small sample size in the Cidofovir and IVIG therapy groups, those patients were not included in further analyses. Graft function measured by the CKD-EPI equation did not differ significantly between the groups over the course of the first 12 months and after 24 months (**Figure 2**). The levels of primary IS are displayed in a **Table 3**.

BK Viremia

Median time to the first detection of any positive BK viremia was 113 (IQR 81–215) days after TX, median time to biopsy proven PyVAN was 181 (IQR 125–317) days. The median viral load at the time of index biopsy was 2.15E+04 (IQR 1.70E+03–1.77E+05) copies/ml and median peak viremia was 3.6E+04 (IQR 2.7E+03–3.3E+05) copies/ml.

TABLE 3 | Level of calcineurin–inhibitors after the diagnosis of BK (PyVAN).

Primary immunosuppression after diagnosis of PyVAN		
Month after diagnosis	**Tacrolimus**	**Cyclosporin A**
0	7.04 ± 2.77	74.54 ± 53.49
1	7.22 ± 2.80	127.20 ± 71.63
3	6.20 ± 2.19	105.15 ± 75.97
6	6.13 ± 2.06	43.67 ± 27.46
9	6.06 ± 2.09	62.50 ± 63.05
12	5.79 ± 2.26	35.57 ± 22.70
24	5.76 ± 2.35	56.00 ± 24.04

Values displayed as trough levels, expressed as mean ± SD in ng/ml. PyVAN, polyomavirus nephropathy.

Neither the timing of first positive BK viremia nor the maximum BK viral load differed significantly between patients in the IS reduction group and patients receiving Leflunomide at any timepoint (**Table 2**). About 40% of patients achieved complete BK virus clearance during the observation time. Patients treated with Leflunomide showed higher rates of complete virus clearance at the last follow-up or after 24 months compared with patients in the IS reduction group (57 vs. 32%; $p = 0.03$).

Graft Survival and Viral Load Kinetics

As shown in **Table 4**, baseline data were not significantly different in subjects with and without graft loss. Moreover, there was no relation regarding diagnosis period (years 2001–2006, 2007–2012, and 2013–2018) and the observed graft loss frequency rate. The overall frequency of rejections in the index biopsy

TABLE 4 | Transplant and demographic patient characteristics in relation to a graft loss.

	All patients *N* = 91	Graft loss *N* = 20	No graft loss *N* = 71	*P*-value*
Donor age mean ± SD	54.8 ± 16.5	51.1 ± 19.1	56.0 ± 15.6	0.30
Recipient age mean ± SD	51.4 ± 15.3	51.4 ± 13.7	51.5 ± 15.8	0.97
Deceased donor *N* (%)	74 (81)	16 (80)	58 (82)	>0.99
Time to diagnosis (days) median/IQR	181 (125–317)	184 (106–241)	181 (129–381)	0.47
Cold ischemia time (h) mean ± SD	12.2 ± 8.2	13.1 ± 8.1	12.2 ± 8.3	0.64
HLA–Mismatch (total) median/IQR	3 (2–4)	2 (3.5–4.5)	3 (2–4)	0.29
CMV recipient status neg/pos *N* (%)	32 (37)	5 (25)	27 (40)	0.29
CMV donor status neg/pos *N* (%)	33 (39)	8 (45)	25 (38)	0.79
Graft function after KTX, CKD–EPI–eGFR, ml/min/1.73 m^2				
Graft function at index biopsy, mean ± SD	37 ± 14	28 ± 10	38 ± 15	0.08
Graft function 3 months after index biopsy, mean ± SD	45 ± 18	42 ± 17	46 ± 18	0.55
Viral load at the index biopsy copies/ml, median (IQR)	2.15E+04 (1.70E+03–1.77E+05)	1.20E+05 (1.00E+03–5.50E+05)	2.00E+04 (1.70E+03–1.20E+05)	0.30
Biopsy findings in index biopsy				
Interstitial fibrosis (ci) mean/SD	1.39 (0.98)	1.40 (0.94)	1.39 (1.00)	0.96
Tubular atrophy (ct) mean/SD	1.07 (0.86)	1.30 (0.92)	1.00 (0.83)	0.17
Total inflamation (ti) mean/SD	1.64 (0.89)	1.38 (0.92)	1.69 (0.89)	0.37
Diagnosis period				0.10
2001–2006 *N* (%)	19 (21)	4 (21)	15 (79)	ND
2007–2012 *N* (%)	37 (41)	12 (32)	25 (68)	ND
2013–2018 *N* (%)	35 (38)	4 (11)	31 (89)	ND

*HLA, human leukocyte antigen; CMV, cytomegalovirus; ND, not done; KTX, kidney transplantation; SD, standard deviation; IQR, Interquartile range; IVIG, intravenous immunoglobulins. T-test for normally distributed continuous data, Mann -Whitney U-test for analysis without normal distribution, Fischer´s exact test for categorized data. *p refers to a comparison of patients with and without graft loss.*

was comparable in the groups with and without graft loss (35 vs. 31%). Neither the frequency of concomitant TCMR (29.6 vs. 30%), nor antibody mediated rejection (5 vs. 1.4%) were significantly related to graft loss in this cohort of PyVAN subjects.

During the follow-up period of 24 months after biopsy, death-censored graft loss occurred in 20 (22%) patients within 350 ± 240 days. The incidence rate for graft loss was 1 per 100 patient months. While baseline virological variables were comparable between patients with and without graft loss (**Table 4**), we observed that patients with graft loss did not have a significant drop of viremia between baseline viremia and viremia at months 3, ($p = 0.9$), 6 ($p = 0.5$), and 12 ($p = 0.25$). In contrast, patients without graft loss experienced a highly significant drop of absolute viremia between baseline and months 3 ($p < 0.001$), 6 ($p < 0.001$), and 12 ($p < 0.001$).

Multivariable Poisson Model

To further model the complex interplay of exposure time and absolute and relative viral load kinetics as risk factors for death-censored graft loss, we applied a multivariable Poisson model. This allowed for correction for multiple variables at distinct time points in relation to exposure time (time intervals: months 0–1,1–3,3–6,6–12, and 12–24). The absolute viral load change was a significant risk factor for graft survival (*IRR* = 0.78 95% *CI* 0.64–0.97, $p = 0.03$), showing that each log unit drop in absolute viral load decreased the risk for graft loss by ∼22%. *IRR* was not different among treatment groups (IS reduction vs. Leflunomide 0.05 vs. 0.04) and was therefore not included in the multivariable model. Even after correction for baseline viral load and baseline eGFR, the absolute viral load change remained an independent protective factor for graft loss (*IRR* = 0.77, 95% *CI* 0.61–0.96, $p = 0.02$) (**Figure 3**). In contrast to absolute viral load changes, relative viral load changes were not significantly associated with graft loss (*IRR* = 0.98 95% *CI* 0.97–1.00, $p = 0.1$). Neither Tacrolimus- nor Cyclosporin A categories of through levels at each timepoint after diagnosis were significantly associated with graft loss [*IRR*: categories low (1.7), medium (0.8), and high (1.9); $p = 0.5$].

Histological Findings

We analyzed the relationship between absolute viral loads at time of diagnosis with biopsy findings: Acute and chronic histological lesions assessed according to BANFF criteria at the time of diagnosis in relation to the absolute viral load at biopsy are displayed in form of a heat map in **Figure 4**. The BANFF single lesions did not differ significantly between patients with and

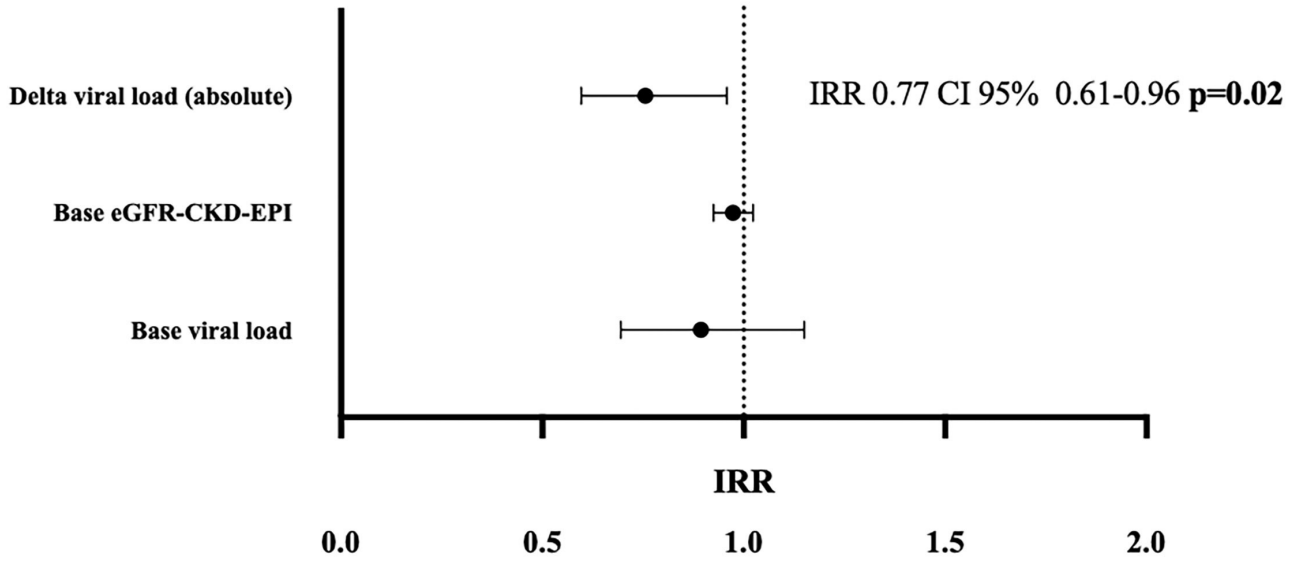

FIGURE 3 | Multivariable Poisson regression model; shows the incidence rate ratios (*IRR*s) for death censored graft loss after 2 years for absolute viral load changes corrected for the baseline eGFR and baseline viral load at biopsy. Absolute delta viral load over 24 months was assessed between months 0–1, 1–3, 3–6, 6–12, and 12–24. Poisson models for estimation of *IRR* with 95% *CI*s; eGFR-CKD-EPI: estimated glomerular filtration rate measured by the CKD-EPI equation, base viral load: BK-Virus viral load in plasma at the time of diagnosis.

without later graft loss. Furthermore, the extent of chronic injury reflected by BANFF lesion scores interstitial fibrosis (ci) and tubular atrophy showed no correlation with graft loss (**Table 4**). Concomitant rejection event rate was similar between the two major therapy groups (**Table 2**).

DISCUSSION

In this large retrospective analysis of a detailly characterized cohort of PyVAN patients, we demonstrate the clinical relevance of absolute BKPyV kinetics as an important prognostic marker for graft loss. Absolute (and not relative) BK viral load reductions were associated with a significantly decreased graft loss rate of 22% per log unit change after adjustment for other established risk factors. Our findings suggest that in clinical routine, attention should not only be given to patients with high viral load or chronic lesions at the index biopsy but also on patients with an insufficient reduction of viremia over time.

Our findings are partly in line with two prior studies by Nickeleit et al. (16, 35), demonstrating associations with worse graft function and high plasma viral load at index biopsy. In addition, we observed that patients with graft loss had a significantly worse graft function already at the time of index biopsy, however, after correction in a time adjusted multivariable model, this finding lost statistical significance. In most of the studies, only a minority of patients reached complete viral clearance (copies/ml below detection limit), a parameter therefore largely unsuitable for treatment surveillance (16–18). In contrast, assessing absolute viral load changes during the whole course of disease may provide an important clinical tool.

In our study, neither BANFF scores for ci or ct nor total inflammation differed significantly between patients with and without graft loss. These findings may be somewhat discrepant to a prior multicenter study (29, 30). We believe that these discrepancies are potentially related to a different study approach (static vs. dynamic) and the previously described problem of sampling errors (17) in PyVAN, which show the limitation of cross-sectionally assessed parameters, such as biopsies and support the use of dynamic assessments like absolute viral load changes for clinical management of PyVAN patients.

There is some evidence that the cumulative viremia is associated with unfavorable outcomes (15, 21). In contrast, a smaller study by Simard-Meilleur et al. demonstrated that the absolute viremia alone was not associated with further eGFR loss in a cohort of patients with PyVAN or significant BK viremia (36). Notably, in contrast with the cited studies, our analysis was focused on patients with biopsy-proven PyVAN only—a patient group commonly underrepresented in prior studies.

The studies analyzing alternative treatment regimens included mostly small sample sizes (21, 23–26, 28, 29). Besides, in our comparably large study we did not find significantly improved graft survival in patients receiving a specific treatment regimen. However, patients under Leflunomide showed higher rates of complete viral clearance, a supposed surrogate for treatment success, which is in line with a recent systematic review (24). The observed viral clearance in our study of 60% suggests that in selected patients, i.e., without relevant change in absolute viremia, Leflunomide may be considered as a valid alternative treatment option.

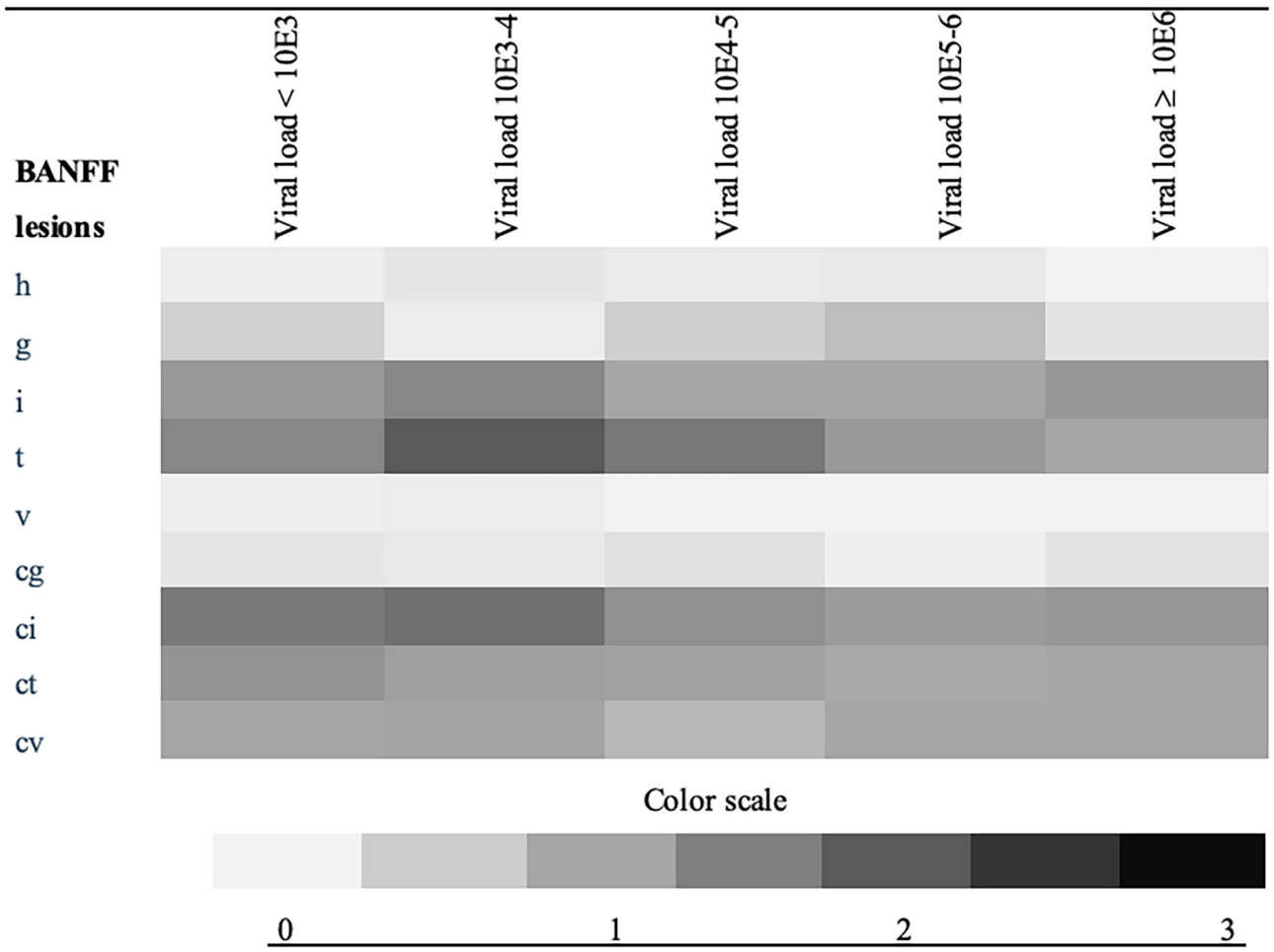

FIGURE 4 | Heat map of BANFF single lesions in the index biopsy in relation to absolute viral load; shows the mean value of BANFF scores (color scaled) at the index biopsy according to the viral load at the time of the diagnosis, (copies/ml), h, arteriolar hyalinosis; g, glomerulitis; i, interstitial inflammation; v, intimal arteritis; t, tubulitis; cg, transplant glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, arterial fibrous intimal thickening, color scale describes the mean score of each group.

There is some evidence that concomitant rejection impacts graft survival in patients with PyVAN (2, 37). However, the discrimination of interstitial inflammation/tubulitis and attribution to concurrent TCMR vs. resolving PyVAN is a remaining clinical challenge and not fully resolvable in renal transplant pathology due to technical aspects (18). In our cohort, however, we did not find a higher frequency of concomitant rejections at index biopsy in patients with graft loss, excluding the possibility that concomitant rejection may have biased our findings. This is in line with a prior study where rejection at index biopsy was not associated with graft loss rates (38).

The use of Tacrolimus compared with CyA is a well-described risk factor for PyVAN (3, 10, 39–41). We assessed semi-quantitatively the cumulative level of CNI based IS by including all serial measurements of CNI levels in a Poisson model. We observed that the degree of CNI based IS after PyVAN diagnosis did not differ between patients with or without graft loss. This suggests that the short-term prognosis of PyVAN patients is more determined by absolute viral kinetics than the level of CNI based IS.

The current study has multiple strengths, most importantly the large sample size and serial assessment of multiple parameters allowing for their inclusion into a longitudinal Poisson model and adjustment of our results to time of exposure. Moreover, this study focused on purely biopsy proven PyVAN, while previous studies have included presumptive and proven PyVAN in variable proportions.

The major inherent limitations of retrospective studies are also applicable to the current study. While the decision for treatment allocation was individual, treatment groups were well-balanced regarding the baseline clinical- and virological risk factors arguing against a treatment related bias. Moreover, a delay in diagnosis and treatment is unlikely as time to first positive viremia was similar between groups. While our analysis covers a large study period of 18 years, we did not find a significantly higher graft loss rate in relation to the timing of diagnosis arguing against a relevant time-related bias. Further, study inclusion

was based on histological diagnosis of PyVAN, therefore, mostly excluding a strong selection bias.

In conclusion, this study provides evidence for the prognostic importance of absolute BK viremia kinetics as a dynamic parameter indicating short-term graft survival independently of other established risk factors. Our findings support serial measurement of absolute BK viremia load changes to early identify patients with persistent viremia levels and consequently higher risk for graft loss.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical University of Vienna Institutional Review Board (Nr.1291/2020). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HO, ME, and ŽK participated in the research design, performance of the research, data analysis, interpretation of results, and writing of the manuscript. HH, JK, SA, TS, GB, KH, BW, JW, RS, and LW participated in performance of research, data analysis, and writing of the manuscript. All authors contributed to the article and approved the submitted version.

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A Prospective Multicenter Trial to Evaluate Urinary Metabolomics for Non-invasive Detection of Renal Allograft Rejection (PARASOL): Study Protocol and Patient Recruitment

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Background: In an earlier monocentric study, we have developed a novel non-invasive test system for the prediction of renal allograft rejection, based on the detection of a specific urine metabolite constellation. To further validate our results in a large real-world patient cohort, we designed a multicentric observational prospective study (PARASOL) including six independent European transplant centers. This article describes the study protocol and characteristics of recruited better patients as subjects.

Methods: Within the PARASOL study, urine samples were taken from renal transplant recipients when kidney biopsies were performed. According to the Banff classification, urine samples were assigned to a case group (renal allograft rejection), a control group (normal renal histology), or an additional group (kidney damage other than rejection).

Results: Between June 2017 and March 2020, 972 transplant recipients were included in the trial (1,230 urine samples and matched biopsies, respectively). Overall, 237 samples (19.3%) were assigned to the case group, 541 (44.0%) to the control group, and 452 (36.7%) samples to the additional group. About 65.9% were obtained from male patients, the mean age of transplant recipients participating in the study was 53.7 ± 13.8 years. The most frequently used immunosuppressive drugs were tacrolimus (92.8%), mycophenolate mofetil (88.0%), and steroids (79.3%). Antihypertensives and antidiabetics were used in 88.0 and 27.4% of the patients, respectively. Approximately 20.9% of patients showed the presence of circulating donor-specific anti-HLA IgG antibodies at time of biopsy. Most of the samples (51.1%) were collected within the first

6 months after transplantation, 48.0% were protocol biopsies, followed by event-driven (43.6%), and follow-up biopsies (8.5%). Over time the proportion of biopsies classified into the categories Banff 4 (T-cell-mediated rejection [TCMR]) and Banff 1 (normal tissue) decreased whereas Banff 2 (antibody-mediated rejection [ABMR]) and Banff 5I (mild interstitial fibrosis and tubular atrophy) increased to 84.2 and 74.5%, respectively, after 4 years post transplantation. Patients with rejection showed worse kidney function than patients without rejection.

Conclusion: The clinical characteristics of subjects recruited indicate a patient cohort typical for routine renal transplantation all over Europe. A typical shift from T-cellular early rejections episodes to later antibody mediated allograft damage over time after renal transplantation further strengthens the usefulness of our cohort for the evaluation of novel biomarkers for allograft damage.

Keywords: kidney transplant rejection, urinary metabolites, biomarker, NMR-spectroscopy, non-invasive test

INTRODUCTION

Despite a steady improvement of patient and organ survival after renal transplantation, allograft rejection continues to pose a risk of graft damage. In the first week and month after transplantation, T-cell-mediated rejection (TCMR) in particular is more common, while later antibody-mediated rejection (ABMR) accounts for the majority of immunological graft damages (1, 2).

Changes in kidney function, a decrease in urine output, or an increase in proteinuria may reflect transplant rejection during routine clinical patient care. Kidney biopsies are still current gold standard for diagnosing an allograft rejection, but as an invasive procedure it carries the risk of bleeding and other complications. The latter limits the routine use of serial biopsies, and the diagnosis of rejection is often made at an advanced stage of irreversible tissue injury. At many transplant units, protocol biopsies have been introduced to potentially detect the acute rejection already in a sub-clinical state (3). However, with serial biopsies, it is unlikely that all rejection episodes will be detected upon onset, not to speak of complications associated with such a costly approach (4).

Biomarkers in the urine could help to detect rejections early and non-invasively, whereby an appropriate sensitivity and specificity as well as a quick diagnosis are necessary for the clinical routine (5, 6).

Recently, we developed a novel, non-invasive method to detect the graft rejection *via* a characteristic constellation of the urine metabolites alanine, citrate, lactate, and urea investigated by NMR spectroscopy (7). In a first monocentric prospective observational (UMBRELLA) study which included 109 patients, the test performance reached an area under the curve (AUC_{ROC}) value of 0.84 when combining metabolomic analysis and corresponding estimated glomerular filtration rate (eGFR) values at time of urine sampling (8).

The subsequently following PARASOL study presented here is an open, international, multicenter, prospective, observational study, in which the diagnostic accuracy for the urinary metabolite constellation initially assessed in the UMBRELLA study will be validated in an independent cohort resembling the routine kidney transplantation programs in six different European transplant centers. The study is based on a reasonable number of patients recruited with their respective urine samples and renal allograft biopsies, such as both protocol and event-driven biopsies.

MATERIALS AND METHODS

Study Design

Urine samples were taken from adult (≥18 years) renal or combined renal and pancreas transplant patients prior to a kidney biopsy that was performed according to local center standards as protocol or event-driven biopsies. The target population is thus within the clinical routine and planned for renal allograft biopsies. The patient population consists of patients recruited at least 14 days after transplantation. To ensure broad real-world spectrum of biopsy results, all patients scheduled for a renal allograft biopsy were screened for eligibility.

Between June 2017 and March 2020, 972 transplant recipients were included (1,230 urine samples and matched biopsies, respectively). According to biopsy results, patients were retrospectively assigned to a case group [Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other findings (other non-rejection changes)], a control group [(no rejection): Banff categories 1 (normal biopsy), 5I (mild interstitial fibrosis and atrophy (IFTA)], and an additional group [Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes)]. Criteria for subject categorization—based on the rules of the Banff classification—are detailed in **Figure 1**.

Abbreviations: ABMR, antibody-mediated rejection; AUC_{ROC}, area under the ROC curve, CKD, chronic kidney disease; DSA, donor-specific anti-HLA IgG antibodies; eGFR, estimated glomerular filtration rate; NMR, nuclear magnetic resonance spectroscopy; ROC, receiver operating characteristic; TCMR, T-cell-mediated rejection; MFI, mean fluorescene intensity; MMF, mycofenolate mofetil; MPA, mycophenolic acid.

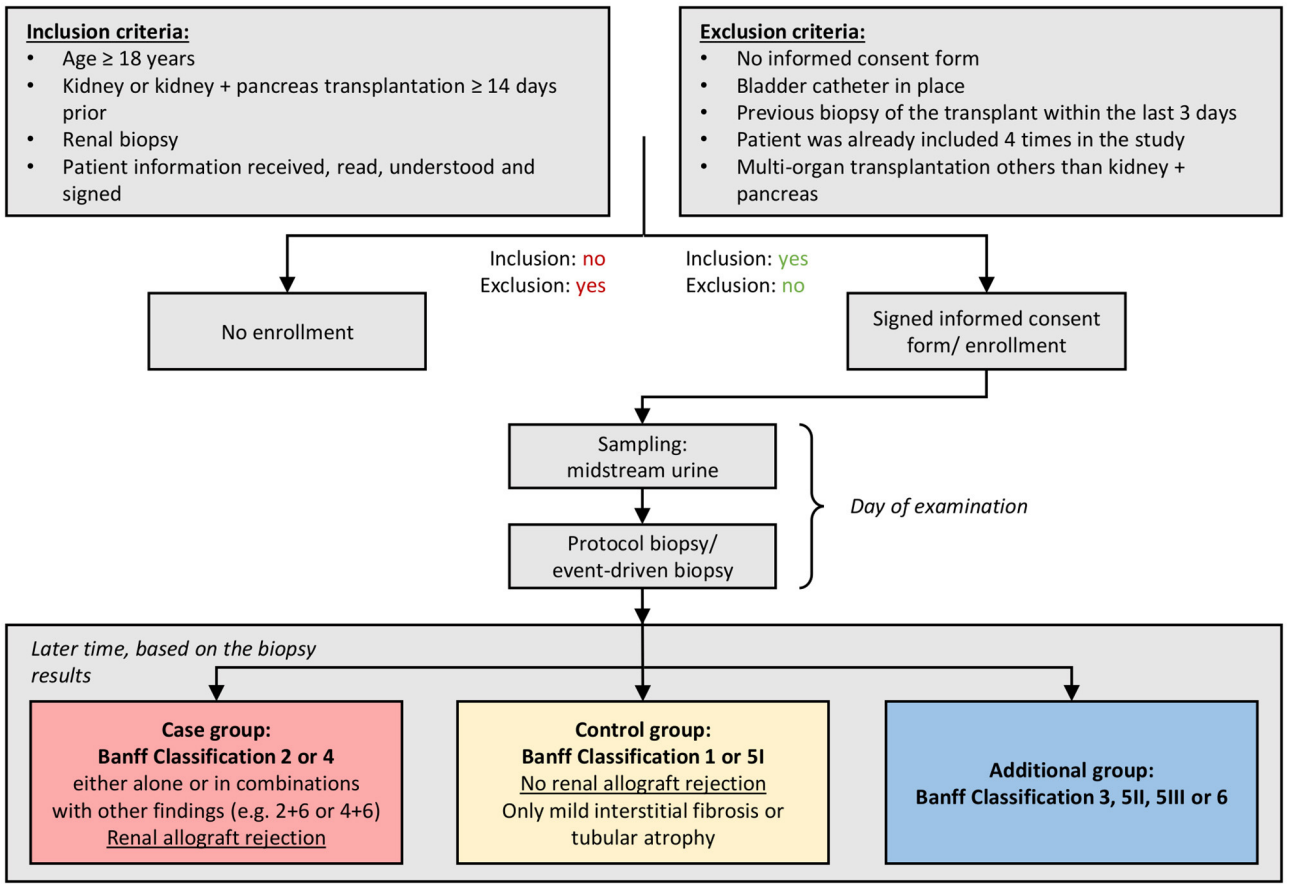

FIGURE 1 | Study sequence. Patients who received a kidney or kidney and pancreas transplantation ≥14 days prior were selected according to the inclusion criteria of the PARASOL study. After informed consent discussion and signed informed consent form, the clinical data were recorded, the midstream urine sample was collected, and the kidney biopsy was taken within the clinical routine. According to biopsy results, patients were assigned either to a case group [Banff category 2 (antibody-mediated rejection [ABMR]) or 4 (T-cell-mediated rejection [TCMR]), either alone or in combination with other findings (other non-rejection changes)], a control group [(no rejection): Banff categories 1 (normal biopsy), 5I (mild interstitial fibrosis and atrophy (IFTA)], and an additional group [Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes)]. The study was then ended for the participant.

Clinical routine shows that patients of the target population may be biopsied several times. In this context, PARASOL collected data on the day of the examination (cross-sectional data), but did not take any data over time (longitudinal data) into account. This resulted in the possibility that some patients were included in the study several times. These patients signed the informed consent forms again and new patient identification numbers were given (at a maximum of four times). The study sequence is similar to an initial recruitment (**Table 1**).

To preclude an interaction between the biopsy procedure and the results of the NMR analysis samples were obtained before biopsies and, in case of two or more sequential biopsies, a sufficiently large interval between the biopsies was defined in the protocol to prevent possible blood residues in urine affecting the NMR analysis of the second sample. In addition, we defined an upper limit for re-recruitment of a patient. The goal was to include an adequate number of patients to get a representative reflection of the biological variance in the study population. Therefore, a maximum of four biopsies per patient could be included in the study. The patients, such as not willing to participate, with a bladder catheter in place, with a previous biopsy of the transplant within the last 3 days, already included four times in the study, and after multi-organ transplantation other than kidney and pancreas, are excluded from the study. Respective clinical data (e.g., date of transplantation, medication, donor-specific anti-HLA IgG antibodies (DSA), former rejections, and infections) were collected on the day of the examination and the urine sample was taken immediately before the planned biopsy. The report of the kidney histopathology analysis, as clinical reference standard, was added to the study documentation after becoming available. Definition of DSA was done individually according to local center standards. The threshold for the positivity was defined as 800–1,000 mean fluorescence intensity (MFI) depending on the local protocol and assay used. The centers used mixed and single antigen (SAB) class I and class II bead assays. No follow-up appointments were planned for the study participants.

TABLE 1 | Examination matrix.

Assessments to be performed	On one day of the regular examinations, ≥14 days after kidney transplantation	Later time	Comment on timing
Informed consent	X		Prior to any study procedure
Demography (a)	X		
Anamnesis (b)	X		Renal transplantation: ≥ 14 days before urine sample collection
			Last renal transplant biopsy: not within the last 3 days before sample collection.
			Last serum creatinine, Cystatin-C (optional), eGFR, CMV/ BKV Infection, Urinary tract infection: at day of (or max. 3 days prior to) urine sampling, during regular care (d)
Inclusion/ exclusion criteria	X		Criteria at day of urine sampling
Sampling of midstream urine	X		≥14 days after renal transplantation, before scheduled transplant biopsy, during regular care
Medication (c)	X		At time of urine sampling
Renal transplant biopsy	X		At same day of urine sampling, at a time point after urine sampling, during regular care
Biopsy result		X	
Final status		X	

(a) Age at enrollment (date of signed ICF), ethnicity, and sex. (b) Date of (last) renal transplantation, date of last renal transplant biopsy, last serum creatinine, last cystatin-C (optional), last estimated glomerular filtration rate (eGFR), existence of donor-specific antibodies (DSA), active CMV infection (based on CMV virus detection via PCR at the time of taking the urine sample), active BKV infection (based on polyoma virus detection in the blood of a patient via PCR testing at the time of taking the urine sample and/or direct staining of polyoma virus in the corresponding renal biopsy), active urinary tract infection, diabetes mellitus, hypertension, and underlying renal disease. (c) Immunosuppressants [tacrolimus, cyclosporine, steroids, mycofenolate mofetil (MMF)/mycophenolic acid (MPA), other immunosuppressants], antibiotics, antihypertensives, and antidiabetics. (d) If status was determined >3 days before urine sampling, it is considered as "N/A".

The study was approved by the local Ethics Committees of all participating centers. The written informed consent was received from participants prior to study inclusion.

Kidney Biopsies

The results of morphologic renal allograft evaluation were documented as the reference standard. The study included protocol biopsies (as per local center protocol), event-driven biopsies, as well as follow-up biopsies performed for monitoring of treatment responses. Specimens were evaluated in the context of center routine by experienced local renal pathologists following the rules of the 2018 Reference Guide to the Banff Classification of Renal Allograft Pathology (9). For analysis in relation to biomarker results, biopsies were grouped according to Banff diagnostic categories as follows: (i) case group (rejection): Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other findings (other non-rejection changes) (ii) control group (no rejection): Banff categories 1 (normal biopsy), 5I (mild IFTA) (iii) additional group: [Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes)], respectively.

NMR-Analysis

Spontaneous mid-stream urine samples were collected and measured by NMR spectroscopy at numares AG (Regensburg, Germany). For this, a volume of 600 µl of each urine sample was mixed with 150 µl of Axinon® urine additive solution in a centrifuge tube. The samples were centrifuged at 20,000 g for 10 min at 20°C, and 600 µl of the supernatant were transferred to 5 mm NMR tubes and kept at 2–6°C until measurement. All measurements were carried out on a Bruker Avance II + 600 MHz NMR-spectrometer as already described before (7). Using the Axinon® renalTX-SCORE® system, urinary metabolite quantification and test results were generated in a fully automated manner. The resulting score correlates with the probability of allograft rejection and ranges between 0 and 100 with low to high probability and is based on an NMR-based pattern encompassing the metabolites alanine, citrate, lactate, and urea.

Statistical Analysis Strategy

Statistical analyses were planned in two ways: a descriptive analysis and a performance analysis. The former was to address the patient data and highlight demographic facts using clinical data derived from the corresponding case report form (CRF). It was planned that important variables, such as sex, age, ethnicity, and various disease factors of the patients would be investigated. Furthermore, all variables were considered to be used for the performance analysis.

It was clear that it may be necessary to exclude patient data from the performance analysis in case that technically no renalTX-SCORE output could be determined by NMR measurement. The performance analysis should use standard methods and assess conventional parameters for evaluating the quality of the diagnostic patterns developed. Particular attention to the area under the receiver operating characteristic (ROC) curve (AUC_{ROC}), specificity, and sensitivity should be paid. In addition, certain subgroup performance analyses should be obtained in concerns of different cut-off values with regard to specificity and sensitivity.

Sample Size Estimation

The number of cases was based on the observed values for the AUC_{ROC} values of the NMR-based patterns obtained in the UMBRELLA (8) study for the detection of acute renal allograft rejection ($AUC_{ROC} = 0.75$). In particular, the number of cases in the multicenter validation should be large enough to prove a difference between the AUC_{ROC} value of the developed NMR-based tests and a minimum AUC_{ROC} (null hypothesis H_0) of 0.675 at the level of significance $\alpha = 5\%$ with a power of 80%. The percentage of positive biopsy results (prevalence of acute Banff 4 renal allograft rejection) in the UMBRELLA collective was 27% (54/203 biopsies). The expected percentage

TABLE 2 | Urine sample characteristics collected by the six different study centers.

		Total	Barcelona Bel.	Barcelona FP.	Grenoble	Praha	Vienna	Regensburg
Number of patients	Total	972	21	49	348	150	219	185
	With one urine sample	790/972 (81.3%)	21/21 (100.0%)	47/49 (95.9%)	256/348 (73.6%)	138/150 (92.0%)	209/219 (95.4%)	119/185 (64.3%)
	With multiple urine samples	182/972 (18.7%)	0/21 (0.0%)	2/49 (4.1%)	92/348 (26.4%)	12/150 (8.0%)	10/219 (5.6%)	66/185 (35.7%)
Number of urine samples	Total	1,230	21	51	479	164	229	286
Urine samples per patient	Mean	1.3	1.0	1.0	1.4	1.1	1.0	1.5
Status	Case	237/1,230 (19.3%)	1/21 (4.8%)	4/51 (7.8%)	73/479 (15.2%)	60/164 (36.6%)	41/229 (17.9%)	58/286 (20.3%)
	Control	541/1,230 (44.0%)	15/21 (71.4%)	23/51 (45.1%)	260/479 (54.3%)	34/164 (20.7%)	96/229 (41.9%)	113/286 (39.5%)
	Additional	452/1,230 (36.7%)	5/21 (23.8%)	24/51 (47.1%)	146/479 (30.5%)	70/164 (42.7%)	92/229 (40.2%)	115/286 (40.2%)
Age	Range	19–84	37–78	26–75	21–84	21–82	20–79	19–84
	Mean ± SD	53.7 ± 13.8	58.7 ± 11.1	55.0 ± 13.3	54.2 ± 14.9	53.2 ± 13.2	54.3 ± 12.8	52.3 ± 13.2
Sex	Male	810/1,230 (65.9%)	12/21 (57.1%)	26/51 (51.0%)	311/479 (64.9%)	116/164 (70.7%)	151/229 (65.9%)	194/286 (67.8%)
	Female	420/1,230 (34.1%)	9/21 (42.9%)	25/51 (49.0%)	168/479 (35.1%)	48/164 (29.3%)	78/229 (34.1%)	92/286 (32.2%)
Ethnicity	African	24/1,230 (2.0%)	0/21 (0.0%)	1/51 (2.0%)	21/479 (4.4%)	0/164 (0.0%)	2/229 (0.9%)	0/286 (0.0%)
	Asian	8/1,230 (0.7%)	0/21 (0.0%)	0/51 (0.0%)	6/479 (1.3%)	1/164 (0.6%)	1/229 (0.4%)	0/286 (0.0%)
	Caucasian	1,184/1,230 (96.3%)	21/21 (100.0%)	45/51 (88.2%)	450/479 (93.9%)	162/164 (98.8%)	226/229 (98.7%)	280/286 (97.9%)
	Other	13/1,230 (1.1%)	0/21 (0.0%)	5/51 (9.8%)	2/479 (0.4%)	0/164 (0.0%)	0/229 (0.0%)	6/286 (2.1%)
	Not specified	1/1,230 (0.1%)	0/21 (0.0%)	0/51 (0.0%)	0/479 (0.0%)	1/164 (0.6%)	0/229 (0.0%)	0/286 (0.0%)
Biopsy reason	Event-driven	536/1,230 (43.6%)	8/21 (38.1%)	15/51 (29.4%)	164/479 (34.2%)	114/164 (69.5%)	97/229 (42.4%)	138/286 (48.3%)
	Follow-up	104/1,230 (8.5%)	0/21 (0.0%)	1/51 (2.0%)	67/479 (14.0%)	0/164 (0.0%)	4/229 (1.7%)	32/286 (11.2%)
	Protocol	590/1,230 (48.0%)	13/21 (61.9%)	35/51 (68.6%)	248/479 (51.8%)	50/164 (30.5%)	128/229 (55.9%)	116/286 (40.6%)
Time after TX	≤6 months	628/1,230 (51.1%)	8/21 (38.1%)	20/51 (39.2%)	238/479 (49.7%)	92/164 (56.1%)	113/229 (49.3%)	157/286 (54.9%)
	[6,12] months	132/1,230 (10.7%)	4/21 (19.0%)	15/51 (29.4%)	60/479 (12.5%)	16/164 (9.8%)	22/229 (9.6%)	15/286 (5.2%)
	[1,4] years	222/1,230 (18.0%)	6/21 (28.6%)	11/51 (21.6%)	86/479 (18.0%)	29/164 (17.7%)	59/229 (25.8%)	31/286 (10.8%)
	>4 years	248/1,230 (20.2%)	3/21 (14.3%)	5/51 (9.8%)	95/479 (19.8%)	27/164 (16.5%)	35/229 (15.3%)	83/286 (29.0%)
Anamnesis	Infections	175/1,230 (14.2%)	1/21 (4.8%)	5/51 (9.8%)	101/479 (21.1%)	16/164 (9.8%)	39/229 (17.0%)	13/286 (4.5%)
	Diabetes	364/1,230 (29.6%)	8/21 (38.1%)	9/51 (17.6%)	123/479 (25.7%)	56/164 (34.1%)	62/229 (27.1%)	106/286 (37.1%)
	Hypertension	1,092/1,230 (88.8%)	17/21 (81.0%)	48/51 (94.1%)	389/479 (81.2%)	149/164 (90.9%)	211/229 (92.1%)	278/286 (97.2%)
Medication	Tacrolimus	1,141/1,230 (92.8%)	20/21 (95.2%)	50/51 (98.0%)	429/479 (89.6%)	152/164 (92.7%)	216/229 (94.3%)	274/286 (95.8%)
	Cyclosporine	31/1,230 (2.5%)	0/21 (0.0%)	0/51 (0.0%)	8/479 (1.7%)	5/164 (3.0%)	11/229 (4.8%)	7/286 (2.4%)
	Steroids	975/1,230 (79.3%)	18/21 (85.7%)	51/51 (100.0%)	317/479 (66.2%)	156/164 (95.1%)	224/229 (97.8%)	209/286 (73.1%)
	MMF/MPA	1,082/1,230 (88.0%)	21/21 (100.0%)	42/51 (82.4%)	389/479 (81.2%)	138/164 (84.1%)	217/229 (94.8%)	275/286 (96.2%)
	Antibiotics	306/1,230 (24.9%)	4/21 (19.0%)	2/51 (3.9%)	107/479 (22.3%)	31/164 (18.9%)	83/229 (36.2%)	79/286 (27.6%)
	Antihyper-tensives	1,083/1,230 (88.0%)	17/21 (81.0%)	48/51 (94.1%)	386/479 (80.6%)	147/164 (89.6%)	209/229 (91.3%)	276/286 (96.5%)
	Antidiabetics	337/1,230 (27.4%)	8/21 (38.1%)	12/51 (23.5%)	123/479 (25.7%)	52/164 (31.7%)	62/229 (27.1%)	80/286 (28.0%)

Overall and for each center respectively, the distribution for different subsets is shown. The numbers and relative frequencies in the categories status, age, sex, ethnicity, biopsy type, time after renal transplantation (TX), anamnesis, and medication refer to the number of urine samples. Infections include active CMV (based on CMV virus detection via PCR at the time of taking the urine sample), active BKV (based on polyoma virus detection in the blood of a patient via PCR testing at the time of taking the urine sample and/or direct staining of polyoma virus in the corresponding renal biopsy) and/or active urinary tract infections. Case group: Banff category 2 (ABMR [antibody-mediated rejection]) or 4 (T-cell-mediated rejection [TCMR]), either alone or in combination with other findings (other non-rejection changes); control group [(no rejection): Banff categories 1 (normal biopsy), 5I (mild interstitial fibrosis and atrophy (IFTA)]; additional group [Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes)]. For further clarification: by study design, at day of urine sample collection a biopsy was performed.

of positive biopsy results in the PARASOL study was therefore estimated to be 30%. Accordingly, 151 rejections (cases) and 334 controls were needed. The indicated number of cases was calculated using the MedCalc v.12.7.7.0-64 bit, observing a two-tailed binomial test with a power of 80%. To compensate for the losses in recruiting, withdrawal of consent, insufficient urine quantities, losses, etc., a 15% safety margin was calculated so that recruiting should include 174 cases: renal allograft rejection Banff 2, 4, either alone or in combination with other findings, e.g., 2 + 6 or 4 + 6, and 384 controls: Banff classification 1 and 5I.

The estimated number of cases was exceeded with 237 cases instead of estimated 174 cases and 541 controls instead of the planned 384.

Primary Endpoint

The primary endpoint of the study is the respective AUC_{ROC} value of the ROC curve for the NMR-based pattern. Acute renal allograft rejection is defined according to the Banff classification and categorized 2 (ABMR), 4 (TCMR), either alone or in combinations with other findings, e.g., 2 + 6 or 4 + 6. The AUC_{ROC} values and their 95% *CI*s are determined as a measure of diagnostic accuracy. It should be tested whether the diagnostic accuracy is to be proven with an AUC_{ROC} value of at least 0.675 under the assumption that the observed AUC_{ROC} value itself is 0.75.

Secondary Endpoint

As a secondary endpoint, the recruited collective will be analyzed with respect to the determination of cut-off values associated with a sensitivity and specificity of 90% and the respective specificity at 90% sensitivity and sensitivity at 90% specificity including the 95% *CI* for the exact definition of the NMR-based pattern to detect renal allograft rejection. In addition, for the cut-off values determined, the sensitivities and specificities in patients with Banff classification 3, 5II, 5III, and 6 will be determined in a subgroup analysis if enough samples are available. Furthermore, the demographic and clinical parameters will be used to examine whether individual parameters or a group of these parameters are suitable for reliably predicting the occurrence of renal allograft rejection in this large, non-stratified patient group.

RESULTS

In the context of PARASOL (multicenter observational prospective trial including six European transplant centers), a total of 1,230 urine samples for metabolite evaluation and corresponding histopathology results (972 patients; 1.3 samples per patient) were collected (recruitment period between June 2017 and March 2020).

Table 2 provides sample and patient characteristics for the overall cohort and individual centers. Among 1,230 biopsy specimens, 237 samples (19.3%) were assigned to the case group, 541 to the control group (44.0%), and 452 (36.7%) samples were assigned to the additional group. Overall, 810 samples (65.9%) were obtained from male patients and 420 (34.1%) from female patients. The mean recipient age at the time of study inclusion was 53.7 ± 13.8 years (range 19–84 years) and most of the patients were Caucasian (96.3%).

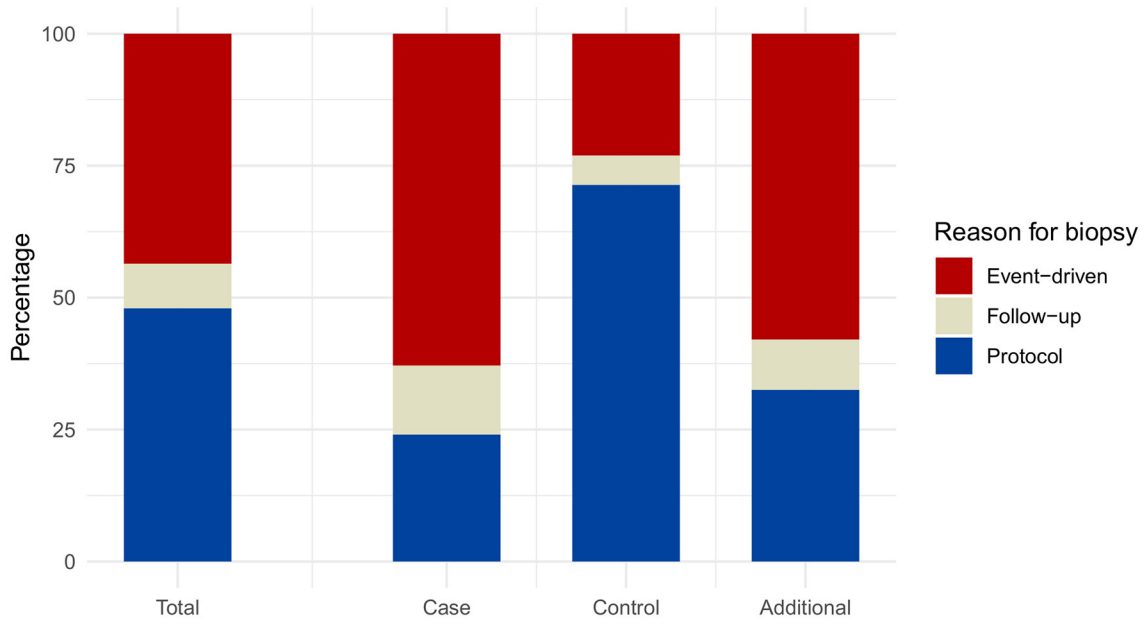

FIGURE 2 | Distribution of the reasons for biopsy. Relative frequencies are displayed for the total number of samples (irrespective of the group assignment), as well as for each of the status groups. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other non-rejection changes; Control group: Banff categories 1 (normal biopsy), 5I (mild IFTA); Additional group: Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes).

Tacrolimus (92.8%), mycophenolate mofetil (88.0%), and steroids (79.3%) were the most frequently used immunosuppressive drugs. Antihypertensives and antidiabetics were used in 88.0 and 27.4% of the patients, respectively. About 88.8% of patients suffered from hypertension, 29.6% were diabetics, and 14.2% had any infection (BKV, CMV, or urinary tract infection) at the time of inclusion into the study.

Of the 1,230 urine samples, 628 (51.1%) were collected within the first 6 months after transplantation, 132 (10.7%) between 6 and 12 months, 222 (18.0%) between 1 and 4 years, and 248 (20.2%) more than 4 years after transplantation. Most of the samples [590 (48.0%)] were in the context of protocol biopsies, followed by event-driven biopsies [536 (43.6%)], and follow-up biopsies [104 (8.5%)].

The distribution of biopsy types for case, control, and additional group differed in the groups. In the case group, 62.9% event-driven biopsies were noted, 24.1% protocol biopsies, and 13.1% follow-up biopsies. In the control group, 71.3% protocol biopsies, 23.1% event-driven biopsies, and 5.5% follow-up biopsies were seen. The additional group consisted of 58.0% event-driven biopsies, 32.5% protocol biopsies, and 9.5% follow-up biopsies (**Figure 2** and **Supplementary Table S1**). Additionally **Supplementary Figure S1** and **Supplementary Table S2** show the distribution of the case, control, and additional groups within each of the three biopsy reasons: event-driven, follow-up, and protocol.

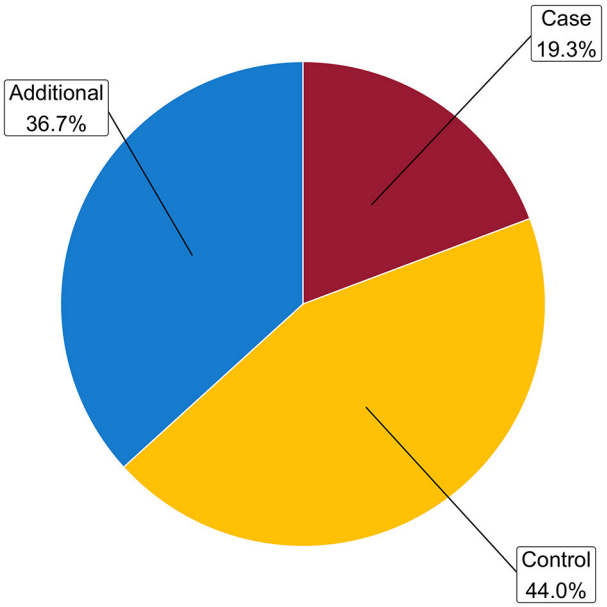

FIGURE 3 | Distribution of the status groups. Relative frequencies are derived from the total number of samples. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other findings (other non-rejection changes); control group [(no rejection): Banff categories 1 (normal biopsy), 5I (mild IFTA); additional group (Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes)].

Distribution of Banff Categories Within Case (Rejection), Control, and Additional Group

Most of the biopsies (44.0%) were assigned to the control group, corresponding histopathology showed classification into the Banff categories 1 (normal biopsy) (28.0% out of all biopsies) and Banff 5I (mild IFTA) (16.0% out of all biopsies) for the largest proportion of findings, followed by other combinations (15.2%) and allograft fibrosis classified as Banff grades 5 II (moderate IFTA) and III (severe IFTA) (10.0%) both belonging to the additional group (**Figure 3** and **Supplementary Table S3**).

Within the group of rejection, ABMR (Banff 2) represented the largest proportion (64.6%), followed by TCMR (Banff 4: 30.4%). Combined ABMR and TCMR was infrequent (5.1%) (**Figure 4** and **Supplementary Table S3**). The control group showed further a higher proportion of Banff 1 (63.6%) than of Banff 5I (36.4%) graded biopsies (**Figure 4** and **Supplementary Table S3**). In the additional group, the highest proportion of pathology findings belongs to other combinations (41.4%), Banff 5II and III (27.2%), followed by Banff 6 (17.0%) and Banff 3 (14.4%) (**Figure 4** and **Supplementary Table S3**).

Type of Renal Allograft Rejections Dependent From Time After Transplantation

Within the case group, ABMR classified as Banff 2 category (either isolated or in combination with other lesions) represented a large group of rejections with 42.0% already within the first 6 months post transplantation. With increasing time post-transplantation, the proportion of antibody mediated rejection increased up to 84.2% (after 4 years). TCMR (Banff 4 and Banff 4 + x) decreased from 50.7 to 11.8% over time. The combination of both stayed stable with 7.3% and 4.0% (**Figure 5A** and **Supplementary Table S4**).

In the control group within the first 6 months, most biopsy samples were assigned to Banff 1 (70.8%), whereas after 4 years, almost 75% of biopsies of the control group were classified as Banff 5I (**Figure 5B** and **Supplementary Table S4**).

In the additional group, the proportion of borderline rejections (Banff 3) decreased from 21.5 to 2.4% over time, whereas the amount of graft fibrosis represented by Banff 5II and III findings increased to 33.6% (**Figure 5C** and **Supplementary Table S4**).

Detection of Donor-Specific Antibodies (DSA) Dependent on Sample Classification and Banff Categories

For 257 of the 1,230 samples, positive corresponding DSA results were documented (20.9%) (**Table 3**). Within the case group for 43.5% of the samples (103/237), DSAs were noted. 84 from these samples (81.6%) showed ABMR (Banff 2). Simultaneous presence of DSA in blood was documented for only 80/541 samples (14.8%) from the control group patients. Therein, in

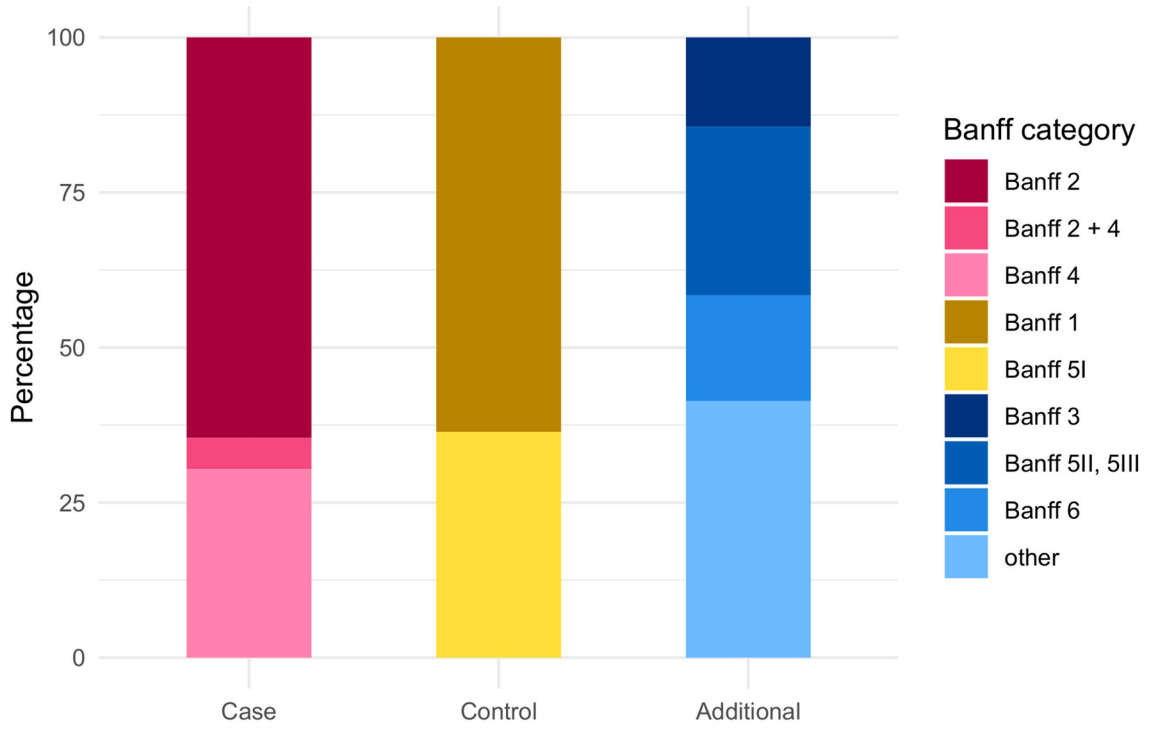

FIGURE 4 | Distribution of Banff categories among the case, control, and additional group. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other non-rejection changes; Control group: Banff categories 1 (normal biopsy), 5I (mild IFTA); Additional group: Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes).

the subgroup associated with Banff 1 findings in the biopsy 46/80 (57.5%) samples corresponded to patients with DSA, in the subgroup with Banff 5I lesion, the rate was 34/80 (42.5%). In the additional group, 74/452 (16.4%) samples were associated with DSA.

Distribution of Chronic Kidney Disease (CKD) Stages Among Status, Time After Renal Transplantation, and Recipient Age

Kidney function was seen to be dependent on the status (case/control/additional), time after transplantation, and the recipient age.

Regarding the severity of CKD of the study population, patients with allograft rejections and from the additional group showed significantly worse kidney function than controls (samples 318 corresponding with CKD stage 4 or 5 32.9% in cases vs. 11.4% in controls vs. 32.6% in the additional group; **Table 4**). Whereas control group samples were associated with good allograft function up to 4 years, case group samples and additional group samples corresponded with worse kidney function (**Table 4**).

With regards to the allograft recipient age, it could be seen that controls showed a better renal function within all age groups compared with cases and samples from the additional group (**Table 5**).

DISCUSSION

For patients with end stage renal disease, kidney transplantation is the renal replacement therapy of choice as it significantly improves the life quality and life expectancy (10). However, by far not all suitable patients can be offered a donor kidney as both post-mortal and living kidney donation are limited. With the observed increase in patients in the majority of countries worldwide suffering from renal disease, the gap between donor kidneys available and renal transplantation needed is more and more widening (11–13). One of the reasons of this observation is that renal allograft survival is limited and often shorter as the survival of recipient. In consequence, increasing numbers of patients with end stage renal disease need more than only one kidney transplantation and in countries with a very high rate of post-mortal organ donors, such as Spain, loss of a previous allograft is nowadays one of the main causes for listing patients for renal transplantation (14–23)[1].

Therefore, one of the major goals in transplant medicine is to prolong the time of allograft survival, especially as more and more allografts with extended donor criteria have to be used to overcome organ shortage (2, 24–28). Despite major improvements in transplant patient care and therein immunosuppressive treatment, acute and chronic rejection processes often irreversibly damaging renal allograft tissues are

[1]http://www.registrorenal.es/

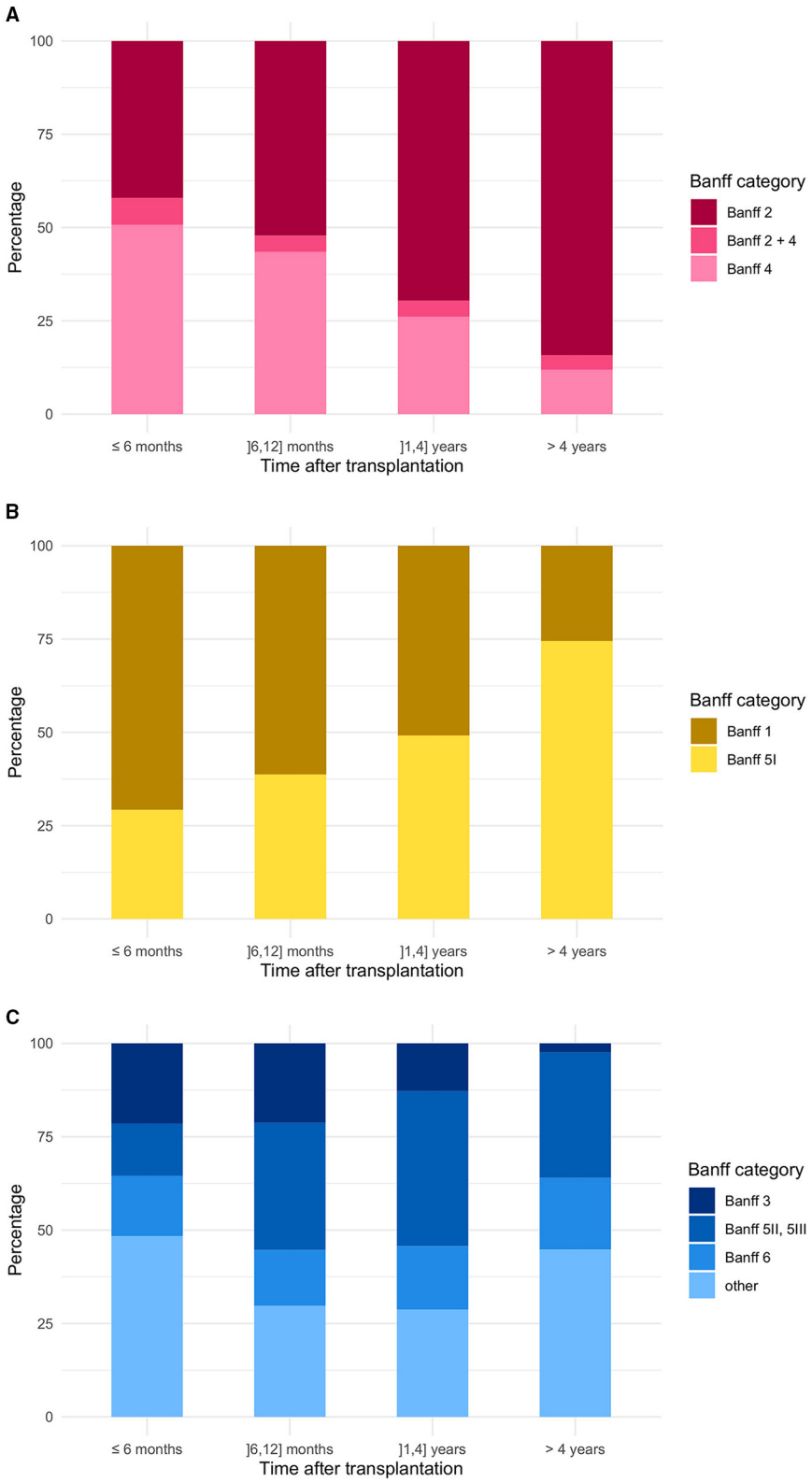

FIGURE 5 | Banff category distribution in concerns of time after renal transplantation **(A)** in the case group, **(B)** in the control group, and **(C)** in the additional group. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other non-rejection changes; Control group: Banff categories 1 (normal biopsy), 5I (mild IFTA); Additional group: Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes).

TABLE 3 | Existence of donor-specific anti-HLA IgG antibodies (DSA) in the status groups and each of the Banff categories.

		Total	DSA negative	DSA positive	DSA not specified
Number of samples		1,230	802	257	171
Status: case	Total	237	106/237 (44.7%)	103/237 (43.5%)	28/237 (11.8%)
	Banff 2	153/237 (64.6%)	45/153 (29.4%)	84/153 (54.9%)	24/153 (15.7%)
	Banff 2 + 4	12/237 (5.1%)	5/12 (41.7%)	7/12 (58.3%)	0/12 (0.0%)
	Banff 4	72/237 (30.4%)	56/72 (77.8%)	12/72 (16.7%)	4/72 (5.6%)
Status: control	Total	541	395/541 (73.0%)	80/541 (14.8%)	66/541 (12.2%)
	Banff 1	344/541 (63.6%)	258/344 (75.0%)	46/344 (13.4%)	40/344 (11.6%)
	Banff 5I	197/541 (36.4%)	137/197 (69.5%)	34/197 (17.3%)	26/197 (13.2%)
Status: additional	Total	452	301/452 (66.6%)	74/452 (16.4%)	77/452 (17.0%)
	Banff 3	65/452 (14.4%)	41/65 (63.1%)	15/65 (23.1%)	9/65 (13.8%)
	Banff 5II, 5III	123/452 (27.2%)	68/123 (55.3%)	27/123 (22.0%)	28/123 (22.8%)
	Banff 6	77/452 (17.0%)	58/77 (75.3%)	4/77 (5.2%)	15/77 (19.5%)
	Other	187/452 (41.4%)	134/187 (71.7%)	28/187 (15.0%)	25/187 (13.4%)

Relative frequencies are derived from the respective total number of samples in the three status groups. The order of the Banff categories is based on the assignment to case, control, and additional. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other non-rejection changes; Control group: Banff categories 1 (normal biopsy), 5I (mild IFTA); Additional group: Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes).

TABLE 4 | Distribution of chronic kidney disease (CKD) stages among status groups and time after renal transplantation (TX).

		Total	CKD Stage 1	CKD Stage 2	CKD Stage 3A	CKD Stage 3B	CKD Stage 4	CKD Stage 5	CKD Stage not specified
Number of samples		1,230	34	193	253	288	235	52	175
Status: case	Total	237	2/237 (0.8%)	31/237 (13.1%)	36/237 (15.2%)	62/237 (26.2%)	58/237 (24.5%)	20/237 (8.4%)	28/237 (11.8%)
	≤6 months	69/237 (29.1%)	1/69 (1.4%)	16/69 (23.2%)	9/69 (13.0%)	19/69 (27.5%)	10/69 (14.5%)	7/69 (10.1%)	7/69 (10.1%)
	[6,12] months	23/237 (9.7%)	1/23 (4.3%)	3/23 (13.0%)	2/23 (8.7%)	7/23 (30.4%)	6/23 (26.1%)	2/23 (8.7%)	2/23 (8.7%)
	[1,4] years	69/237 (29.1%)	0/69 (0.0%)	9/69 (13.0%)	11/69 (15.9%)	19/69 (27.5%)	20/69 (29.0%)	2/69 (2.9%)	8/69 (11.6%)
	>4 years	76/237 (32.1%)	0/76 (0.0%)	3/76 (3.9%)	14/76 (18.4%)	17/76 (22.4%)	22/76 (28.9%)	9/76 (11.8%)	11/76 (14.5%)
Status: control	Total	541	22/541 (4.1%)	115/541 (21.3%)	137/541 (25.3%)	116/541 (21.4%)	52/541 (9.6%)	10/541 (1.8%)	89/541 (16.5%)
	≤6 months	373/541 (68.9%)	15/373 (4.0%)	88/373 (23.6%)	110/373 (29.5%)	83/373 (22.3%)	28/373 (7.5%)	7/373 (1.9%)	42/373 (11.3%)
	[6,12] months	62/541 (11.5%)	4/62 (6.5%)	12/62 (19.4%)	12/62 (19.4%)	12/62 (19.4%)	7/62 (11.3%)	1/62 (1.6%)	14/62 (22.6%)
	[1,4] years	59/541 (10.9%)	3/59 (5.1%)	10/59 (16.9%)	10/59 (16.9%)	10/59 (16.9%)	6/59 (10.2%)	1/59 (1.7%)	19/59 (32.2%)
	>4 years	47/541 (8.9%)	0/47 (0.0%)	5/47 (10.6%)	5/47 (10.6%)	11/47 (23.4%)	11/47 (23.4%)	1/47 (2.1%)	14/47 (29.8%)
Status: additional	Total	452	10/452 (2.2%)	47/452 (10.4%)	80/452 (17.7%)	110/452 (24.3%)	125/452 (27.7%)	22/452 (4.9%)	58/452 (12.8%)
	≤ 6 months	186/452 (41.2%)	7/186 (3.8%)	26/186 (14.0%)	35/186 (18.8%)	54/186 (29.0%)	46/186 (24.7%)	9/186 (4.8%)	9/186 (4.8%)
	[6,12] months	47/452 (10.4%)	3/47 (6.4%)	5/47 (10.6%)	13/47 (27.7%)	7/47 (14.9%)	9/47 (19.1%)	4/47 (8.5%)	6/47 (12.8%)
	[1,4] years	94/452 (20.8%)	0/94 (0.0%)	7/94 (7.4%)	17/94 (18.1%)	27/94 (28.7%)	25/94 (26.6%)	3/94 (3.2%)	15/94 (16.0%)
	>4 years	125/452 (27.7%)	0/125 (0.0%)	9/125 (7.2%)	15/125 (12.0%)	22/125 (17.6%)	45/125 (36.0%)	6/125 (4.8%)	28/125 (22.4%)

The relative frequencies are derived from the respective total number of samples in each status group. However, the relative frequencies for each time after transplantation category in the respective status groups are computed using the total number of samples in the respective time after transplantation category. Samples that did not have an eGFR value assigned are included in the "CKD not specified" group. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other non-rejection changes; Control group: Banff categories 1 (normal biopsy), 5I (mild IFTA); Additional group: Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes).

still a major risk for premature allograft loss (1, 2, 29). Strategies to completely prevent allograft rejection are still missing, and diagnostic procedures for early detection of rejection are still suboptimal.

Until now, a histopathological evaluation of a renal allograft biopsy is the gold standard to detect the cause of an allograft malfunction after extra renal problems, such as perfusion deficits or urinary obstruction could be ruled out (1, 3, 4). Molecular analyses of renal tissue samples become more and more important and look very promising to become routine in the future, however, so far in many countries, this technique is not available due to its costs not routinely covered by medical insurance companies.

Several biomarkers have been previously described mainly in blood samples to detect allograft damage without performing renal biopsies, and costs and insufficient proof of usefulness under routine conditions are among the reasons for only limited use in daily clinical practice so far (5, 6, 30–35).

TABLE 5 | Distribution of CKD stages in different age groups shown for case, control and additional group.

		Total	19–29 years	30–39 years	40–49 years	50–59 years	60–69 years	70–79 years	80–84 years
Number of samples		1,230	73	141	243	306	297	158	12
Status: case	Total	237	27/237 (11.4%)	27/237 (11.4%)	66/237 (27.8%)	48/237 (20.3%)	48/237 (20.3%)	18/237 (7.6%)	3/237 (1.3%)
	CKD Stage 1	2/237 (0.8%)	0/2 (0.0%)	1/2 (50.0%)	1/2 (50.0%)	0/2 (0.0%)	0/2 (0.0%)	0/2 (0.0%)	0/2 (0.0%)
	CKD Stage 2	31/237 (13.1%)	9/31 (29.0%)	3/31 (9.7%)	12/31 (38.7%)	4/31 (12.9%)	2/31 (6.5%)	1/31 (3.2%)	0/31 (0.0%)
	CKD Stage 3A	36/237 (15.2%)	6/36 (16.7%)	6/36 (16.7%)	11/36 (30.6%)	6/36 (16.7%)	6/36 (16.7%)	1/36 (2.8%)	0/36 (0.0%)
	CKD Stage 3B	62/237 (26.2%)	7/62 (11.3%)	7/62 (11.3%)	14/62 (22.6%)	12/62 (19.4%)	16/62 (25.8%)	5/62 (8.1%)	1/62 (1.6%)
	CKD Stage 4	58/237 (24.5%)	5/58 (8.6%)	8/58 (13.8%)	12/58 (20.7%)	12/58 (20.7%)	12/58 (20.7%)	7/58 (12.1%)	2/58 (3.4%)
	CKD Stage 5	20/237 (8.4%)	0/20 (0.0%)	0/20 (0.0%)	8/20 (40.0%)	6/20 (30.0%)	5/20 (25.0%)	1/20 (5.0%)	0/20 (0.0%)
	CKD Stage not specified	28/237 (11.8%)	0/28 (0.0%)	2/28 (7.1%)	8/28 (28.6%)	8/28 (28.6%)	7/28 (25.0%)	3/28 (10.7%)	0/28 (0.0%)
Status: control	Total	541	22/541 (4.1%)	64/541 (11.8%)	96/541 (17.7%)	140/541 (25.9%)	139/541 (25.7%)	75/541 (13.9%)	5/541 (0.9%)
	CKD Stage 1	22/541 (4.1%)	3/22 (13.6%)	7/22 (31.8%)	2/22 (9.1%)	5/22 (22.7%)	5/22 (22.7%)	0/22 (0.0%)	0/22 (0.0%)
	CKD Stage 2	115/541 (21.3%)	6/115 (5.2%)	22/115 (19.1%)	25/115 (21.7%)	27/115 (23.5%)	23/115 (20.0%)	10/115 (8.7%)	2/115 (1.7%)
	CKD Stage 3A	137/541 (25.3%)	4/137 (2.9%)	16/137 (11.7%)	22/137 (16.1%)	46/137 (33.6%)	33/137 (24.1%)	15/137 (10.9%)	1/137 (0.7%)
	CKD Stage 3B	116/541 (21.4%)	5/116 (4.3%)	7/116 (6.0%)	17/116 (14.7%)	25/116 (21.6%)	37/116 (31.9%)	24/116 (20.7%)	1/116 (0.9%)
	CKD Stage 4	52/541 (9.6%)	2/52 (3.8%)	4/52 (7.7%)	10/52 (19.2%)	10/52 (19.2%)	15/52 (28.8%)	10/52 (19.2%)	1/52 (1.9%)
	CKD Stage 5	10/541 (1.8%)	0/10 (0.0%)	0/10 (0.0%)	1/10 (10.0%)	2/10 (20.0%)	4/10 (40.0%)	3/10 (30.0%)	0/10 (0.0%)
	CKD Stage not specified	89/541 (16.5%)	2/89 (2.2%)	8/89 (9.0%)	19/89 (21.3%)	25/89 (28.1%)	22/89 (24.7%)	13/89 (14.6%)	0/89 (0.0%)
Status: additional	Total	452	24/452 (5.3%)	50/452 (11.1%)	81/452 (17.9%)	118/452 (26.1%)	110/452 (24.3%)	65/452 (14.4%)	4/452 (0.9%)
	CKD Stage 1	10/452 (2.2%)	3/10 (30.0%)	1/10 (10.0%)	3/10 (30.0%)	2/10 (20.0%)	1/10 (10.0%)	0/10 (0.0%)	0/10 (0.0%)
	CKD Stage 2	47/452 (10.4%)	4/47 (8.5%)	11/47 (23.4%)	6/47 (12.8%)	16/47 (34.0%)	6/47 (12.8%)	4/47 (8.5%)	0/47 (0.0%)
	CKD Stage 3A	80/452 (17.7%)	4/80 (5.0%)	10/80 (12.5%)	22/80 (27.5%)	20/80 (25.0%)	13/80 (16.2%)	11/80 (13.8%)	0/80 (0.0%)
	CKD Stage 3B	110/452 (24.3%)	4/110 (3.6%)	8/110 (7.3%)	19/110 (17.3%)	30/110 (27.3%)	32/110 (29.1%)	17/110 (15.5%)	0/110 (0.0%)
	CKD Stage 4	125/452 (27.7%)	3/125 (2.4%)	10/125 (8.0%)	20/125 (16.0%)	32/125 (25.6%)	37/125 (29.6%)	20/125 (16.0%)	3/125 (2.4%)
	CKD Stage 5	22/452 (4.9%)	0/22 (0.0%)	4/22 (18.2%)	3/22 (13.6%)	4/22 (18.2%)	6/22 (27.3%)	4/22 (18.2%)	1/22 (4.5%)
	CKD Stage not specified	58/452 (12.8%)	6/58 (10.3%)	6/58 (10.3%)	8/58 (13.8%)	14/58 (24.1%)	15/58 (25.9%)	9/58 (15.5%)	0/58 (0.0%)

Within each of the status groups, the bold numbers and relative frequencies refer to the total number of samples in that group. However, the relative frequencies for each age group in the respective CKD stages are calculated using the total number of samples in the respective age group within the status group. Samples that did not have an eGFR value assigned are included in the CKD "not specified" group. Case group: Banff category 2 (ABMR) or 4 (TCMR), either alone or in combination with other non-rejection changes; Control group: Banff categories 1 (normal biopsy), 5I (mild IFTA); Additional group: Banff categories 3 (suspicious for TCMR), 5II (moderate IFTA), 5III (severe IFTA), or 6 (other non-rejection changes).

A characteristic urinary metabolite constellation for the detection of TCMR in renal allografts has been previously described by our group (7, 8). In combination with the eGFR values of patient, this test was previously proposed as a valuable support in biopsy decision-making and routine follow-up after renal transplantation. However, so far two main reasons limit the use of this test system in the clinic. The one is that all results generated so far were based on single center studies. The second reason is that the original cohort used to develop the test system was dominated by cellular rejections as these could be easily diagnosed in corresponding kidney biopsies at the time of development.

To overcome these limitations, the PARASOL study was initiated as a further completely independent validation study. Major aims of the PARASOL study were evaluation of the test system completely under routine conditions in six large, different European transplant centers. All centers were completely free in including suitable patients transplanted with donor organs retrieved as usually done in the respective country. Additionally, all treatment strategies, i.e., for immunosuppressive therapy, concomitant medication, routine follow-up visits, and renal biopsy, were on purpose not standardized to recruit a study population typical for renal transplant recipients all over Europe.

Successful recruitment of the study is described here along with an in-depth analysis of the study population, urine samples collected and correlation of these with biopsy findings, and analyses for the presence of donor specific antibodies in blood samples of the allograft recipients at the time of renal biopsies. All biopsies were evaluated by experienced renal pathologists according to the Banff classification systematics. Taking clinical findings, laboratory values, and histopathological results into account, the patients were grouped by the investigators into so called "cases," "controls," and "additional," respectively. This will allow not only to discriminate rejecting from non-rejecting allografts. With this strategy, changes in the urine metabolome can be also correlated with pre-existing, i.e., donor-derived and *de novo* allograft damages over time and of all types,

such as cellular and antibody mediated acute and chronic allograft lesions.

The complete documentation of 1,230 urine samples along with corresponding histopathology reports and blood analyses for donor specific antibodies from 972 different patients (approximately one third of them with rebiopsies during the study), will allow to evaluate the urinary metabolite changes for a wide variety of different allograft damages alone or in combination and with different severity, all being representative for typical patient courses after renal transplantation.

Future goals for the test progress are the validation of previous findings in an actual multicenter setting, detection and differentiating antibody-mediated rejection from cellular rejection and recognition of potential disruptive factors other causes of allograft damage.

In conclusion, the clinical part of the PARASOL study as an international, multicenter study representative for typical renal transplant patients in Europe could be finished successfully. All urine samples, renal biopsy pathology results, and biographic and clinical patient data are validated and now available for further spectroscopic and metabolomic analyses.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Regensburg (principal investigator #16-315-101) and Local Ethics Committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MB, GB, OV, LR, SN, ES, and BB designed the study. LG, OB, and H-JG gave conceptual advice to the study protocol and the enrollment strategy. MB, GB, OV, LR, LG, OB, and BB were local principal investigators for the participating transplant centers and enrolled together with their study personnel all participating patients. H-JG and KK gave advice on histopathological evaluation and classification of renal biopsy findings. MB, SN, VR, AS, TJ, CF, DZ, TB, FP, VH, and ES collected study material and data and were responsible for raw data controls. SN, VR, AS, and ES supervised and evaluated metabolomic analyses and performed statistical evaluations. MB and BB wrote the manuscript. All authors evaluated study data and contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.780585/full#supplementary-material

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Hemodynamics and Metabolic Parameters in Normothermic Kidney Preservation Are Linked With Donor Factors, Perfusate Cells, and Cytokines

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Kidney transplantation is the best renal-replacement option for most patients with end-stage renal disease. Normothermic machine preservation (NMP) of the kidney has been studied extensively during the last two decades and implemented in clinical trials. Biomarker research led to success in identifying molecules with diagnostic, predictive and therapeutic properties in chronic kidney disease. However, perfusate biomarkers and potential predictive mechanisms in NMP have not been identified yet. Twelve discarded human kidneys ($n = 7$ DBD, $n = 5$ DCD) underwent NMP for up to 24 h. Eight were perfused applying urine recirculation (URC), four with replacement of urine (UR) using Ringer's lactate. The aim of our study was to investigate biomarkers (NGAL, KIM-1, and L-FABP), cells and cytokines in the perfusate in context with donor characteristics, perfusate hemodynamics and metabolic parameters. Cold ischemia time did not correlate with any of the markers. Perfusates of DBD kidneys had a significantly lower number of leukocytes after 6 h of NMP compared to DCD. Arterial flow, pH, NGAL and L-FABP correlated with donor creatinine and eGFR. Arterial flow was higher in kidneys with lower perfusate lactate. Perfusate TNF-α was higher in kidneys with lower arterial flow. The cytokines IL-1β and GM-CSF decreased during 6 h of NMP. Kidneys with more urine output had lower perfusate KIM-1 levels. Median and 6-h values of lactate, arterial flow, pH, NGAL, KIM-1, and L-FABP correlated with each other indicating a 6-h period being applicable for kidney viability assessment. The study results demonstrate a comparable cytokine and cell profile in perfusates with URC and UR. In conclusion, clinically available perfusate and hemodynamic parameters correlate well with donor characteristics and measured biomarkers in a discarded human NMP model.

Keywords: kidney transplantation, organ preservation, normothermic, urine recirculation, *ex-situ* perfusion

INTRODUCTION

Improving the quality and duration of donor kidney preservation prior to transplant may increase utility and potentially improve outcomes. Machine perfusion is at the forefront of this field (1–4), but accurate monitoring and evaluation of the kidney is essential to optimize outcomes. Currently, no biomarkers exist that can predict the usability or quality of a kidney during perfusion. Broad hemodynamic parameters such as arterial flow during hypothermic machine perfusion (HMP) with DGF and higher levels of NGAL and/or L-FABP over time have been inversely associated with estimated glomerular filtration rate (eGFR) together with an increasing intrarenal resistance (IRR) (5). Critical criteria consisting of arterial flow, macroscopic appearance of the kidney and volume of excreted urine have been incorporated into a decision-making score during normothermic machine perfusion (NMP) (6), but clearly, a prognostic marker of post-transplant function would represent a stepwise improvement to perfusion.

The kidney is equipped with a sophisticated immune compartment, hosting a plethora of non-hematopoetic cell types and a variety of both transient and resident leukocytes (7, 8). This leukocyte population remains in a steady state until an immunological challenge occurs. In the transplant setting this consists of death in the donor, surgical intervention in the donor, preservation, and finally reperfusion in the recipient. A potent inflammatory cascade ensues involving severe cytokine activation and cellular extravasation (9–11). Given this inflammatory response is initiated in the donor and continues throughout preservation, evaluating leukocyte diapedesis and cytokine secretion may identify novel biomarkers during kidney perfusion.

On these grounds, the aim of this study was to investigate if perfusion inflammatory profiles correlate with (i) donor factors, (ii) perfusion hemodynamics, (iii) the type of volume management, applying either urine recirculation (URC) or urine replacement (UR), and (iv) biomarkers of renal injury (NGAL, KIM-1, and L-FABP) in a blood-based perfusate of long-term NMP (12, 13) of discarded human kidneys.

MATERIALS AND METHODS

Donor Factors

Human kidney grafts, deemed not transplantable by all kidney transplant centers in the United Kingdom were included in this study. All organs were retrieved for the purpose of transplantation but discarded during post-procurement assessment. Donor and retrieval characteristics, kidney function parameter, and ischemia times were collected.

Abbreviations: CIT, cold ischemia time; DBD, donation after brain death; DCD, donation after circulatory death; Δ, delta; ECD, extended criteria donor; ECMO, extracorporeal membrane oxygenation; ESRD, end stage renal disease; HMP, hypothermic machine perfusion; IRR, intrarenal resistance; KIM-1, kidney injury molecule-1; LDH, lactate dehydrogenase; L-FABP, liver-type fatty acid-binding protein; NADH, nicotinamide adenine dinucleotide; NGAL, neutrophil gelatinase-associated lipocalin; NMP, normothermic machine perfusion; SCS, static cold storage; WIT, warm ischemia time.

NMP Perfusion

After being sent to Oxford, perfusions were performed at the Institute of Biomedical Engineering, University of Oxford. The technique of NMP using discarded human kidneys was reported previously (12, 13). Briefly, hemodynamic (arterial blood flow, mean arterial pressure and IRR) and biochemical perfusion parameters were recorded. Perfusate samples were collected and processed by centrifugation at 4,000 rpm for 15 min at 4°C. The supernatant was aliquoted, snap-frozen and stored at −80°C. The study was evaluated and approved by the National Ethics Review Committee of the United Kingdom (REC reference 12/EE/0273 IRAS project ID 106793).

Volume Management

Continuous urine recirculation or urine replacement with Ringer's lactate was applied as reported previously (13, 14). Ringer's lactate was infused to replace the excreted urine as a 1:1 volume replenishment in 20 mL intervals (14). The pH was adjusted through titration with sodium bicarbonate 8.4% (5–15 mL) to the physiological level of 7.3 before kidney NMP was started. No additional sodium bicarbonate was given at any point during perfusion after kidney connection.

Biomarkers of Renal Injury

Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and liver fatty acid-binding protein (L-FABP) levels in the perfusate samples were measured. Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and liver-type fatty acid-binding protein (L-FABP) levels in the perfusate samples were measured by a quantitative sandwich enzyme immunoassay technique using NGAL and KIM-1 Quantikine ELISA kits (R&D systems, USA) and Human FABP1/L-FABP ELISA Kit (CMIC Co., Ltd., supplied by R&D systems) according to manufacturers' instructions (12).

Inflammatory Profiling

Luminex® Analysis

A commercially available human 13-plex magnetic bead panel (Merck Millipore, Billerica, Massachusetts, USA) was used, following the manufacturer's protocol. The plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, United Kingdom). Thirteen cytokines and chemokines were assessed: Interferon (IFN)-γ, Interleukin (IL)-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, granulocyte macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor (TNF)-α (12).

Flow Cytometry

Samples of perfusate (4 ml) were collected into EDTA vacutainers, 0.4 ml dimethyl sulfoxide (DMSO) was added and well-mixed; 2 ml of this solution was then transferred into a cryogenic storage vial, moved to a CoolCell® Cell Freezing Container and stored in a −80°C freezer. Immunophenotyping of the human perfusate samples was performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, United Kingdom). Leukocytes were identified and gated

as CD45+ and their viability assessed using an eFluor™ 506 viability dye (eBioscience, California, USA). Following this, a panel of antibodies was utilized to characterize T helper cells (CD3ε+CD4α+), cytotoxic T cells (CD3ε+CD8α+), double-positive T cells (CD3ε+CD4α+CD8α+), double-negative T cells (CD3ε+CD4α-CD8α-), γδ T cells (γδ+), B cells (CD3ε-CD21+), classical monocytes (CD14+CD163-), non-classical monocytes (CD14+CD163+), immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), and natural killer cells (CD335+). Cells were treated with red blood cell lysing solution (BD Biosciences, United Kingdom), washed, and resuspended in 0.3 ml of staining buffer. A 20 ml quantity of e123count beads (eBioscience, California, USA) was added and samples were analyzed for 3 min. All gating strategies and analysis were performed using FlowJo version 10.0.6 (12).

Data Analysis

The statistical testing was done with Graph Pad Prism 7 and IBM® SPSS® Statistics Version 25. A p-value of <0.05 was considered as statistically significant. Biomarker, Luminex® and flow results, donor and perfusion factors were analyzed using parametric and non-parametric tests, including Spearman rank correlation. The Bonferroni method was applied to correct for multiple testing in the correlation analyses.

RESULTS

Twelve discarded human kidneys, seven from donors after brain death (DBD) and five from donors after circulatory death (DCD), were perfused for a median (min-max) of 12.8 (6.1–24.1) h. Volume management was facilitated by replacement of the urine (UR) with Ringer's lactate in four (4/12, 33.3%) NMP kidneys, and urine recirculation (URC) was applied in eight (8/12, 66.7%) kidney perfusions.

Table 1A illustrates the demographics for perfused DBD and DCD kidneys including donor risk indices. The median (min-max) UK kidney donor risk index [UKKDRI, (16)] was 1.9 (1.1–2.87); only three kidneys had a UKKDRI < 1.35 (**Table 1**). Median (min-max) CIT was 20.5 (12.7–46.9) h, median (min-max) WIT was 12 (9–15) min for DCD kidneys. DBD kidneys experienced a shorter median (IQR) CIT with 17.5 (5.9) h compared to 22 (24.1) h in DCD kidneys, $p = 0.05$. Median (min-max) donor urine output prior to retrieval was 60 (10–350) ml/h. Median (min-max) donor serum creatinine and eGFR at the time point of retrieval were 64.5 (32–208) μmol/l and 81 (29–247) ml/min/1.73 m^2.

The median (min-max, IQR) duration of NMP was 12.8 (6.1–24.1, 17.2) h. The median (min-max) hourly urine output during NMP was 54.4 (1.7–471.9) ml/h and the median arterial flow was 370.8 (100–787) ml/min. The median (min-max) perfusate lactate during NMP was 12.8 (4.4–20) mmol/l and the median (min-max) perfusate pH throughout the preservation period was 7.36 (7.16–7.62). A significantly longer NMP period could be achieved in kidneys with URC ($n = 3$ URC kidneys 12 h, $n = 1$ URC kidney 18 h, $n = 4$ URC kidneys 24 h); median (IQR) NMP time of 21 (11.4) hours with URC vs. 7.2 (3) hours with UR ($n = 2$ UR kidneys 6 h, $n = 1$ UR kidney 8 h, $n = 1$ UR kidney 9 h), $p = 0.01$.

To compare similar adequate time points between URC and UR kidneys, the time interval for perfusate analyses was the start of NMP until hour 6 of NMP as all of the perfused kidneys reached at least 6 h of NMP. **Table 1B** summarizes the hemodynamic and metabolic function parameters for the individual kidneys. A total of 135 perfusate samples were analyzed; 45 per assessment of NGAL/KIM-1/L-FABP, cytokines, and cells.

Table 2 shows the first and the 6-h measurements, as well as the Δvalues for NGAL, KIM-1, and L-FABP in absolute numbers. **Table 3A** displays the flow cytometry analyses results for all ($n = 12$) perfused NMP kidneys for time points 30 min, hours 1 and 6 after NMP-start. There were significantly more T-cells, CD4+ and NKT-cells in the perfusate after 6 h of NMP compared to 30 min after NMP-start. The content of monocytes, the intermediate type, was also significantly higher at hour 6 compared to the early measurements after initiation of NMP (**Table 3A**). Perfusate volume in NMP-kidneys was either managed with URC ($n = 8$) or UR ($n = 4$); we compared the potential efflux of cells between these two groups, shown in **Table 3B**. In regards to cell type and number of cells in the perfusate, there were no significant differences detectable between URC and UR kidneys throughout 6 h of NMP. **Supplementary Table 1A** displays cells in the perfusate of URC kidneys up to 24 h. To visualize changes of the cell count in the perfusate over time, a heatmap comprising all 12 NMP kidneys is pictured in **Figure 1A**; the changes of the number of cells in perfusates of kidneys undergoing different volume management (URC or UR) are depicted in **Figure 1B**.

Table 4A shows the cytokine concentration measured in the perfusate after 1 and 6 h of NMP in the overall investigated cohort of 12 kidneys. Interleukin 8 was the only cytokine which increased significantly over time; $p = 0.003$, 95% CI of difference −21,204 to −2,633. **Table 4B** gives an overview of the potential effect of the type of perfusate volume management, URC or UR, on the concentration of cytokines in the perfusate. In both settings, URC and UR, IL-1ß decreased over time. There were no significant differences of ΔIL-1ß in perfusates of URC or UR kidneys between NMP-start and hour 6, $p = 0.09$. **Supplementary Table 1B** shows cytokines in the perfusate of URC kidneys up to 24 h. For visualization purposes, heatmaps for the cytokine concentration changes over time, were configured and are shown in **Figures 2A,B**. **Figure 2A** displays the development of cytokine changes over time in the overall cohort of 12 NMP kidneys, **Figure 2B** illustrates the stratification for URC and UR kidneys.

Donor Factors in Context With Perfusate Measurements

Perfusates of DBD kidneys had a significant lower number of leukocytes (median, IQR) after 6 h of NMP compared to DCD kidneys; 99,862 (178,223) in DBD vs. 466,163 (239,703) in DCD, $p = 0.02$. There were less T cells (median, IQR), CD4+ and CD8+ T cells, in the perfusates

TABLE 1A | Organ donor characteristics, procurement parameters, and reasons for discard of kidneys.

	Age in years	Sex	BMI in kg/m^2	Donor type	Serum creatinine @retrieval in μmol/L	eGFR @retrieval in ml/min/1.73 m^2	WIT in minutes	CIT in hours + minutes	Hypertension	UKKDRI/KDRI	Reason for discard
Kidney 1	59	Male	35.1	DCD	32	247	15	21 + 16	Yes	1.10/1.21	Arteriosclerosis
Kidney 2	60	Male	35.1	DCD	114	71	14	42 + 17	Yes	1.50/1.60	Poor perfusion
Kidney 3	44	Male	41.4	DCD	63	120	12	46 + 59	Yes	1.22/1.13	Biopsy findings
Kidney 4	66	Female	31.2	DBD	208	41	n.a.	15 + 9	No	1.96/1/49	Patchy perfusion
Kidney 5	70	Female	24.3	DBD	44	119	n.a.	17 + 30	Yes	2.02/1.83	Stenosis of renal artery
Kidney 6	74	Female	24.8	DCD	57	90	11	22	Yes	2.07/2.17	Lesion on partner kidney (monomorphic cell infiltration)
Kidney 7	71	Female	28.1	DBD	86	56	n.a.	46 + 47	Yes	1.85/1.98	Anatomy, long CIT
Kidney 8	78	Female	25.4	DBD	79	61	n.a.	18 + 22	Yes	2.87/2.38	Vascular damage
Kidney 9	71	Female	29.1	DBD	66	77	n.a.	21 + 4	No	2.02/1.67	Organ size
Kidney 10	47	Female	39.1	DBD	152	29	n.a.	12 + 41	No	1.21/1.09	Vascular damage, patchy perfusion
Kidney 11	62	Female	23.5	DCD	62	85	9	19 + 52	No	1.67/1.50	Suspicion of cancer
Kidney 12	76	Female	24.5	DBD	36	152	n.a.	15 + 26	Yes	1.97/1.28	Long CIT

BMI, body mass index; DCD, donation after circulatory death; DBD, donation after brain death; eGFR, estimated glomerular filtration rate MDRD (modification of diet in renal disease); WIT, warm ischemia time; CIT, cold ischemia time. UKKDRI (15): Watson et al. (16); KDRI, OPTN KDRI/KDPI calculator.

TABLE 1B | Hemodynamic and metabolic function parameters.

	Kidney 1	Kidney 2	Kidney 3	Kidney 4	Kidney 5	Kidney 6
Arterial pressure in mmHg (mean, SD)*	84.6 ± 1.1	82.5 ± 7.8	91.3 ± 6.4	90.3 ± 1.3	90.4 ± 2.1	88.9 ± 1.7
Arterial flow in ml/min (mean, SD)*	650 ± 191.6	294.5 ± 74	325 ± 127.7	271 ± 54.6	383.9 ± 88.3	474.1 ± 149.7
IRR in ml/min/mmHg (mean, SD)*	0.15 ± 0.08	0.3 ± 0.09	0.39 ± 0.36	0.36 ± 0.18	0.25 ± 0.08	0.2 ± 0.09
pH (mean, SD)*	7.39 ± 0.12	7.21 ± 0.1	7.24 ± 0.17	7.33 ± 0.06	7.4 ± 0.04	7.66 ± 0.2
Arterial pO_2 in kPa (mean, SD)*	15 ± 1.6	14.7 ± 0.7	12.6 ± 2	12 ± 1.5	13.8 ± 0.6	15 ± 3
Venous pO_2 in kPa (mean, SD)*	7.7 ± 1.9	7.6 ± 0.8	6.4 ± 1.1	7 ± 2.4	8 ± 0.9	7.4 ± 0.9
Arterial pCO_2 in kPa (mean, SD)*	4.3 ± 0.9	5.1 ± 0.4	5.9 ± 0.7	4.6 ± 0.7	5.1 ± 0.9	4.6 ± 0.6
Lactate level in mmol/l (mean, SD)*	13.4 ± 1.62	13.27 ± 1.32	12.19 ± 3	18.52 ± 2.16	5.65 ± 3.8	9.62 ± 4
Total glucose given in gram**	2.4	3	1.2	0.75	3.2	0.55
Total urine output in ml	828	286	10	105	1,285	445
Total urine output in ml/hour	63.7	15.9	1.7	8.8	53.5	74.2
Urine recirculation yes/no	Yes	Yes	Yes	Yes	Yes	No
Time on the device (hours + min)	13 + 1	18 + 3	6 + 20	12 + 35	24 + 5	6 + 10
	Kidney 7	**Kidney 8**	**Kidney 9**	**Kidney 10**	**Kidney 11**	**Kidney 12**
Arterial pressure in mmHg (mean, SD)*	92.4 ± 2.9	92.2 ± 2.8	89.8 ± 0.5	91.64 ± 2.3	89.2 ± 2.2	90.3 ± 2.1
Arterial flow in ml/min (mean, SD)*	148.2 ± 46.3	123.5 ± 79.16	339.6 ± 83.4	240.7 ± 120.9	468.8 ± 82.8	664.9 ± 228.1
IRR in ml/min/mmHg (mean, SD)*	0.76 ± 0.5	1.2 ± 1.02	0.3 ± 0.12	0.5 ± 0.3	0.19 ± 0.05	0.17 ± 0.13
pH (mean, SD)*	7.18 ± 0.08	7.2 ± 0.1	7.6 ± 0.2	7.33 ± 0.1	7.39 ± 0.04	7.43 ± 0.02
Arterial pO_2 in kPa (mean, SD)*	13.6 ± 1.1	14.6 ± 2	14 ± 3.2	13.5 ± 2.9	15.4 ± 3.6	12.45 ± 0.8
Venous pO_2 in kPa (mean, SD)*	8.3 ± 1.5	7.8 ± 1.8	6.4 ± 1.3	6.7 ± 1.3	7.1 ± 1.6	7.6 ± 1.3
Arterial pCO_2 in kPa (mean, SD)*	5.4 ± 0.9	4.8 ± 1.1	5 ± 0.7	4.5 ± 0.9	4.7 ± 0.6	4.6 ± 0.4
Lactate level in mmol/l (mean, SD)*	16.18 ± 2.14	14.28 ± 4.5	16.34 ± 2.9	18.84 ± 2.2	9.19 ± 2	7.04 ± 1.46
Total glucose given in gram**	1	1.5	0.5	5	3	4.55
Total urine output in ml	50	920	675	11,325	1,325	1,223
Total urine output in ml/hour	8.3	102.2	84.4	471.9	55.2	51
Urine recirculation yes/no	No	No	No	Yes	Yes	Yes
Time on the device (hours + min)	6 + 5	9 + 25	8 + 10	24+5	24	24

**Time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion.*

***Circulating perfusate volume of 500 ml.*

Parts of donor and perfusion characteristics of kidneys 6, 8 and 9 without urine recirculation have been published previously (13).

TABLE 2 | Observed perfusate biomarker concentrations.

NGAL in ng/mL with urine recirculation	First time point*	Last time point**	Delta	Median (IQR)****
Kidney 1	28.3	37.2	8.9	37.2 (9.6)
Kidney 2	67.1	102.1	35	97.9 (32.1)
Kidney 3	19.1	43.1	24	31 (24)
Kidney 4	60.2	54.3	−5.9	59.8 (6)
Kidney 5	8.9	7.7	−1.2	12 (24.7)
Kidney 10	59.1	122	62.9	182.2 (152.2)
Kidney 11	10.4	32.9	22.5	36.8 (34.1)
Kidney 12	10.7	1.6	−9.1	16.1 (47.7)
NGAL in ng/mL without urine recirculation				
Kidney 6	17.3	7.4	−9.9	7.4 (10.1)
Kidney 7	100.9	117.2	16.3	103.5 (16.3)
Kidney 8	88.5	4.1	−84.4	4.2 (84.4)
Kidney 9	16.2	7.5	−8.7	6.9 (12.1)
KIM-1 in pg/mL with urine recirculation				
Kidney 1	348.5	518.1	169.7	518.1 (389.3)
Kidney 2	1,132.7	1,206.7	74.1	1,213 (77)
Kidney 3	397.6	932.1	552.5	655.8 (552.5)
Kidney 4	731.1	1,144.3	413.2	1,144 (438.9)
Kidney 5	170.5	429.8	259.4	724.2 (618.8)
Kidney 10	211.8	267.1	55.3	305.7 (168)
Kidney 11	73.9	436.5	362.6	518.2 (489.4)
Kidney 12	55.6	545.6	490	828.3 (892.4)
KIM-1 in pg/mL without urine recirculation				
Kidney 6	75.3	151.5	76.3	151.5 (99.5)
Kidney 7	244.5	635.9	391.4	521.8 (391.4)
Kidney 8	258.3	404.4	146.1	404.4 (211)
Kidney 9	401.6	518.9	150.7	489.1 (128)
L-FABP in ng/mL with urine recirculation				
Kidney 1	15	<detection limit***	−15	0*** (15)
Kidney 2	47.9	2.5	−45.4	4.8 (37.1)
Kidney 3	31.1	0.7	−30.4	15.9 (30.4)
Kidney 4	862.8	877.3	14.5	862.8 (21.7)
Kidney 5	<detection limit***	13.6	13.6	26.9 (39.7)
Kidney 10	70.2	41.8	−28.4	56 (20.1)
Kidney 11	88.4	138	49.6	138 (36.6)
Kidney 12	154.8	<detection limit***	−154.8	10.4 (111.6)
L-FABP in ng/mL without urine recirculation				
Kidney 6	289.2	369.5	80.3	357.2 (79.7)
Kidney 7	852.5	859.4	6.9	855.2 (6.9)
Kidney 8	1.8	27.3	25.5	22.4 (25.5)
Kidney 9	39.6	<detection limit***	−39.6	4.2 (39.6)

**1 h after perfusion start.*
***6 h after perfusion start.*
****Minimum detectable dose for assay <6.25 ng/ml (L-FABP).*
*****Time-averaged longitudinal value compiled from all measurements over the course of the perfusion; perfusate volume = 500 ml.*
Some NGAL, KIM-1 and L-FABP measurements of kidneys 6, 8, and 9 without urine recirculation have been published previously (13).

TABLE 3A | Flow cytometry results* of $n = 12$ NMP kidneys.

	30 min**	1 h**	6 h**	*p*-value***	*p*-value****
Total leukocytes	166,023, 158,326	259,628, 190,892	268,659, 355,959	0.10	0.15
Total T Cells	33,434, 30,009	53,971, 35,611	58,032, 74,672	0.14	0.03
CD4 T cells	11,716, 20,791	18,821, 18,157	24,095, 49,548	0.28	0.03
CD8 T cells	15,124, 15,919	27,610, 26,454	27,612, 25,313	0.13	0.10
NK T cells	4,332, 5,681	4,997, 4,677	7,932, 8,762	0.25	0.03
B cells	17,692, 27,406	17,736, 27,009	23,732, 40,729	0.82	0.31
Monocytes					
Classical	7,480, 6,085	18,017, 19,586	5,547, 10,488	0.04	0.96
Intermediate	4,099, 4,603	11,412, 11,400	1,363, 1,696	0.01	0.03
Non-classical	2,452, 2,423	5,341, 6,669	2,320, 4,276	0.01	0.97
Eosinophils	393, 786	439, 742	711, 1,473	0.68	0.23
Neutrophils	157, 249	359, 217	242, 1,263	0.84	0.99
NK cells	11,780, 40,888	26,342, 49,070	10,883, 41,349	0.46	0.88
Macrophages	3,529, 6,142	5,929, 18,386	3,883, 5,279	0.12	0.81

*Number of cells in cells/ml; overall perfusate volume = 500 ml.
**Time after start of NMP, values in median and IQR (interquartile range).
***Comparison 30 min with 1 h values.
****Comparison 30 min with 6 h values.

TABLE 3B | Flow cytometry results* stratified for urine recirculation and urine replacement.

	Kidneys with urine recirculation ($n = 8$)			Kidneys without urine recirculation ($n = 4$)			*p*-value*** 30 min	*p*-value*** 1 h	*p*-value*** 6 h
	30 min**	1 h**	6 h**	30 min**	1 h**	6 h**			
Total leukocytes	194,687, 169,462	301,435, 173,632	285,198, 314,757	112,250, 125,008	173,140, 224,651	131,250, 371,186	0.37	0.21	0.68
Total T Cells	39,778, 42,889	54,203, 18,377	79,875, 79,263	21,809, 28,131	26,523, 47,966	28,027, 69,184	0.28	0.15	0.28
CD4 T cells	14,579, 24,059	24,088, 14,444	30,468, 54,969	6,123, 9,731	8,402, 11,934	9,112, 40,426	0.28	0.07	0.57
CD8 T cells	16,913, 13,757	33,008, 19,555	31,977, 21,867	11,074, 18,395	13,174, 32,824	12,905, 23,162	0.15	0.15	0.11
NK T cells	2,943, 5,681	4,595, 3,800	8,050, 11,446	6,254 ± 3,241	7,870, 5,795	7,932, 5,671	0.68	0.37	0.93
B cells	17,692, 29,619	18,707, 26,760	31,089, 43,484	18,933, 64,538	16,183, 60,774	19,205, 271,378	0.68	0.99	0.93
Monocytes									
Classical	6,310, 10,731	18,017, 19,859	7,188, 11,711	7,480, 2,978	15,596, 19,757	5,547, 5,020	0.99	0.93	0.99
Intermediate	3,462, 5,310	9,255, 11,880	1,363, 2,799	4,180, 3,842	13,508, 13,273	1,292, 1,749	0.81	0.46	0.93
Non-classical	2,743, 3,285	7,667, 6,711	3,790, 3,645	1,810, 3,074	2,960, 2,963	1,114, 1,364	0.15	0.05	0.05
Eosinophils	545, 12,346	613.4, 5,528	1,122, 24,421	332, 764	296, 853	711, 653	0.57	0.28	0.81
Neutrophils	157, 202	379.1, 156.2	210.2, 351.4	105, 392	296, 438	1,039, 2,564	0.88	0.49	0.20
NK cells	11,780, 56,253	34,918, 63,636	26,187, 48,707	10,316, 12,633	19,607, 22,801	3,549, 5,731	0.57	0.21	0.07
Macrophages	3,073, 9,088	4,433, 11,636	3,709, 4,719	6,469, 6,217	21,035, 42,213	5,988, 15,180	0.68	0.21	0.46

*Number of cells in cells/ml; overall perfusate volume = 500 ml.
**Time after start of NMP, values in median and IQR (interquartile range).
***P-value result of comparison with and without urine recirculation.

of DBD kidneys after 6 h of NMP compared to DCD organs; 30,071 (22,961) in DBD vs. 102,356 (56,002) in DCD, $p = 0.01$.

Donor hypertension did not correlate with any of the perfusate markers measured.

Estimated glomerular filtration rate (eGFR) of the kidney donors and their corresponding serum creatinine were related with changes of IFN-γ and IL-6 over 6 h NMP without reaching significance. Perfusate NGAL after 6 h of NMP was insignificantly higher in kidneys from a donor with higher serum creatinine at the time of retrieval.

Duration of CIT did not correlate with any of the biomarkers (NGAL, KIM-1, and L-FABP), neither with number of leukocytes nor with cytokines in the perfusate. The correlations coefficients (Spearman's rho) and associated *p*-values are shown in **Table 5**; $p < 0.001$ considered significant according to Bonferroni correction.

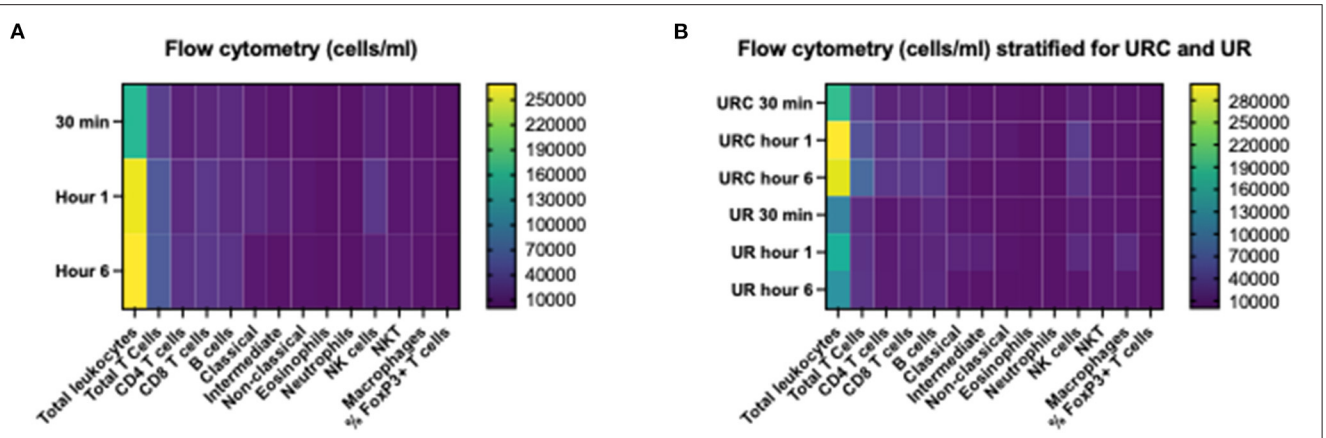

FIGURE 1 | (A) Heatmap visualizing results of flow cytometry analyses (cells/ml perfusate, 500 ml overall volume) of $n = 12$ NMP kidneys; time points investigated: 30 min, 1 and 6 h after NMP start. Median of cell count per measured entity per time point is displayed. **(B)** Heatmap visualizing results of flow cytometry analyses (cells/ml perfusate, 500 ml overall volume) stratified for urine recirculation (URC) and urine replacement (UR) kidneys; time points investigated: 30 min, 1 and 6 h after NMP start. Median of cell count per measured entity per time point is displayed.

TABLE 4A | Luminex results* of $n = 12$ NMP kidneys.

	1 h**	6 h**	*p*-value
GM-CSF	10,000, 9,979	27, 208	0.77
IFN-γ	10,000, 0	10,000, 7,476	>0.9
IL-10	2,159, 9952	1,743, 3,646	>0.9
IL-12p40	10,000, 0	10,000, 7,481	>0.9
IL-12p70	10,000, 0	10,000, 0	>0.9
IL-1RA	453, 1,845	980, 1,997	>0.9
IL-1α	10,000, 7,472	10,000, 7,387	>0.9
IL-1β	10,000, 9,985	14, 164	0.94
IL-2	10,000, 7,497	20, 9,999	0.94
IL-4	10,000, 0	10,000, 0	>0.9
IL-6	45, 9,007	6,943, 4,882	0.89
IL-8	24, 872	17,625, 196,167	0.003
TNF-α	10, 214	398, 295	>0.9

*Number of cells in pg/ml; overall perfusate volume = 500 ml.
**Time after start of NMP, values in median and IQR (interquartile range).
GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

NMP Perfusion Hemodynamics

Renal arterial flow in ml/min after 6 h of NMP correlated insignificantly with the CD8+ cell count. Overall, perfusates of kidneys with higher arterial flow had lower TNF-α levels. Kidneys from donors with higher eGFR at time of retrieval developed a significantly better arterial flow until hour 6 of NMP.

Figure 3A displays a comparison of CIT and donor characteristics stratified for median (IQR) arterial flow, 303.5 (186) ml/min at hour 6 after NMP start. There was no significant difference in duration of CIT ($p = 0.7$) and donor age ($p = 0.8$) for NMP kidneys reaching higher or lower arterial flow than the median of 303.5 ml/min. Donor eGFR ($p = 0.002$) was higher and donor serum creatinine lower ($p = 0.002$) in NMP kidneys reaching a higher arterial flow than the median.

Arterial flow after 6 h of NMP and median arterial flow correlated inversely. Perfusate lactate measured at hour 6 after NMP start was significantly lower in NMP kidneys reaching an arterial flow higher than the median of 303.5 ml/min; $p = 0.004$, shown in **Figure 3B**. The duration of NMP had no impact on reaching higher or lower median arterial flow; $p = 0.6$, depicted in **Figure 3B**.

Perfusate pH measured at hour 6 after NMP-start correlated significantly with median pH over time. There was a trend toward a more physiological pH and lower NGAL levels of kidney perfusates with higher arterial flow after 6 h of NMP. **Figure 3B** displays the relation of NGAL, pH and lactate, all measured at hour 6, with arterial flow. Kidneys with arterial flows higher than the median of 303.5 ml/min had non-significantly lower NGAL-perfusate levels ($p = 0.07$) and a more physiological pH ($p = 0.06$) but significantly lower perfusate lactate levels ($p = 0.004$).

NMP kidneys with higher arterial flow had non-significantly higher volume of hourly urine output; $p = 0.7$, shown in **Figure 3B**. There was also a link between KIM-1 levels after 6 h of NMP and the change of KIM-1 in the perfusate within the first 6 h of NMP. The correlations coefficients (Spearman's rho) and associated *p*-values are shown in **Table 5**; $p < 0.001$ considered significant according to Bonferroni correction.

Volume Management

The application of URC led to significantly longer periods of NMP; $p = 0.01$, shown in **Figure 3C**. There were no other significant correlations of type of volume management, URC or UR, in regard to donor (type of donor, age, cause of death, serum creatinine, and eGFR), preservation (CIT, WIT), and hemodynamic factors (arterial flow during NMP). Perfusate lactate after 6 h of NMP was comparable between URC and UR kidneys; $p = 0.6$, displayed in **Figure 3C**.

TABLE 4B | Luminex results* stratified for urine recirculation and urine replacement.

	Kidneys with urine recirculation ($n = 8$)		Kidneys without urine recirculation ($n = 4$)		*p*-value*** 1 h	*p*-value*** 6 h
	1 h**	6 h**	1 h**	6 h**		
GM-CSF	420.6, 9,993	53.5, 2,300	10,000, 0	12.6, 27.7	0.11	0.21
IFN-γ	10,000, 0	10,000, 9,982	10,000, 0	10,000, 0	>0.9	0.42
IL-10	2,160, 8196	1,890, 3,727	5,023, 9,988	1,214, 3,607	>0.9	>0.9
IL-12p40	10,000, 0	10,000, 7,481	10,000, 0	10,000, 7,499	>0.9	0.83
IL-12p70	10,000, 0	10,000, 0	10,000, 0	10,000, 7,493	>0.9	>0.9
IL-1RA	1,214, 2,229	1,933, 2,710	453.7, 419.7	192.6, 969	0.68	0.26
IL-1α	10,000, 0	10,000, 9,935	5,010, 9,986	10,000, 0	0.09	0.42
IL-1β	5,082, 9,986	113.7, 7,534	10,000, 7,499	1.2, 3	0.67	0.02
IL-2	10,000, 9,998	19.7, 7,518	10,000, 0	5,001, 9,999	0.42	0.53
IL-4	10,000, 0	10,000, 0	10,000, 0	10,000, 0	>0.9	>0.9
IL-6	4,493, 9,040	8,496, 4,829	19.6, 47.7	5,224, 14,384	0.11	0.68
IL-8	66.1, 10,943	16,501, 35,438	23.9, 55.5	17,625, 9,744	0.77	0.89
TNF-α	18.4, 560.8	459.7, 1,277	8.9, 9.4	354.7, 221.3	0.46	0.37

Number of cells in pg/ml; overall perfusate volume = 500 ml.
*** Time after start of NMP, values in median and IQR (interquartile range).*
**** P-value result of comparison with and without urine recirculation.*
GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

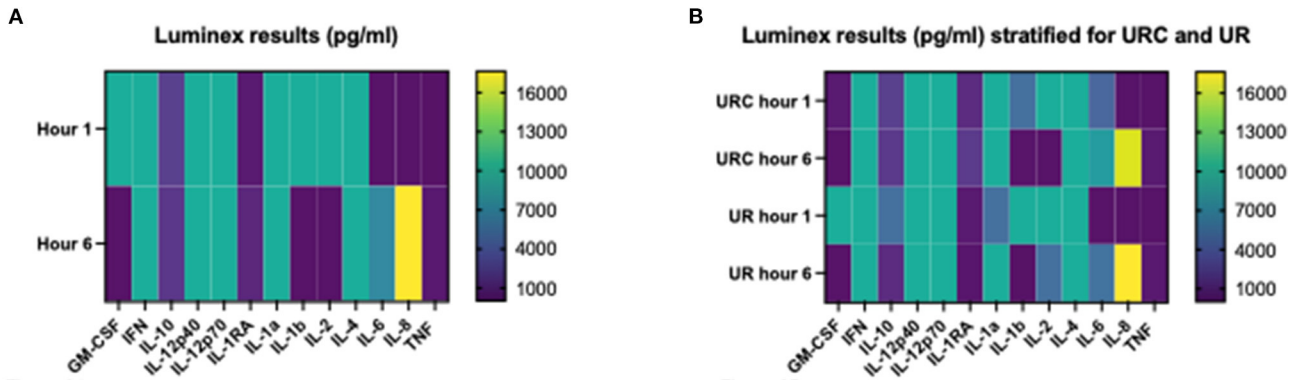

FIGURE 2 | (A) Heatmap visualizing Luminex results (pg/ml) of $n = 12$ NMP kidneys; time points investigated: 1 and 6 h after NMP start. Median cytokine concentration per measured entity per time point is displayed. **(B)** Heatmap visualizing Luminex results (pg/ml) stratified for urine recirculation (URC) and urine replacement (UR) kidneys; time points investigated: 1 and 6 h after NMP start. Median cytokine concentration per measured entity per time point is displayed.

The correlation coefficients (Spearman's rho) and associated *p*-values are shown in **Table 5**; $p < 0.001$ considered significant according to Bonferroni correction.

Biomarkers of Renal Injury

After application of the Bonferroni correction for multiple testing, there were no significant correlation of NGAL, KIM-1, and L-FABP with other parameters measured in the perfusate. There was a connection between NGAL levels after 6 h of NMP and the concentration of GM-CSF and its change over time, ΔGM-CSF, in the perfusate. The concentration of IL-1β was non significantly higher in perfusates with higher 6-h NGAL levels. Perfusates with higher KIM-1 levels after 6 h of NMP had a higher count of non-classical monocytes. Perfusates with higher L-FABP levels at hour 6 after NMP start had also non-significantly more macrophages in the perfusate. The correlations coefficients (Spearman's rho) and associated *p*-values are shown in **Table 5**; $p < 0.001$ considered significant according to Bonferroni correction.

DISCUSSION

We herein report for the first time the results of measuring several biomarkers, including cytokines and leukocytes, in a normothermic human kidney perfusion model comparing a novel approach of urine recirculation (URC) to facilitate perfusate homeostasis and volume control (12, 13) with the technique of replacement of excreted urine using Ringer's lactate. Urine recirculation led to NMP durations up to 24 h and biomarker could be detected and analyses throughout these

TABLE 5 | Correlation of hemodynamic and metabolic parameters with perfusate biomarker, cells, and cytokines.

Characteristic	Measured in perfusate	Spearman's rho	*p*-value
Donation after brain death	Leukocytes hour 6	−0.710	0.01
	Total T cells hour 6	−0.759	0.004
Donor age	GM-CSF hour 6	−0.655	0.021
	IFN-γ hour 6	0.588	0.04
	IL-1α hour 6	0.588	0.04
	IL-1β hour 6	−0.709	0.01
	NGAL hour 6	−0.718	0.009
	ΔNGAL*	−0.869	<0.001*
CVA as cause of death	ΔIL-1β	−0.641	0.03
	ΔIL-2	−0.599	0.04
	Non-classical monocytes hour 6	−0.717	0.009
Donor eGFR	ΔIFN-γ	0.624	0.03
	ΔIL-6	−0.629	0.03
	L-FABP hour 6	−0.662	0.02
	Δneutrophils	−0.615	0.03
	Median arterial flow	0.832	0.001
	Arterial flow hour 6*	0.860	<0.001*
	pH hour 6	0.592	0.04
Donor serum creatinine	ΔIFN-γ	−0.661	0.02
	NGAL hour 6	0.629	0.03
	ΔNK cells	0.671	0.02
	Median arterial flow	−0.839	0.001
	Arterial flow hour 6	−0.776	0.003
URC	Perfusion time	0.720	0.008
	IL-1β hour 6	0.718	0.009
Arterial flow hour 6	CD8+ cells hour 6	0.615	0.03
	Δneutrophils	−0.751	0.005
	%FoxP3 hour 6	−0.629	0.03
	TNF-α hour 6	0.627	0.03
	Median arterial flow*	0.881	<0.001*
	Lactate hour 6	−0.590	0.04
	Median lactate	−0.720	0.008
	pH hour 6	0.669	0.02
Perfusate pH hour 6	**Median pH***	0.947	<0.001*
	Median arterial flow	0.746	0.005
	NGAL hour 6	−0.627	0.03
Hourly urine output	IL-1RA hour 6	−0.82	0.001
	Δclassical monocytes	−0.594	0.04
	KIM-1 hour 6	−0.804	0.002
	ΔKIM-1	−0.727	0.007
NGAL hour 6	ΔNGAL	0.839	0.001
	GM-CSF hour 6	0.657	0.02
	ΔGM-CSF	0.629	0.03
	IL-1β hour 6	0.599	0.04
	median lactate	*0.664*	0.02
ΔNGAL	IL-1α hour 6	−0.624	0.03
KIM-1 hour 6	IL-1RA hour 6	0.627	0.03
	IL-1α hour 6	0.624	0.03
	ΔIL-1β hour 6	0.609	0.04

(Continued)

TABLE 5 | Continued

Characteristic	Measured in perfusate	Spearman's rho	*p*-value
	Δintermediate monocytes	0.594	0.04
	Δnon-classical monocytes	0.720	0.008
	ΔNK cells	0.608	0.04
L-FABP hour 6	IL-2 hour 6	−0.599	0.04
	ΔL-FABP	0.711	0.009
	eosinophils hour 6	−0.592	0.04
	macrophages hour 6	0.641	0.03
ΔL-FABP	IL-2 hour 6	−0.626	0.03
	Δeosinophils	−0.650	0.02
	Δ non-classical monocytes	−0.594	0.04

Δ, delta (difference start-6-h perfusion value); URC, urine recirculation; CVA, cerebrovascular accident; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; IFN, interferon; NK, natural killer; NGAL, neutrophil gelatinase-associated lipocalin; L-FABP, liver-type fatty acid-binding protein; KIM-1, kidney injury molecule 1; TNF, tumor necrosis factor. We applied the Bonferroni method to correct for multiple testing in the correlation analyses. N = 64 correlations were tested for statistical significance, consequently, the adjusted significance level is 0.05 divided by 64.

**P-values < 0.00078 (<0.001) were considered statistically significant.*

perfusions. The focus of this manuscript, however, was on the time point "hour 6" post perfusion start, as this was the latest comparable time point both, URC and UR NMP kidneys reached (17). Moreover, we focused on correlations with perfusion parameters and donor characteristics which are readily available at the time of decision making if a kidney is deemed transplantable after normothermic preservation and evaluation or declined for clinical use. This approach was chosen to examine some possible surrogate parameters, captured during perfusion, for organ viability. Our analyses were performed in a model of discarded human kidneys, therefore the important link to outcomes after successful transplantation is not available and no statements can be made in regard to estimating probable occurrence of delayed or primary non-function in the clinical setting.

The Cambridge group of Clatworthy, Ferdinand et al. (11), reported on NMP kidneys with higher inflammatory gene expression detectable in recipients who experienced prolonged DGF after receiving an NMP kidney. In their analyses, the course of 2 h NMP led to an upregulation of oxidative phosphorylation, but also an upregulation of a number of genes important for immune and inflammatory processes with NFkB induced TNF-α signaling as the major part of it (11). We detected an increase of TNF-α in perfusates in URC and UR kidneys, but the change over time was insignificant and more importantly, there was no difference at any time of perfusate TNF-α between the URC and UR kidneys. However, interestingly renal arterial flow (in URC and UR kidneys) was higher in kidneys with lower perfusate TNF-α which implicates a link between inflammatory potential of the perfusate and one of the best-studied hemodynamic parameters, especially in a pressure-fixed system in which increasing arterial flow over time is a parameter of kidney function (6, 18). In concordance with Ferdinand et al. (11), we also saw an increase of IL-8 in our perfusates over time. In line with IL-8, IL-6 increased over time in URC and UR kidneys without significant differences, but was not associated directly with any parameters identifying organ function during kidney NMP. However, both interleukins are known to be inflammatory with possible negative effects on renal parenchyma (19–21) and could be a future target for *ex-situ* organ treatment to prevent detrimental effects for the organ recipient. Our data revealed that IL-1β was higher in perfusates with higher content of NGAL and KIM-1 which are well-known markers for impaired kidney function and, NGAL at least, available to be measured in the clinical routine (2, 22–24). Another cytokine which could become of interest in future in a dynamic, normothermic preservation setting is GM-CSF. It is an immunregulatory cytokine which has been studied extensively recently due to its potential association with hyperinflammation in COVID-19 (25). GM-CSF is pro-inflammatory and plays a role in activation of macrophages and antigen-presenting cells (26). Whereas, in patients, as described in the literature, increased GM-CSF levels are associated with increased cytokines IL-6, TNF-α, IFN-γ, and IL-18 (25, 27), in our perfusates GM-CSF decreased over time. In perfusates with higher NGAL, GM-CSF was also higher compared to perfusates with lower NGAL which could potentially indicate less organ damage.

The Cambridge group did not detect any differences between DBD and DCD organs in terms of inflammatory gene signature they described. The only difference we could observe between DBD and DCD organs was the significant higher number of leukocytes, CD4+ and CD8+ T-cells in DCD-perfusates after 6 h of NMP compared to DBD organs; independent of URC or UR. This finding could be representing the cessation of blood flow in DCD organs and warm ischemia time prior to the start of retrieval, as the donor leukocytes have still a "route out" in DBD kidneys. In future, it will be interesting to compare DBD kidneys not only with DCD ones, but also with kidneys procured after normothermic regional perfusion.

The most relevant findings of our analyses were the clear connection of perfusate lactate, perfusate pH and urine output with several kidney function parameters in the donor as well as

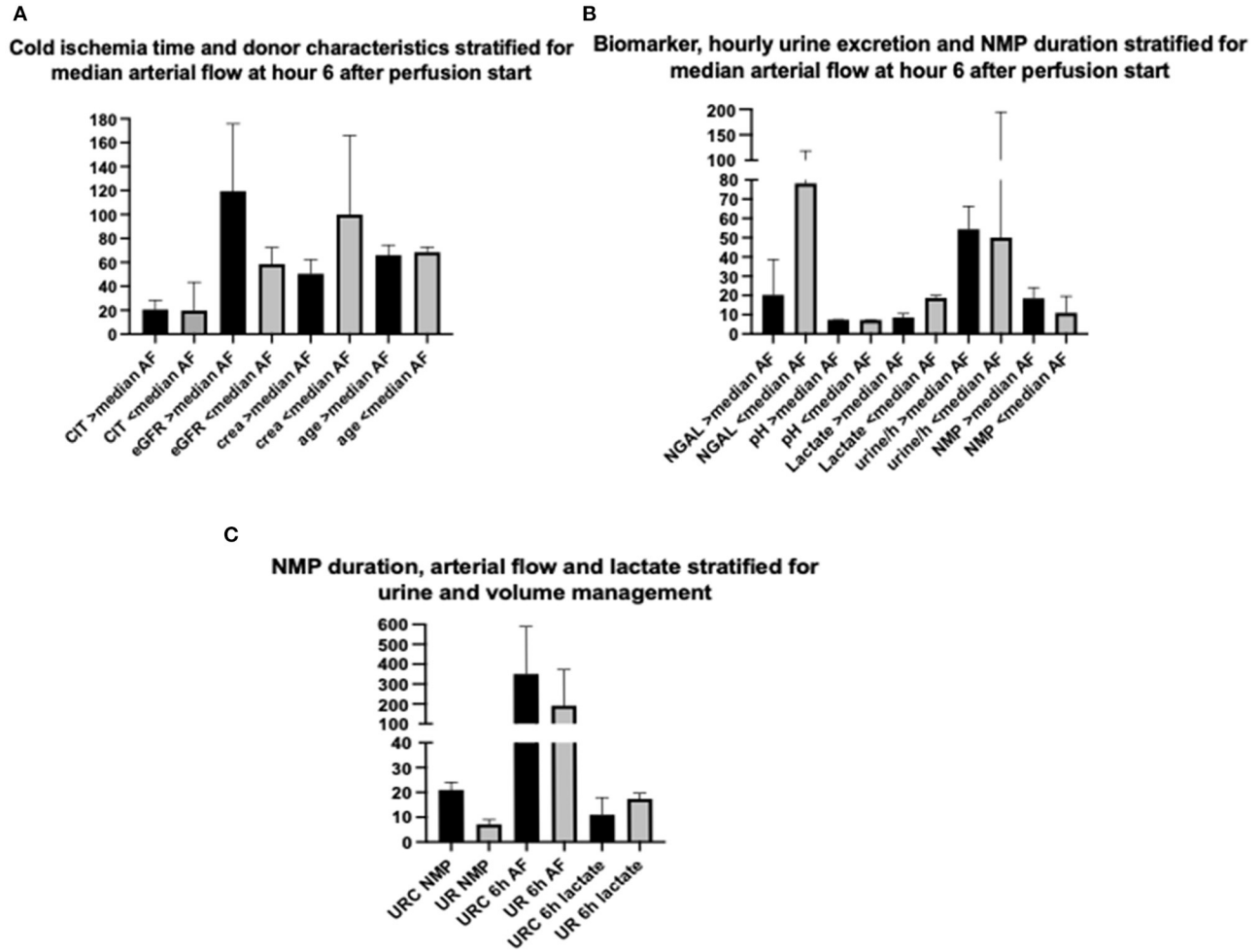

FIGURE 3 | (A) Comparison of cold ischemia time (CIT), estimated donor glomerular filtration rate (eGFR), donor serum creatinine (crea) at time point of retrieval, and donor age (age) stratified for median arterial flow (303.5 ml/min) after 6 h of NMP start; depicted values (column) of parameters are in median and IQR. **(B)** Comparison of perfusate NGAL, perfusate pH, perfusate lactate at hour 6 after NMP start, hourly urine excretion, and NMP duration stratified for median arterial flow (303.5 ml/min) after 6 h of NMP start; depicted values (column) of parameters are in median and IQR. **(C)** Comparison of NMP duration, arterial flow at hour 6 after NMP start and perfusate lactate at hour 6 after NMP start stratified for perfusate volume management (urine recirculation URC of urine replacement UR); depicted values (column) of parameters are in median and IQR.

with published kidney injury markers as NGAL, KIM-1, and L-FABP (2). Perfusate lactate was lower the higher the arterial flow was and also, potentially important for defining timing for *ex-situ* organ assessment, the 6-h values of lactate, renal arterial flow and pH correlated with their respective median values. NGAL, a biomarker we would define as a routinely available biomarker (22), was also higher in perfusates with higher lactates and could be a surrogate biomarker for the donor kidney function on the circuit as it correlated significantly with donor creatinine and eGFR. Urine output during NMP, a marker implemented in the Hosgood and Nicholson score already, was associated positively with KIM-1 levels in the perfusate. In addition to our finding that biomarkers can be measured and correlated with transplant factors, overall NMP time itself did not correlate with any of the parameters measured. In particular, there was no association of a preservation period of 6 h and beyond with arterial flow and inflammatory potential of the perfusate. Such a finding could be crucial for implementing longer-term kidney NMP in the clinical routine.

Our results, gained from an *ex-situ* NMP setting cannot be correlated with clinical study results yet, but different cytokine and immune cell patterns do offer an important target to invest more research, particularly in forthcoming clinical use of the NMP device. The downside of performing cell and cytokine analyses solely in the perfusate and not in the tissue or from any other components in the circuit, is a limitation of our investigation, is the missing answer to the question of where the immune cells were possibly migrating to.

To summarize, clinically available perfusion parameters as perfusate lactate, pH and NGAL correlate well with donor

characteristics, renal arterial flow, cytokines, immune cell changes, and KIM-1 in a discarded human NMP model. Potentially, lactate, pH and NGAL become a trinity to support decisions and fulfill the criteria to be diagnostic, predictive, and therapeutic biomarkers (28) in future for longer-term kidney NMP. In a non-transplant model long-term perfusion by applying URC was feasible and safe and also 6 h of NMP with UR seem to be applicable in a clinical transplant setting. Kidney NMP beyond 1 or 2 h might be helpful and instrumental in screening and discover markers indicating primary non-function of suboptimal organs (29). Therefore, these parameters should be considered as additional viability markers expanding the current decision-making score developed by Hosgood et al. (6).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AW designed the study, involved in the development of the perfusion device, performed the perfusions, collected and analysed the data, and wrote the manuscript. JS and JF performed the Luminex and FACS analyses. ML, JH, and RP were involved in data analyses, interpretation, and revision of the manuscript. CC and PF were instrumental for the study set-up, the development of the perfusion device, and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.801098/full#supplementary-material

Supplementary Table 1A | Flow cytometry results for urine recirculation (URC) for 30 min, 1, 6, 12, and 24 h after start of normothermic perfusion; 8 URC kidneys reached 12 h, 5 URC kidneys 18 h, and 4 URC kidneys 24 NMP h.

Supplementary Table 1B | Luminex results for urine recirculation (URC) for 1, 6, 12, and 24 h after start of normothermic perfusion; 8 URC kidneys reached 12 h, 5 URC kidneys 18 h, and 4 URC kidneys 24 NMP h.

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Conflict of Interest: PF and CC are co-founders of OrganOx R Limited, receive consultancy payments as non-executive medical and technical directors of OrganOx R Limited, and are shareholders. JF is the Chief Scientific Officer of the ex-vivo Research Center CIC, but receives no payments and has no shares and is an executive director of Perfusion Biotechnology Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Sex Differences in Kidney Transplantation: Austria and the United States, 1978–2018

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Background: Systematic analyses about sex differences in wait-listing and kidney transplantation after dialysis initiation are scarce. We aimed at identifying sex-specific disparities along the path of kidney disease treatment, comparing two countries with distinctive health care systems, the US and Austria, over time.

Methods: We analyzed subjects who initiated dialysis from 1979–2018, in observational cohort studies from the US and Austria. We used Cox regression to model male-to-female cause-specific hazard ratios (csHRs, 95% confidence intervals) for transitions along the consecutive states dialysis initiation, wait-listing, kidney transplantation and death, adjusted for age and stratified by country and decade of dialysis initiation.

Results: Among 3,053,206 US and 36,608 Austrian patients starting dialysis, men had higher chances to enter the wait-list, which however decreased over time [male-to-female csHRs for wait-listing, 1978–1987: US 1.94 (1.71, 2.20), AUT 1.61 (1.20, 2.17); 2008–2018: US 1.35 (1.32, 1.38), AUT 1.11 (0.94, 1.32)]. Once wait-listed, the advantage of the men became smaller, but persisted in the US [male-to-female csHR for transplantation after wait-listing, 2008–2018: 1.08 (1.05, 1.11)]. The greatest disparity between men and women occurred in older age groups in both countries [male-to-female csHR for wait-listing after dialysis, adjusted to 75% age quantile, 2008–2018: US 1.83 (1.74, 1.92), AUT 1.48 (1.02, 2.13)]. Male-to-female csHRs for death were close to one, but higher after transplantation than after dialysis.

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Conclusions: We found evidence for sex disparities in both countries. Historically, men in the US and Austria had 90%, respectively, 60% higher chances of being wait-listed for kidney transplantation, although these gaps decreased over time. Efforts should be continued to render kidney transplantation equally accessible for both sexes, especially for older women.

Keywords: chronic kidney disease, dialysis, kidney transplantation, sex, gender, USRDS, ADTR

INTRODUCTION

According to the United States Renal Data System (USRDS) Annual Data Reports from the years 1994 (1) to 2018 (2), and at least six non-USRDS based, original articles from the United States (3–8), women with kidney failure requiring kidney replacement therapy (KRT), formerly entitled end stage kidney disease (ESKD) (9), have lower kidney transplant rates than men every year. This observation has been placed in context with gender disparity (10, 11). Compared with US men, US women are also more frequently living kidney donors (12–14). Systematic analyses from outside the United States, however, are scarce (15, 16), hindering international comparisons.

The absolute numbers of deceased and living donor kidney transplantations between the sexes should not be directly compared, as they have to be interpreted relative to the underlying dialysis population. Describing the relative sex proportions is indispensable because the dialysis population is dominated by men, at an approximate, historically consistent rate (17–20) of 60 to 40 percent (21, 22). Realizing that kidney transplantation is a stepwise process is another important prerequisite for adequately interpreting sex differences in transplantation, because wait-listing may be influenced by gender disparities (23), while sex differences in transplantation rates after wait-listing have previously been explained by biological factors, specifically higher levels of preformed antibodies among women (24). Hence, besides transplant rates alone, wait-listing rates represent an important factor in measuring fair organ distribution in kidney transplantation.

Austria is a central European country with a population of 9 million (25), with a socially funded health insurance model, in contrary to the federal and out of pocket health insurance system of the US. Austria participates in the Eurotransplant donor organ allocation system (26) and has an efficient kidney transplant (and dialysis) registry with consistent follow-up (19, 27). In the US all dialysis patients and kidney transplantations are documented by the US Renal Data System (USRDS) (28). In the present analysis, we aimed at filling part of the international knowledge gap on sex differences in kidney transplantation by investigating wait-listing and kidney transplantation rates in the US and Austria, between 1978 and 2018. Our aim was to determine the evidence, if any, for sex disparities, past and present, and to compare trends in two countries with different health care models (2).

MATERIALS AND METHODS

Origin of the Study Population and Data Sources

In the US, all patients who start dialysis or receive kidney transplantation, regardless of insurance coverage and age, are documented in the US Renal Data System (USRDS), which is maintained since 1960 and made available to the nephrological community (28). The Austrian Dialysis and Transplant Registry (ADTR) is based on the voluntary cooperation of all 79 Austrian medical centers which cover the Austrian territory and offer kidney replacement therapy by hemodialysis or peritoneal dialysis and/or pre-KRT care and/or post-transplant care. In practice, these centers most often operate functional dialysis units, the majority of which (N = 51) are hospital-based (29). The Austrian medical centers also register their patients on the wait-list for kidney transplantation in one of the four transplant centers. All patients receiving hemodialysis or peritoneal dialysis and all kidney transplant recipients in Austria from the year 1964 forward have been entered into the ADTR database. For the present analysis, data from the ADTR were merged with the Eurotransplant database, a non-profit organization which was established in 1969 and is responsible for encouraging and coordinating donor organ allocation across 8 European countries, including Austria (30).

In the ADTR, pre-emptive transplantation can be deduced when a patient appears as having been transplanted without having a prior record as a dialysis patient (these patients were excluded from the present analysis, as further specified below). Similarly, in the USRDS data both the starting date of dialysis as well as the date of the first kidney transplantation are available, hence pre-emptive transplantation can be excluded in the same manner. In both countries, no age-restrictions regarding eligibility for kidney transplants are in place. Dates of dialysis initiation, wait-listing, transplantation and death were available in the same manner for both countries.

In the Austrian data, the precise date of wait-listing was documented only for those patients who subsequently received a donor organ. However, for all wait-listed patients a consecutive registration number from the Eurotransplant system was available. Wait-listing dates for listed patients who did not get a transplant yet were estimated by interpolation based on the consecutively awarded registration number and the known wait-listing dates of transplant patients. The accuracy of the interpolation was high, as for 7493 patients with known wait-listing dates, the deviation between actual and interpolated date was less or equal to 2 days in 75% of cases and less or equal 21

days in 95% of cases. Patients with implausibly early interpolated wait-listing dates of more than 1 year before start of dialysis were excluded, as specified below, in the section on the definitions of the study population.

Data in the ADTR (29) are nearly complete (<1% of patients lost to follow-up) and were extracted from local medical records by the responsible physicians in the various Austrian medical centers, as previously described (19, 27, 31). The present study was approved by the Ethics Committee of the Medical University of Vienna (EK No. 1363/2016).

Definitions of Study Population, Time Periods, and Key Events

After merging data from the ADTR and Eurotransplant, we obtained a database with records on all 39,678 patients who received kidney replacement therapy in Austria, from January 1964 through 31 August 2018. USRDS dialysis and transplant data consisted of 3,228,324 records, from May 1960 to August 2018. Due to sparse data in the early years of both datasets, for the present analysis we examined the last four decades with respect to dialysis initiation, hence USRDS and ADTR records with dialysis initiation before 1978 were excluded. Further, in both datasets we excluded patients who were aged below 18 years at dialysis initiation (US 3.3%, AUT 1.7%), those with missing data on the starting date of dialysis (US 2.1%, AUT 0%), missing information on sex (US 0.01%, AUT 0%), subjects who received a kidney transplant before dialysis initiation (US 2.9%, AUT 1.9%) and those who died on the day of dialysis initiation (US 2.1%, AUT 0.1%). Furthermore, for the Austrian data we excluded patients for whom the wait-listing date based on the Eurotransplant registration number was more than one year before the start of dialysis (1.4%). After these exclusions, the study population consisted of $N = 3{,}053{,}026$ subjects in the US (55.6% men, 44.4% women) and $N = 36{,}608$ in Austria (61.4% men, 38.6% women). A flowchart of the study population and the excluded data is shown in **Figure 1**. As sex was our exposure of primary interest, we depicted the sex distributions before and after the exclusion criteria.

To investigate time trends of sex-specific differences, we defined four periods of approximately one decade (1978–1987, 1988–1997, 1998–2007, and 2008–2018) with respect to the year of initiating dialysis. The last period encompassed 10 years and 8 months due to the last follow up date in August 2018. For analyses stratified by age at dialysis initiation, we defined three age categories, from 18 to 55 years, from 56 to 70 years, and above 70 years. The cut-points at 55 and 70 years corresponded approximately to tertiles of the patients' age distribution pooled over both countries and all decades.

We analyzed the time course of KRT, based on the recorded dates of the following events: start of dialysis, first wait-listing for transplantation, first receipt of a kidney transplant, and death.

Statistical Analysis

For descriptive purposes, we calculated the median and interquartile range of the patients' age distribution at dialysis initiation, first wait-listing and first kidney transplantation, and absolute and relative frequencies for categorical variables, overall, and by sex. We summarized person years, mean follow-up and crude event rates per 1,000 person years of the sequential states in CKD treatment, by country and decade of dialysis initiation and per sex.

To asses sex differences in the risk (or chance) of proceeding from one state (on dialysis, wait-listed, having received kidney transplant, deceased) to another, we estimated male-to-female cause specific hazard ratios (csHRs) and the corresponding 95% confidence intervals (CIs) using Cox proportional hazard models. Cox models were fitted with the sample of all patients who had entered the respective starting state, using the individual time-point of entering the target state as baseline. The dependent variable was the time until transition to the respective target state. If applicable, the transition to another state than the considered target state of the respective model was regarded as censoring event. To allow for unbiased comparisons of the decades, all transition times were censored at 10 years. For an individual patient who started dialysis during any one decade and was subsequently followed forward for 10 years, the starting point of the analyses in some cases reached well into the next decade. To quantify the sex differences in KRT, for each transition we estimated male-to-female csHRs adjusted for age at the starting point and the interaction of age and sex. Age was incorporated via restricted cubic spline terms to account for non-linear effects. Cox models were stratified for each country and decade of dialysis initiation. We reported male-to-female csHRs adjusted to the overall median age of 64 years, as well as the 25% and 75% age quantiles (q25: 52 years, q75: 74 years) (**Figure 2**; **Supplementary Figure 1**). Further, we depicted csHRs and 95% CIs as a function of age for men and women, with median aged women as the reference group (**Figure 3**; **Supplementary Figure 2**). Note that both visualizations represent the same models, but from a different point of view. Hazard rates by the subjects' age visualize the modification of the sex effect by age.

Additionally, for each year from 1995 to 2018 we calculated the crude wait-listing and transplant rates per 100 patient years in both countries per sex, overall and within the age groups 18–55, 56–70, and 70+ years. The number of respective events were divided by the sum of observed person years within the given calendar year and multiplied by 100.

All analyses were performed using the statistical software R, version 4.0.4.

Recording of Patient Sex

Recording of patient sex in both datasets occurred in the form of a binary variable. To our best knowledge, neither dataset differentiated between sex (male vs. female) and gender (man vs. woman) or transgender (32). Throughout the current manuscript, individuals of male and female sex are referred to as men and women, respectively, in order to remain consistent with previous work (20, 21). For reasons of legibility, hazard rates for men, divided by respective hazard rates for women are referred to as male-to-female HRs (rather than men-to-women HRs).

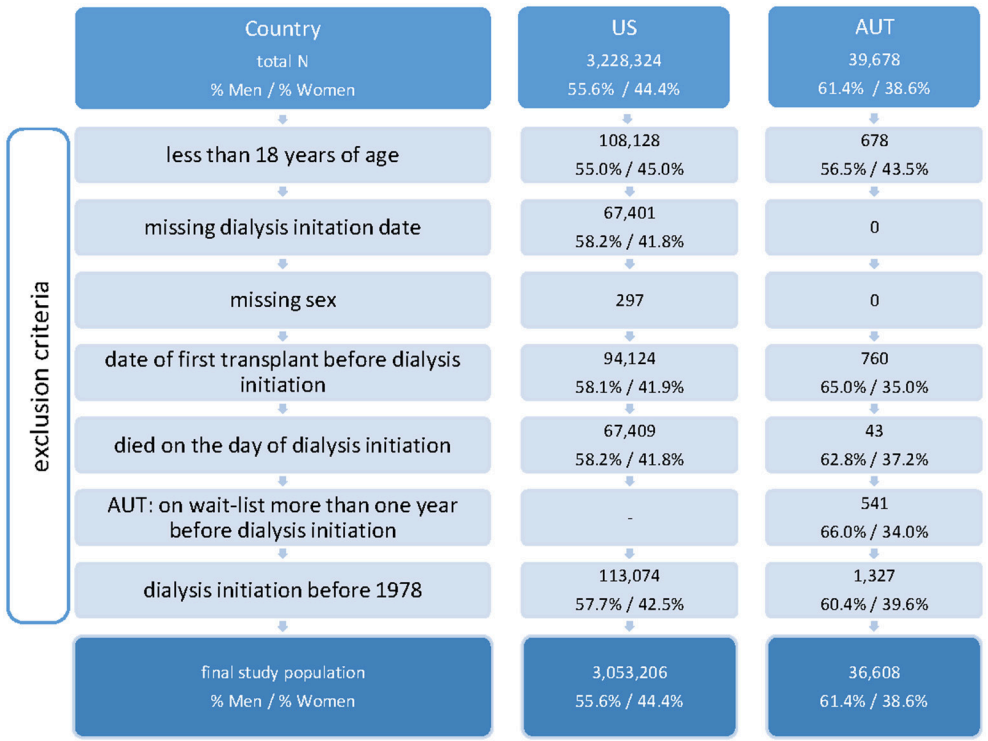

FIGURE 1 | Flowchart of study population. Total number of records, number or records meeting exclusion criteria (non-exclusive), and final study population, per country.

RESULTS

Sex Differences in Patient Characteristics: Age and Type of Kidney Disease

During the study period, 3,053,206 US and 36,608 Austrian patients met our inclusion criteria (US: 55.5% men, 44.5% women, AUT: 61.4% men, 38.6% women) (**Figure 1**). The sex distribution in both the ADTR and USRDS did not change after excluding non-eligible subjects. In **Table 1**, we present time trends of patient characteristics at their start of dialysis, by country and sex. Through the decades, men who initiated dialysis were younger than women in both countries, with the differences in median age ranging from 2 to 5 years. Overall, age at dialysis initiation increased steadily over time: In 1978–1987, in the US the median age at dialysis initiation was 54.1 years for men and 55.8 years for women, while in 2008–2018 the median age at dialysis initiation was 63.3 years for men and 64.3 years for women. In Austria the respective median ages were 49.5 years (men) and 51.6 years (women) in 1978–1987, and 64.9 years (men) and 66.3 years (women) in 2008–2018. The distribution of the type of kidney disease that necessitated KRT also changed over time. In both countries, glomerulonephritis as one of the main drivers of KRT decreased and diabetes and hypertension became the most common primary diseases in the more recent decades. In both countries, the proportion of women among all patients initiating dialysis was relatively stable. In the US, relative frequencies ranged from 42.4 to 46.7%. In Austria, in the first three decades the relative frequencies ranged from 39.2 to 42.4%, while in the most recent decade the proportion of women was somewhat smaller (34.4%).

Sex Differences in Kidney Recipient and Donor Characteristics

Throughout the study period, 385,642 US and 9,966 Austrian patients in the study population received their first kidney transplant; in the US 61.5% and in Austria 64.9% of the transplant recipients were men. In **Table 2**, we summarize the respective donor characteristics for each decade. Throughout the years, the proportion of living donor kidneys increased, especially the proportion of living kidney donation from men donors. Deceased donor kidneys continued to be more frequently available from men than from women, however with decreasing tendency toward the most recent decade.

Sex Differences in the Event Course of KRT

Time and Age Trends in Wait-Listing, Respectively, Transplantation

Table 3 shows crude event data per country and decade for the transition from dialysis initiation to being waitlisted, from waitlist entrance to receive a transplant, and from dialysis

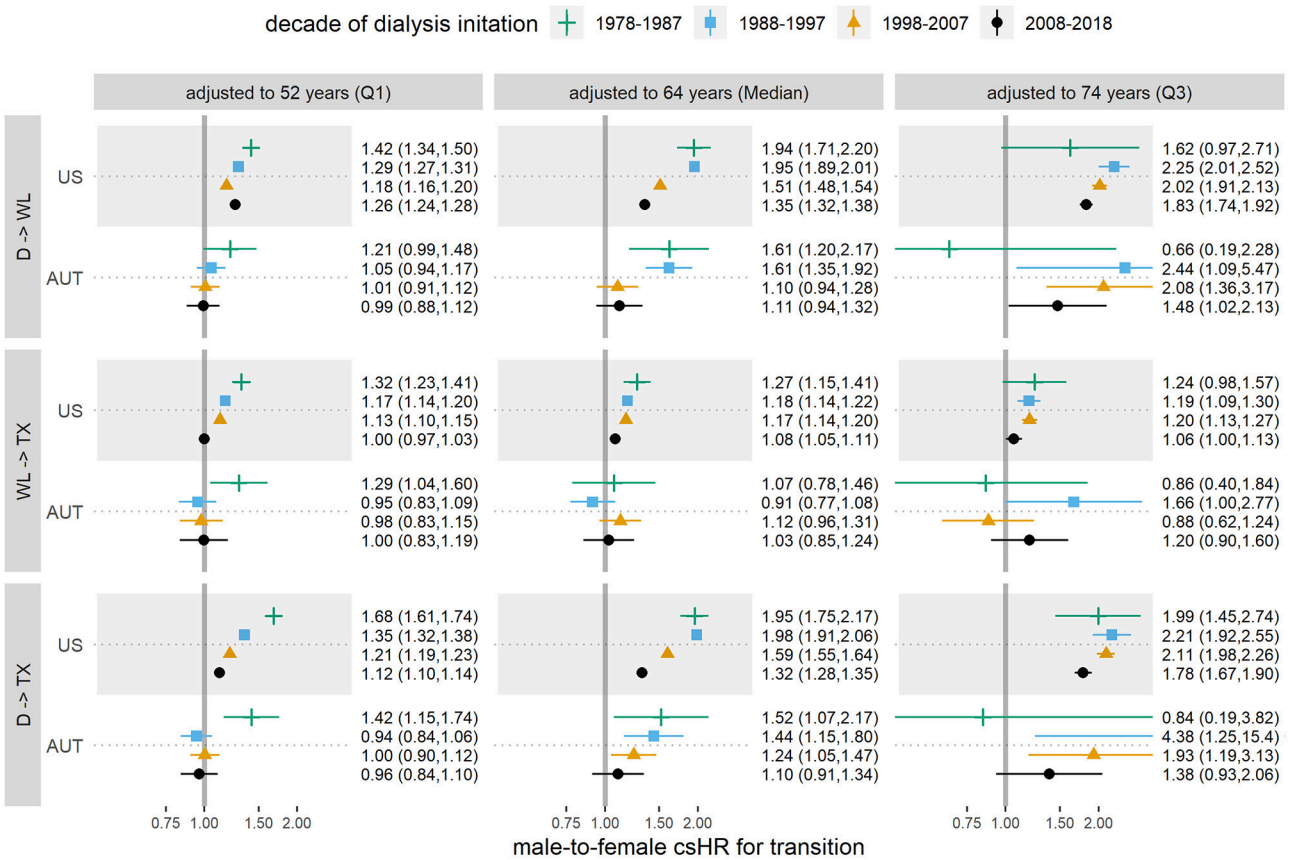

FIGURE 2 | Male-to-female cause specific hazard ratios (csHRs) with 95% confidence intervals for each state transition and decade, adjusted to median age (64 years) and 25% (52 years) and 75% (74 years) age quantiles, in Austria (AUT) and the USA. (D) dialysis initiation, (WL) first entry in wait-list, (TX) first kidney transplantation. The considered transitions are dialysis to wait-list (1st row), wait-list to TX (2nd row), dialysis to TX with wait-list as intermediate state (3rd row). Age is incorporated via restricted cubic splines. Results are based on data from the ADTR/Eurotransplant (AUT) (26, 29) and USRDS (US) (28).

initiation to receive a transplant (with wait-listing as intermediate step). **Figure 2** reports the respective male-to-female csHRs for the transitions in CKD treatment. We observed a lower chance for women on dialysis to enter the wait-list, compared to men, albeit it increased over time. Specifically, in 1978–1987 the male-to-female csHR of getting wait-listed in the US was 1.94 [95% CI 1.71, 2.20] and decreased to 1.35 [95% CI 1.32, 1.38] in the most recent decade. In Austria, in the first decade men also had significantly higher chances of being wait-listed [1978–1987 csHR 1.61 [95% CI 1.20, 2.17)] than women, however this advantage vanished in the last two decades [2008–2018 csHR 1.11 [95% CI 0.94, 1.32)]. To visualize the effect modification by age, **Figure 3** depicts the same models, but with respect to the subject's age at the starting state. The chances of wait-listing decreased with older age for both sexes. In both countries, age modified the sex-specific wait-listing chances, but the effect modification decreased throughout the decades, especially for younger patients. In the US, effect modification persisted over all ages and throughout the decades (US: $p_{interaction} < 0.001$ for all decades, AUT: $p_{interaction} < 0.05$ for all decades except for the most recent). In both countries, the advantage of men for wait-listing was more distinct in older age.

Once on the wait-list in Austria, chances to receive a donated kidney did not differ between men and women. In the US however, men on the wait-list had significantly higher chances of receiving a kidney transplant in the past [1978–1087 male-to-female csHR 1.27 (95% CI 1.15, 1.41)], although both the age and the sex effect diminished over time [2008–2018 male-to-female csHR 1.08 (95% CI 1.05, 1.11)]. Hence, the main disparity between the sexes occurred in the initial step of entering the wait-list in both countries, and was to a smaller extent driven by unbalanced sex-specific kidney transplantation. Overall, differences between the sexes regarding wait-listing and transplantation were gradually reduced throughout the study period. However, especially for patients of older age, sex differences were still prominent in the most recent decade, as can best be noted from the male-to-female csHR adjusted to the 75% age quantile (74 years) in 2008–2018, in comparison to the adjustment for median and low age. For wait-listing in this age group, we obtained a male-to-female csHR of 1.83 [95% CI 1.74–1.92] in the US and 1.48 [95% CI 1.02–2.13] in Austria.

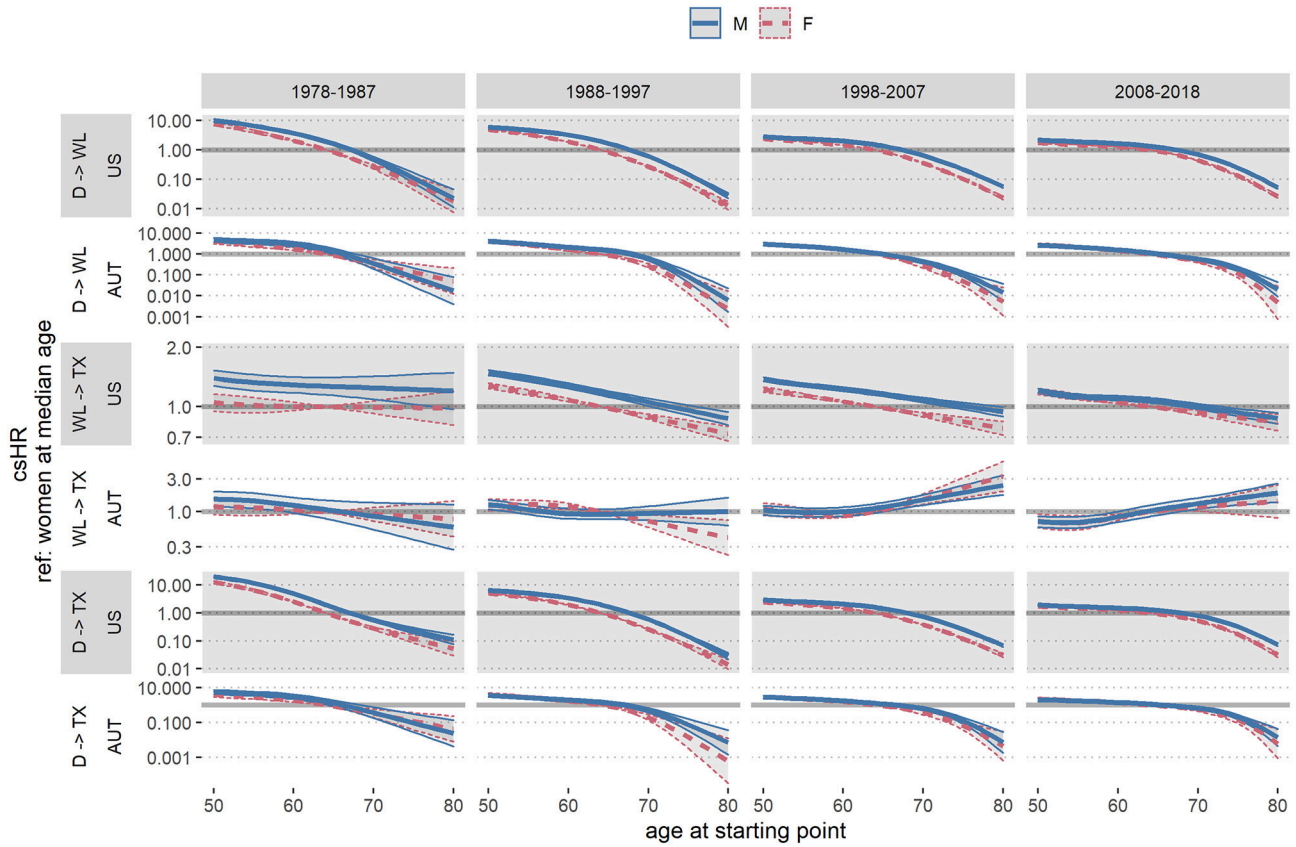

FIGURE 3 | Austrian (AUT) and US cause specific hazard ratios (csHRs) and 95% confidence intervals by sex and age at the respective starting state, with median aged females as reference group, per country and decade. (D) dialysis initiation, (WL) first entry in wait-list, (TX) first kidney transplantation. Age is incorporated via restricted cubic splines. Results are based on data from the ADTR/Eurotransplant (AUT) (26, 29) and USRDS (USA) (28).

Mortality

Male-to-female csHRs for death from the two starting points dialysis initiation and wait-listing are shown in the **Supplementary Table 1**, **Supplementary Figures 1**, **2**. Male-to-female mortality hazards after dialysis initiation were rather similar across sexes and decades in both countries. Overall, there were tendencies for higher mortality in men. Age-adjusted mortality after transplantation was higher for men than for women throughout most decades and consistent over age groups, although confidence bands in Austria were very wide (**Supplementary Figure 1**).

Wait-Listing and Transplant Rates per 100 Patient Years

In **Figures 4**, **5** we show crude wait-listing and transplant rates per 100 dialysis patient years, by sex and calendar year in each country, overall (**Figure 4**) and by age group (**Figure 5**). The overall crude event rates were consistently higher for men than for women and declined within the considered time frame. In the first age category (up to 55 years), wait-listing and transplant rates in Austria were similar for men and women. In all other groups, crude event rates for both wait-listing and transplantation were higher in men. This finding is in line with the age-adjusted male-to-female csHRs in **Figures 2**, **3**. When comparing the two countries, although both the wait-listing as well as the transplant event rates were about twice as high in Austria than the US, the trends over time were very similar. However, as can be deduced from **Figure 5**, for the second age group (56–70 years) in the US both event rates increased from 1995 onward up to ∼2010 and decreased thereafter. In Austria, event rates within this age group were rather constant.

DISCUSSION

In this study with historical data through 2018 from the US and Austria, we found that men had a higher chance than women of being placed on the wait-list for kidney transplantation. The age-adjusted probability for women to enter the transplant wait-list was smallest in earlier decades and among older patients, when compared to men (**Figure 2**). Sex differences in wait-listing decreased over time, but were still observed at all ages in the US, and especially for patients in old age. In both the US and Austria, once patients had entered the wait-list, the probability of receiving a donor organ was very similar for men and women, although in the

TABLE 1 | Patient characteristics who initiated dialysis in the US and Austria (AUT), by decade.

Country		1978–1987		1988–1997		1998–2007		2008–2018	
AUT		**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
		***N* = 2,498 (57.6%)**	***N* = 1,839 (42.4%)**	***N* = 4,755 (57.6%)**	***N* = 3,505 (42.4%)**	***N* = 7,061 (60.8%)**	***N* = 4,544 (39.2%)**	***N* = 8,136 (65.6%)**	***N* = 4,270 (34.4%)**
	Age at dialysis initiation								
		49.5 (14.5)	51.6 (15.1)	56.8 (14.8)	60.1 (15.3)	62.1 (14.4)	65.0 (14.5)	64.9 (14.1)	66.3 (14.7)
	Age at wait-listing								
		52.1 (15.7)	54.8 (16.7)	59.1 (15.8)	62.8 (16.5)	64.9 (15.1)	68.2 (15.5)	67.2 (14.5)	68.8 (15.1)
	Age at first transplant								
		53.1 (15.3)	55.8 (16.2)	60.1 (15.2)	63.6 (15.9)	65.7 (14.5)	68.8 (14.8)	67.6 (14.1)	69.1 (14.7)
	Primary disease (ERA group)								
	Diabetes	337 (13.5%)	227 (12.3%)	1156 (24.3%)	927 (26.4%)	2274 (32.2%)	1494 (32.9%)	2352 (28.9%)	1106 (25.9%)
	Glomerulonephritis/sclerosis	941 (37.7%)	421 (22.9%)	1053 (22.1%)	448 (12.8%)	1091 (15.5%)	457 (10.1%)	971 (11.9%)	373 (8.7%)
	Hypertension	79 (3.2%)	30 (1.6%)	280 (5.9%)	105 (3%)	625 (8.9%)	297 (6.5%)	865 (10.6%)	400 (9.4%)
	Miscellaneous	347 (13.9%)	348 (18.9%)	655 (13.8%)	755 (21.5%)	957 (13.6%)	815 (17.9%)	1362 (16.7%)	841 (19.7%)
	Polycystic kidney, adult type	157 (6.3%)	152 (8.3%)	268 (5.6%)	242 (6.9%)	361 (5.1%)	310 (6.8%)	390 (4.8%)	321 (7.5%)
	Pyelonephritis	295 (11.8%)	416 (22.6%)	343 (7.2%)	418 (11.9%)	267 (3.8%)	277 (6.1%)	262 (3.2%)	187 (4.4%)
	Renal vascular disease	72 (2.9%)	31 (1.7%)	321 (6.8%)	155 (4.4%)	760 (10.8%)	386 (8.5%)	1051 (12.9%)	519 (12.2%)
	Unknown	270 (10.8%)	214 (11.6%)	679 (14.3%)	455 (13%)	726 (10.3%)	508 (11.2%)	883 (10.9%)	523 (12.2%)
US		**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
		***N* = 136,162 (55.1%)**	***N* = 110,737 (44.9%)**	***N* = 328,612 (53.3%)**	***N* = 287,601 (46.7%)**	***N* = 536,918 (54.6%)**	***N* = 446,855 (45.4%)**	***N* = 694,565 (57.6%)**	***N* = 511,576 (42.4%)**
	Age at dialysis initiation								
		54.1 (17.1)	55.8 (16.5)	59.1 (16.5)	60.9 (15.8)	62.5 (15.6)	63.8 (15.3)	63.3 (14.8)	64.3 (14.9)
	Age at wait-listing								
		58.1 (16.6)	60.1 (16.2)	62.2 (16.5)	64.1 (15.9)	65.7 (15.4)	67.1 (15.3)	65.6 (14.7)	66.7 (14.9)
	Age at first transplant								
		57.4 (17.3)	59.7 (16.8)	62.6 (16.2)	64.5 (15.6)	66.3 (14.9)	67.7 (14.8)	66.1 (14.4)	67.1 (14.6)
	Primary disease								
	Cystic kidney	4,423 (3.2%)	4,104 (3.7%)	8,760 (2.7%)	7,471 (2.6%)	10,896 (2%)	9,329 (2.1%)	12,553 (1.8%)	10,366 (2%)
	Diabetes	24,695 (18.1%)	26,268 (23.7%)	102,670 (31.2%)	113,769 (39.6%)	222,766 (41.5%)	209,973 (47%)	305,433 (44%)	236,206 (46.2%)
	Glomerulonephritis	22,328 (16.4%)	14,963 (13.5%)	44,696 (13.6%)	34,524 (12%)	53,301 (9.9%)	43,096 (9.6%)	47,282 (6.8%)	38,388 (7.5%)
	Hypertension	28,961 (21.3%)	20,369 (18.4%)	92,198 (28.1%)	680,15 (23.6%)	150,151 (28%)	114,135 (25.5%)	200,308 (28.8%)	140,085 (27.4%)
	Missing cause	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (0%)	0 (0%)	226 (0%)	114 (0%)
	Other cause	11,510 (8.5%)	10,758 (9.7%)	25,679 (7.8%)	17,741 (6.2%)	52,892 (9.9%)	36,075 (8.1%)	67,402 (9.7%)	44,816 (8.8%)
	Other urologic	2,892 (2.1%)	975 (0.9%)	6,711 (2%)	3,649 (1.3%)	10,003 (1.9%)	5,244 (1.2%)	10,279 (1.5%)	4,518 (0.9%)
	Unknown cause	7,815 (5.7%)	5,940 (5.4%)	14,488 (4.4%)	10,832 (3.8%)	22,848 (4.3%)	16,424 (3.7%)	15,796 (2.3%)	10,783 (2.1%)
	Missing	33,538 (24.6%)	27,360 (24.7%)	33,410 (10.2%)	31,600 (11%)	14,060 (2.6%)	12,579 (2.8%)	35,286 (5.1%)	26,300 (5.1%)

Diagnosis data are displayed as absolute and relative frequencies. Ages are displayed as median (interquartile range).

TABLE 2 | Donor and recipient characteristics, by decade of dialysis initiation.

Country	Donor sex	Donor type	1978–1987		1988–1997		1998–2007		2008–2018	
AUT			**M** ***N* = 1,136**	**F** ***N* = 689**	**M** ***N* = 1,796**	**F** ***N* = 1,057**	**M** ***N* = 2,020**	**F** ***N* = 1,032**	**M** ***N* = 1,513**	**F** ***N* = 723**
	M	Cadaver	52.10%	50.10%	51.60%	51.80%	46.70%	48.60%	45.40%	44.50%
		Living	0.80%	0.60%	0.80%	1.10%	2.60%	3.30%	3.70%	6.60%
	F	Cadaver	39.70%	41.40%	39.40%	40.90%	40.40%	41.00%	35.80%	39.70%
		Living	0.80%	1.30%	1.30%	1.40%	4.90%	3.00%	12.50%	6.80%
	Unknown	Cadaver	6.50%	6.50%	5.80%	4.50%	5.10%	3.60%	2.00%	2.20%
		Living	0.10%	0.10%	1.10%	0.30%	0.30%	0.50%	0.60%	0.10%
US			**M** ***N* = 38,581**	**F** ***N* = 23,796**	**M** ***N* = 66,629**	**F** ***N* = 43,645**	**M** ***N* = 83,881**	**F** ***N* = 52,652**	**M** ***N* = 57,104**	**F** ***N* = 34,256**
	M	Cadaver	46.00%	45.10%	46.00%	43.80%	41.30%	39.30%	40.90%	41.20%
		Living	9.80%	9.60%	9.80%	10.80%	12.20%	13.40%	11.80%	11.50%
		Unknown	1.10%	1.00%	0.20%	0.20%	0.00%	0.00%	0.00%	0.00%
	F	Cadaver	24.60%	24.70%	29.90%	29.70%	26.90%	27.80%	25.10%	27.70%
		Living	9.40%	10.30%	12.20%	13.20%	17.70%	17.30%	20.80%	18.00%
		Unknown	0.70%	0.80%	0.20%	0.20%	0.00%	0.00%	0.00%	0.00%
	Unknown	Cadaver	4.30%	4.40%	1.00%	1.10%	1.20%	1.40%	0.90%	1.10%
		Living	1.30%	1.30%	0.30%	0.40%	0.60%	0.70%	0.50%	0.40%
		Unknown	2.70%	2.80%	0.50%	0.50%	0.10%	0.10%	0.00%	0.10%

Results refer to the first kidney transplantation of a patient.

TABLE 3 | Crude time-to-event data per country, decade of dialysis initiation, and sex.

Event	Country	Variable	1978–1987 M	1978–1987 F	1988–1997 M	1988–1997 F	1998–2007 M	1998–2007 F	2008–2018 M	2008–2018 F
D –> WL	US	Person years	452,618	397,614	900,419	848,463	1,569,883	1,362,920	1,603,818	1,248,679
		Mean (SD) follow-up	3.33 (3.1)	3.60 (3.2)	2.80 (2.9)	3.00 (2.9)	3.00 (3.0)	3.12 (3.0)	2.42 (2.3)	2.55 (2.3)
		Events	16,847	11,434	68,527	45,739	99,507	63,903	97,914	55,229
		Events per 1,000 PY	37.2	28.8	76.1	53.9	63.4	46.9	61.1	44.2
	AUT	Person years	5,109	4,571	9,234	7,843	18,229	13,094	18,947	10,378
		Mean (SD) follow-up	2.16 (2.6)	2.60 (2.9)	2.05 (2.5)	2.34 (2.7)	2.63 (2.9)	2.93 (3.2)	2.36 (2.2)	2.47 (2.3)
		Events	1,152	704	1,945	1,076	2,186	1,099	1,913	863
		Events per 1,000 PY	225.5	154.0	210.6	137.2	119.9	83.9	101.0	83.2
WL –> TX	US	Person years	19,739	19,182	156,066	118,890	347,189	248,271	364,866	225,662
		Mean (SD) follow-up	1.88 (2.4)	2.42 (2.7)	2.11 (2.3)	2.39 (2.5)	3.06 (2.8)	3.34 (3.0)	2.83 (2.3)	2.96 (2.4)
		Events	8,209	5,876	57,490	36,928	75,717	47,011	55,497	33,241
		Events per 1,000 PY	415.9	306.3	368.4	310.6	218.1	189.4	152.1	147.3
	AUT	Person years	2,383	1,665	4,605	2,547	5,191	2,785	3,231	1,549
		Mean (SD) follow-up	1.93 (2.2)	2.18 (2.4)	2.12 (2.0)	2.09 (2.0)	2.24 (2.2)	2.40 (2.3)	1.61 (1.7)	1.69 (1.7)
		Events	1,027	616	1,711	1,032	1,949	996	1,449	695
		Events per 1,000 PY	431.0	370.0	371.5	405.2	375.5	357.6	448.5	448.6
D –> TX	US	Person years	395,685	368,027	1,019,753	940,367	1,877,517	1,580,278	1,930,922	1,446,097
		Mean (SD) follow-up	2.91 (2.9)	3.32 (3.1)	3.10 (2.9)	3.27 (2.9)	3.50 (3.1)	3.54 (3.2)	2.78 (2.4)	2.83 (2.4)
		Events	37,929	23,243	65,164	42,447	81,387	50,874	57,074	34,240
		Events per 1,000 PY	95.9	63.2	63.9	45.1	43.3	32.2	29.6	23.7
	AUT	Person years	7,365	6,148	13,759	10,339	23,306	15,827	22,134	11,905
		Mean (SD) follow-up	2.95 (2.8)	3.34 (3.1)	2.89 (2.6)	2.95 (2.7)	3.30 (2.9)	3.48 (3.1)	2.72 (2.2)	2.79 (2.3)
		Events	1,111	661	1,778	1,051	2,008	1,029	1,513	723
		Events per 1,000 PY	150.8	107.5	129.2	101.7	86.2	65.0	68.4	60.7

Person years (PY), mean (SD) follow-up, event counts and events per 1,000 person years, by event [Dialysis (D), first wait-listing (WL) and first transplant (TX)], country and decade of dialysis initiation and per sex.

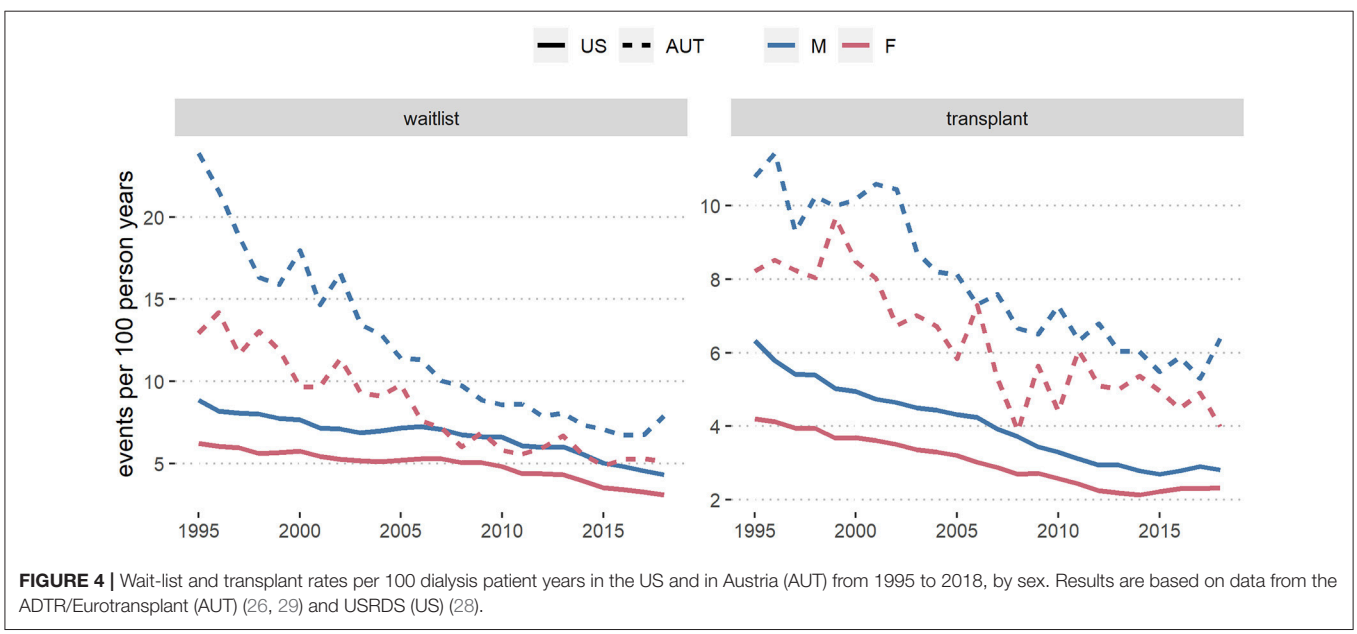

FIGURE 4 | Wait-list and transplant rates per 100 dialysis patient years in the US and in Austria (AUT) from 1995 to 2018, by sex. Results are based on data from the ADTR/Eurotransplant (AUT) (26, 29) and USRDS (US) (28).

US the advantage for men disappeared only within the most recent decade (2008–2018). Wait-listing rates by sex moved closer together in Austria than in the United States, indicating that gender or sex disparities in transplant access in Austria might have been reduced to a greater degree than in the United States.

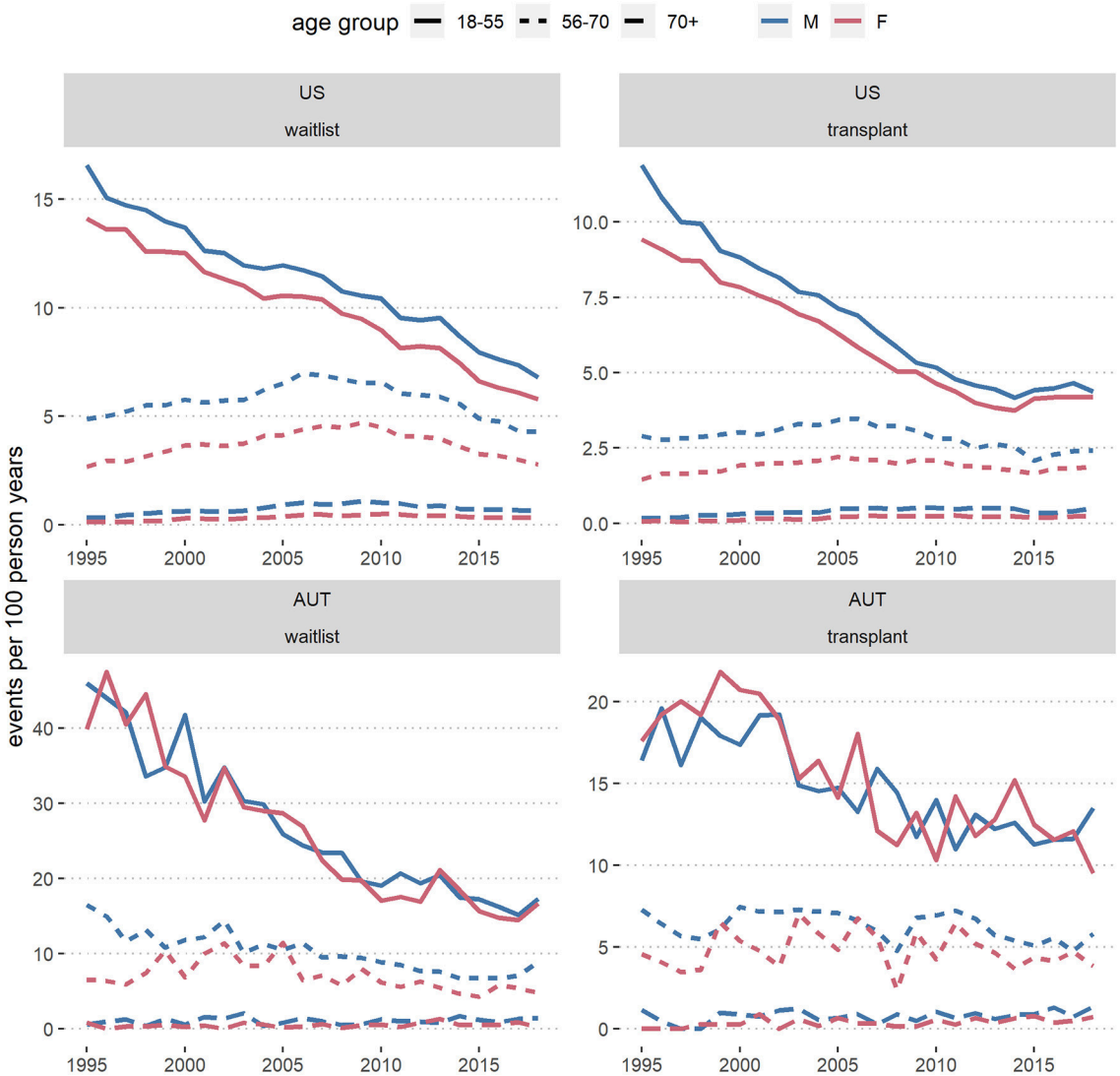

FIGURE 5 | Wait-listing and transplant rates per 100 dialysis patient years in the US and Austria (AUT), from 1995 to 2018, by sex and age group. Results are based on data from the ADTR/Eurotransplant (AUT) (26, 29) and USRDS (US) (28).

Understanding the impact of age is important for correctly interpreting our study findings. Adjusted to the 75% age quantile, in the most recent decade the male-to-female csHR for wait-listing was 1.83 [95% CI 1.74, 1.92] in the US and 1.48 [95% CI 1.02, 2.13] in Austria (**Figure 2**). Further, older age was associated with a reduced probability to receive a donor organ and an increased risk of death (**Figures 2**, **3**). In addition, women were on average older than men in the studied population (**Table 1**). These observations emphasize the need of accounting for age as a potential confounder in the analysis. We accomplished this task by adjusting the male-to-female csHRs within each decade for age (as continuous variable), including an interaction for sex and age. To visualize the results we chose to depict the male-to-female cause specific hazard ratio at the median age as well as 25 and 75% age quantiles (**Figure 2**). This summarizes the csHR at three age levels, yet the underlying model still contains age as continuous variable, represented by restricted cubic splines to account for non-linear age effects, as shown by the csHRs of men and women by age, referenced to median aged women, in **Figure 3**. In the most recent decade the age difference between men and women became smaller, hence the effect of age on sex differences in wait-listing may have become smaller in this decade than in earlier decades, in Austria more so compared to the US. The fact that age is an effect modifier of gender disparity in kidney transplantation has also been shown in another USRDS-based analysis (33).

To a large part, the incidence of dialysis initiation was stable throughout the study period in both countries, and consistent with previously reported sex distributions of roughly 60% men

and 40% women in CKD cohorts (20). The seemingly higher proportion of men starting dialysis in Austria in the last decade (2008–2018: 65.6% men) is likely to be an artifact of the grouping over time, as previous research based on the ADTR data with a different study period and different stratification of the time intervals, did not show significant time trends by sex in dialysis initiation (19). Nevertheless, future monitoring of the ADTR should be sensible to potential trends in the sex-distribution of incident dialysis patients in Austria.

Once wait-listed, the allocation systems of the two countries theoretically do not have gender or sex-specific aspects, meaning that in principle, every listed patient has the same change to receive an organ. Yet it is known that women have higher levels of preformed antibodies, linked to pregnancy. Wolfe et al. (24) showed that sex differences in transplantation rates after wait-listing disappeared when adjusted for panel-reactive antibodies (PRA). Unfortunately, we did not have PRA data for our datasets to confirm this. In any case, as we have shown in this work, the sex differences *after* wait-listing were less pronounced than *for* wait-listing itself.

The age-adjusted male-to-female HR for death after transplantation was >1 in most decades. As the male-to-female mortality rate ratio in adults of the general population remained consistently >1 throughout age groups (21), a higher mortality risk in transplanted men compared to transplanted women might not be surprising. If men have a higher chance of being wait-listed than women, however, then the consequence might be that men who are altogether sicker than women actually receive a transplant, and the comorbidities of these patients might carry over into the post-transplant time, where men die at a higher rate than women. Consistent with this hypothesis, the age-adjusted male-to-female HR for death in the dialysis population of the present study was not as high as it was in the transplant population (although also >1 in some decades and at some ages, see **Supplementary Figure 1**).

Gender disparity in kidney transplantation is often mentioned in context with the perceived unfairness that women are more often donors than they are recipients of living donor transplants (15). In our analysis of US and Austrian data, and as was previously shown for the US (12–14), more living donor kidneys originated from women rather than men (**Table 2**). Many analyses on sex-specific differences in kidney transplantation are not based on registry data, but simply report crude (mostly living related and often single center) transplantation rates which are always shifted toward more women being donors and more men being recipients (34, 35). A wide range of explanations haven been given to the predominance of women in living kidney donation, including better health or a higher degree of responsibility in women and financial obligations of men, all of which remain speculative (11, 13, 15).

The most fundamental difference between the US and the Austria with respect to kidney disease management lies in the distinctive funding of the healthcare systems of the two countries, and thus access to dialysis and subsequent KRT. Austrian's socially funded health care system provides full coverage for its population (99.9%) (36). The majority of dialysis centers are administered by the public sector, private dialysis centers can reimburse a large part of their costs following fixed rates set by the Austrian health fund. In the US, in 2000–2016 88% of dialysis patients were treated in profit-driven facilities, 66.5% of all patients underwent dialysis at only two large, privately owned, for-profit dialysis facility chains. Gander et al. showed that patients under treatment in for-profit dialysis facilities vs. non-profit facilities had lower chances of entering the waitlist and receiving a living or deceased kidney transplant (37). In their analysis, the proportion of women in for-profit facilities was higher compared to women in non-profit facilities. It has previously been hypothesized that for-profit dialysis providers may cut costs in counseling or refrain to refer patients to KRT, since this is in contrast to their financial interests (38). A gender bias in the type of dialysis facility (for-profit vs. non-profit) thus could be a partial explanation of both, the more pronounced advantages for men in KRT in the US compared to Austria, and why gender disparities in wait-listing and transplantation still persisted in the US in the most recent decade, in contrast to Austria.

Among the limitations of this analysis, we acknowledge that it is unclear whether the sex variable was assigned by an investigator or reported by a patient. The sheer size of the dataset implies that 100% correctness cannot be assumed. Further, stratification over time did not follow any significant events in kidney disease management or policy changes, but rather split the data uniformly across the time axis, in order to reveal possible time trends. Moreover, our study cannot provide causality and therefore needs not only to be followed up in additional countries, but also by analyses of socioeconomic differences and other factors, for example obesity (39), which might explain the observed differences between the sexes, on top of age and comorbidities (33).

In summary, the present USRDS and ADTR/Eurotransplant data shed light on the sex differences for various transitions after initiation of kidney replacement therapy, with consideration of trends over four decades. Our analysis follows a recently articulated request (16) to start focusing on non-North American cohorts in examining how sex and gender affect transplantation and compared them with the US. In accordance with previous data from the US (1–8, 24, 33, 40), Canada (41), France (42), Australia (43) and Germany (44), in our analysis predominantly older women have lower access to kidney transplantation than men. Knowing the development in the US and Austria over the last four decades is informative, and this development renders it likely that gender disparity is the root cause of the observed sex differences in kidney transplantation. Future analyses should examine sex-discrepancies in dialysis providers, and also qualitatively address the perspectives of patients (45, 46) and caretakers (47), which might help establish reasons for sex and gender differences, and ways to overcome them. The items in question might include differences in generosity regarding kidney donation, differences in the perception of life, different moral values, and finally, different priorities between men vs. women, including their ability to endorse relationships, despite being affected by kidney disease.

DATA AVAILABILITY STATEMENT

The US based data reported here have been supplied by the United States Renal Data System (USRDS) and are available from USRDS upon request. The interpretation and reporting of these data are the responsibility of the authors and in no way should be seen as an official policy or interpretation of the U.S. government. The Austrian data reported here have been supplied by the Austrian Dialysis and Transplant Registry (ADTR) and are available from the Austrian Society of Nephrology upon request.

ETHICS STATEMENT

This study was reviewed and approved by the Ethics Committee of the Medical University of Vienna (EK No. 1363/2016). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SH conceptualized the analysis, analyzed the data, wrote, revised, and reviewed the manuscript. TG analyzed the data. MA, JC, KJ, and AT conceptualized the analysis and reviewed the manuscript. AK, ML, SK RP-F, WW, and ES reviewed the manuscript. RK provided the data, interpreted the data, and reviewed the manuscript. FP conceptualized the analysis, discussed the data, and reviewed the manuscript. MP conceptualized the analysis. MH provided funding, conceptualized analysis, wrote, revised, and reviewed the manuscript. RR conceptualized the analysis, analyzed the data, wrote, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.800933/full#supplementary-material

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ORIGINAL RESEARCH
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Differential Treatment Effects for Renal Transplant Recipients With DSA-Positive or DSA-Negative Antibody-Mediated Rejection

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Background: Antibody-mediated rejection (ABMR) is the main cause of renal allograft loss. The most common treatment strategy is based on plasmapheresis plus the subsequent administration of intravenous immunoglobulin (IVIG). Unfortunately, no approved long-term therapy is available for ABMR. The current study was designed to analyze the effect of various ABMR treatment approaches on allograft survival and to compare treatment effects in the presence or absence of donor-specific antibodies (DSAs).

Methods: This single-center study retrospectively analyzed 102 renal allograft recipients who had biopsy-proven ABMR after transplant. DSA was detectable in 61 of the 102 patients. Initial standard treatment of ABMR consisted of plasmapheresis (PS) or immunoadsorption (IA), followed by a single course of IVIG. In case of nonresponse or recurrence, additional immunosuppressive medications, such as rituximab, bortezomib, thymoglobulin, or eculizumab, were administered. In a second step, persistent ABMR was treated with increased maintenance immunosuppression, long-term therapy with IVIG (more than 1 year), or both.

Results: Overall graft survival among transplant patients with ABMR was <50% after 3 years of follow-up. Compared to the use of PS/IA and IVIG alone, the use of additional immunosuppressive medications had no beneficial effect on allograft survival ($p = 0.83$). Remarkably, allografts survival rates were comparable between patients treated with the combination of PS/IA and IVIG and those treated with a single administration of IVIG ($p = 0.18$). Renal transplant patients with ABMR but without DSAs benefited more from increased maintenance immunosuppression than did DSA-positive patients with ABMR ($p = 0.01$). Recipients with DSA-positive ABMR exhibited significantly better allograft survival after long-term application of IVIG for more than 1 year than did recipients with DSA-negative ABMR ($p = 0.02$).

Conclusions: The results of our single-center cohort study involving kidney transplant recipients with ABMR suggest that long-term application of IVIG is more favorable for DSA-positive recipients, whereas intensification of maintenance immunosuppression is more effective for recipients with DSA-negative ABMR.

Keywords: antibody-mediated rejection, donor-specific antibody, treatment, IVIG (intravenous immunoglobulin) administration, plasmapheresis, maintenance immunosuppression

INTRODUCTION

Despite all efforts, long-term renal allograft survival is limited to an average of 11 to 15 years (1). The cause of allograft failure is multifactorial. However, antibody-mediated rejection (ABMR) is the main factor contributing to progressive deterioration of allograft function and subsequent allograft loss (1).

Pathophysiological knowledge of the ABMR process has been increasing in recent years. One of the key elements for the diagnosis of ABMR is the formation of donor-specific antibodies (DSAs) (2). DSAs directed against mismatched human leukocyte antigens (HLA) class I and II attach to the endothelium, triggering complement activation via the classic pathway and inducing Fc gamma receptor–dependent effects on the activation of natural killer cells and macrophages. Membrane attack complex (MAC) activated by C1q is responsible for inflammation in the vascular endothelium, generating direct irreversible injury of the allograft (3). Histologic features, such as glomerulitis and peritubular capillaritis, as well as chronic glomerulopathy, indicate endothelial damage. In addition, microvascular injury stimulates platelet activation, resulting in the development of microthrombi (4, 5). C4d is a specific correlate of complement cascade activation initiated by DSAs. As a degradation product of C4, C4d binds to endothelium (3); often rendering C4d deposits detectable in biopsy samples from allografts in patients with ABMR (6). Thus, C4d deposition in renal allografts is one diagnostic criterion for acute and chronic ABMR (7).

Preformed DSAs are present in one third of recipients in whom early acute ABMR takes a severe course (5, 8). However, *de novo* DSAs can develop in nonsensitized patients after transplant. Although ABMR is more common in the late posttransplant course, early ABMR can occur within the first 6 months after transplant (3, 9, 10). ABMR appears in the context of under immunosuppression and is related to the production of DSAs (9, 10).

However, several reports have documented the histologic picture of ABMR in the absence of detectable DSAs (11–17). Circulating DSAs in the presence of ABMR-compatible histologic lesions were absent in as many as 27% of patients with ABMR (12). In addition to the possibility that current techniques cannot detect some anti–HLA-DSA antibodies, DSA-negative ABMR cases may be explained by the occurrence of non-HLA antibodies after renal transplant, the presence of HLA-specific memory B cells, and intragraft deposition of DSAs (14–17). Although published information about DSA-negative ABMR is still limited, the comparison of allograft outcomes between DSA-negative ABMR and DSA-positive ABMR has yielded controversial results (11–13).

Therapy for ABMR is one of the main challenges facing transplant medicine. Currently, no approved treatments for chronic ABMR exist (1, 5). Plasmapheresis (PS) in combination with high-dose intravenous immunoglobulin (IVIG) has proved to be effective in several trials and is the current standard of care for acute ABMR (1, 5). However, the quality of the evidence supporting this treatment regimen is low (1, 18). Therefore, there is an unmet need for new, innovative therapeutic approaches. Recent studies compared the ability of three potential ABMR therapies (the B cell–depleting antibody rituximab; an inhibitor of proteasome, bortezomib, which interferes with alloantibody-producing plasma cells; and an antibody targeting a terminal component of complement pathway, eculizumab) to prevent the genesis of MAC (1, 3, 5). Unfortunately, the results of these therapeutic strategies were disappointing (1, 3, 5). The quality of the existing data is also moderate because most of the studies were small pilot studies and were consequently underpowered (1, 5). Additionally, a proper comparison of previous studies is difficult because intervention protocols vary strongly between centers and because the studies did not account for the various heterogeneous phenotypes of ABMR that have been established in recent years by the evolution of the Banff classification of ABMR (1, 19).

The study reported here analyzed the effect of a stepwise treatment approach for ABMR on allograft survival and compared treatment effects in the presence or absence of DSAs. Additionally, we estimated the importance of various factors associated with ABMR for allograft loss.

MATERIALS AND METHODS

Study Population

Between January 2014 and September 2017, 438 adult recipients of renal allografts, most of whom were treated at the University Hospital Essen, underwent a total of 833 renal transplant biopsies. All biopsies were performed for cause, and samples were analyzed according to the latest available Banff grading criteria (19, 20). Several patients underwent more than one renal biopsy during the study period (on average, 2 renal

Abbreviations: ABMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific antibody; CNI, calcineurin-inhibitor; HLA, human leukocyte antigen; HR, hazard ratio; IA, immunoadsorption; IgG, immunoglobulin; IQR, interquartile range; IVIG, intravenous immunoglobulin; MAC, membrane attack complex; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PRA, panel-reactive antibodies; PS, plasmapheresis; SAB, single-antigen bead; Tx, transplantation.

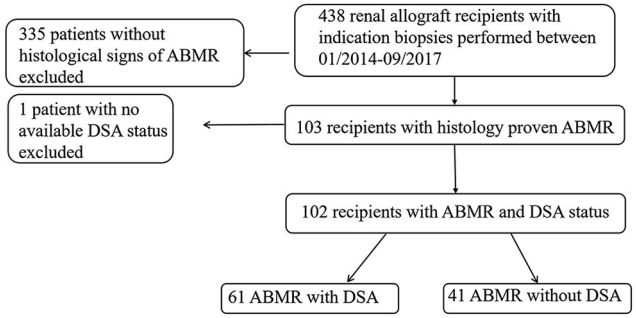

FIGURE 1 | Study population flow chart. ABMR, antibody-mediated rejection; DSA, donor-specific antibody.

biopsies were performed per patient). In such cases we considered the earliest and most representative biopsy. No protocol biopsies were performed during the study period. As shown in **Figure 1**, biopsy-proven ABMR was detected in 103 recipients. For one patient no report on DSAs was available, and this patient was excluded from further analysis. Therefore, 102 recipients with ABMR and available DSA status were included in the current study. Control biopsies performed after the completion of standard treatment of ABMR were used to evaluate the effectiveness of previous treatment. Experienced nephropathologists examined all renal transplant specimens with light microscopy and immunohistochemical analyses. The retrospective single-center study was approved by the institutional ethics board (19-8097-BO).

Clinical and laboratory data were collected by a review of the electronic medical record. Allograft survival was followed up for at least 3 years after the performance of the first biopsy that showed evidence of ABMR. Clinical data on all therapy approaches that were used to cure ABMR were collected for as long as 3 years after the initial biopsy.

Treatment Approaches for ABMR

In our stepwise treatment approach, the first step of initial standard therapy for acute ABMR involved 3–5 runs of PS, immunoadsorption (IA), or both, with subsequent intravenous administration of IVIG at a dosage of 0.5–0.8 g/kg over 3 days (**Table 1**). If there was no response to initial standard therapy, or if ABMR was detected by a follow-up biopsy, additional immunosuppressive drugs such as rituximab, bortezomib, eculizumab, or thymoglobulin were administered off-label (**Table 1**). The second step, for treatment of biopsy-proven persistent ABMR, involved an increase in maintenance immunosuppression, long-term therapy with IVIG for more than 1 year, or both (**Table 1**).

Immunological Analyses

Pretransplant lymphocytotoxic crossmatches were negative for all recipients. For HLA typing of recipients and donors, DNA was isolated from peripheral blood samples with spin columns (Qiagen, Hilden, Germany) or with an automated system using magnetic separation technology (Chemagic, Chemagen PerkinElmer, Baesweiler, Germany). HLA-class I (HLA-A, -B, -C) and II (HLA-DRB1, -DQB1) typing was performed at the first field resolution level with sequence-specific primers (polymerase chain reaction sequence-specific primer method) or alternatively with sequence-specific oligonucleotides (LABType SSO method, both provided by One Lambda/Thermo Fisher Inc., Canoga Park, CA, USA) (21).

All patients were screened for anti-HLA antibodies before transplant. Anti-HLA antibody status after transplant was monitored at months 3, 6, and 12 after transplant and annually thereafter. Additional screening was performed if allograft dysfunction occurred. Pretransplant sensitization status was determined with the standard immunoglobulin G (IgG) complement-dependent cytotoxicity test in combination with a Luminex-based LABScreen Mixed beads assay (One Lambda, Thermo Fisher Scientific, Inc.), which was used to identify the antibodies against HLA classes I and II. If the results of the LABScreen Mixed beads assay were positive, anti-HLA antibodies were further characterized in terms of IgG

TABLE 1 | Overview of therapies used to treat biopsy-proven antibody-mediated rejection among 102 renal allograft recipients.

Primary therapy of ABMR	All patients	Patients with DSA^+ ABMR	Patients with DSA^- ABMR	*p*-value
No therapy	1	0	1	n.c.
Thymoglobulin alone	1	1	0	n.c.
Eculizumab alone	1	1	0	n.c.
Intensification of maintenance immunosuppression + eculizumab	2	0	2	n.c.
IVIG alone	13	6	7	0.285
IVIG + IA/PS	84	53	31	0.145
IVIG + PS	*75*	*46*	*29*	*n.c.*
IVIG + IA	*9*	*7*	*2*	*n.c.*
IVIG + IA/PS without add-on therapy	*46*	*27*	*19*	*0.837*
IVIG + IA/PS + add-on therapy	*38*	*26*	*12*	*0.174*
IVIG + IA/PS + rituximab	*10*	*8*	*2*	*n.c.*
IVIG + IA/PS + bortezomib	*11*	*8*	*3*	*n.c.*
IVIG + IA/PS + thymoglobulin	*9*	*5*	*4*	*n.c.*
IVIG + IA/PS + eculizumab	*3*	*1*	*2*	*n.c.*
IVIG + IA/PS + multiple add-on therapies	*5*	*4*	*1*	*n.c.*
Secondary therapy of ABMR				
Long-term IVIG	**36**	**27**	**9**	**0.021**
Long-term IVIG alone	6	5	1	0.228
Long-term IVIG + IA/PS without add-on therapy	11	8	3	0.357
Long-term IVIG + IA/PS with add-on therapy	19	14	5	0.173

ABMR, antibody-mediated rejection; DSA, donor-specific antibody; IA, immunoadsorption; IVIG, intravenous immune globulin; n.c., not calculated; PS, plasmapheresis.
Bold values are significant values ($p < 0.05$).

alloantibody specificity for HLA-A, -B, -C, -DR, -DP, and -DQ with LABScreen single-antigen bead (SAB) assays (One Lambda, Thermo Fisher Scientific Inc.) according to the manufacturer's protocols. All beads with normalized median fluorescence intensity (MFI) values higher than 1.000 were considered to be positive for anti-HLA antibodies. To address a potential effect of interfering antibodies or prozone effects on our MFI analyses, all patient sera included in this study have been treated with ethylenediaminetetraacetic acid (EDTA) prior to Luminex-based assay testing (22).

Statistical Analyses

Categorical variables were expressed as numbers and percentages. Comparisons between groups were made with the χ^2 test for categorical variables and with the Mann–Whitney test for continuous variables. Allograft survival among patients with ABMR was illustrated with Kaplan–Meier survival curves and analyzed with the log-rank test. To assess independent factors influencing renal allograft survival, we performed a multivariable Cox regression analysis and calculated the hazard ratio (HR) and the 95% confidence interval (CI). Variables for multivariate analysis were selected based on the results of univariate analysis. For all tests, statistical significance was set at the level of $p \leq 0.05$. All data analyses were performed with GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA) and IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA).

RESULTS

Patient Characteristics

Of the 438 renal allograft recipients who underwent allograft biopsy between January 2014 and September 2017, 102 were found to have biopsy-proven ABMR with known DSA status, one patient with ABMR was excluded due to missing DSA status. The remaining 335 renal allograft recipients who received a primary biopsy for cause showed the following results: Borderline rejection (Banff category 3) in 60 and T-cell mediated rejection (Banff category) in 46, Banff category 6 in 201 (with CNI-toxicity in 51, polyoma virus nephropathy in 12, acute reversible tubular necrosis in 94, signs of infection in 10, recurrence of primary renal disease in 34), Banff category 5 in 1, normal renal tissue in 4, non-representative renal biopsy in 23.

Clinical and demographic characteristics of these 102 patients with histologic features of ABMR are summarized in **Table 2**. Of these patients, 24 (24%) underwent renal allograft biopsy within the first year after transplant. At the time of biopsy, 101 of the 102 patients were receiving maintenance immunosuppression including steroids; 76 patients (75%) were treated with tacrolimus as a calcineurin inhibitor, 16 (16%) with cyclosporin A. Most patients (80; 78%) were receiving maintenance immunosuppression with mycophenolic acid (MMF) or mycophenolate mofetil (MPA) and 12 (12%) with everolimus. DSAs were detected in 61 (60%) patients with histologic evidence of ABMR. The remaining 41 patients with ABMR had a negative DSA status at the time of diagnosis. Among DSA-positive patients, 14 (14%) exhibited anti–HLA-DSA class I antibodies and 35 (34%) exhibited DSA class II antibodies. Twelve (12%) recipients exhibited anti–HLA-DSA class I and class II antibodies simultaneously.

Comparing patients with DSA-positive ABMR and patients with DSA-negative ABMR, we noted that a larger proportion of patients in the DSA-negative group had undergone previous transplants (**Table 2**). Recipients without DSA were older and were more likely to have undergone a renal biopsy during the first year after transplant at first evidence of ABMR (**Table 2**). Mismatches in HLA class I and class II were more frequent in DSA-positive patients with ABMR than in ABMR patients without DSA finding (**Table 2**). Immunosuppressive regimens at the time of biopsy were mainly comparable except that the use of everolimus and belatacept was more common among DSA-negative patients (**Table 2**).

Overall allograft survival among all kidney transplant patients with ABMR was <50% at the 3-year follow-up.

Late ABMR and Positive C4d Status Were the Main Risk Factors for Allograft Failure

First, we analyzed the effect of several variables associated with ABMR on allograft survival among the 102 recipients with biopsy-proven ABMR. We selected several relevant factors, such as DSA status, C4d positivity, occurrence of ABMR within the first year after transplant, and histologic signs of various Banff categories in addition to ABMR so that we could evaluate the potential effect of these variables on allograft outcome.

Renal allograft survival rates were comparable between recipients with DSA-positive or DSA-negative ABMR, a finding suggesting that DSA status had no significant effect on allograft loss in the present cohort ($p = 0.72$, **Figure 2A**).

Renal allograft biopsies found evidence of C4d deposition among 26 patients in our cohort. C4d positivity was associated with significantly worse allograft survival at the follow-up 3 years after transplant ($p = 0.004$, **Figure 2B**).

As expected, the occurrence of biopsy-proven ABMR within the first year after transplant, referred as early ABMR, reflected an advantage in terms of allograft survival compared to late ABMR ($p = 0.01$, **Figure 2C**). However, many recipients with early ABMR exhibited no DSAs (**Table 2**).

Besides humoral rejection within Banff category 2, a proportion of patients showed additional histologic signs related to other Banff categories. There were no significant differences in allograft survival rates between patients with ABMR alone and those with co-occurrence of ABMR and T cell–mediated rejection or borderline changes, although we observed a slight trend toward earlier occurrence of allograft loss among patients with characteristics of other Banff categories in addition to Banff category 2 (**Figure 2D**).

Univariate and subsequent multivariate analyses found that late ABMR and C4d were independent risk factors for allograft failure among patients with ABMR after renal transplant (**Table 3**).

TABLE 2 | Baseline characteristics of 102 renal allograft recipients with biopsy-proven antibody-mediated rejection.

Variables	All patients $n = 102$	Patients with DSA$^+$ ABMR $n = 61$	Patients with DSA$^-$ ABMR $n = 41$	*p*-value
Characteristics at the time of Tx/biopsy				
Number of men, *n* (%)	47 (46.1)	31 (50.8)	16 (39.0)	0.241
Recipient age, median (IQR)	**43 (26,75–55.25)**	**37 (21.5–52.0)**	**49 (39.0–62.0)**	**<0.001**
Recipient age at the time of biopsy, median (IQR)	**50.5 (32.25–60.25)**	**47 (27.0–56.5)**	**54 (43.5–65.0)**	**0.008**
Time between Tx and biopsy in days, median (IQR)	**1,550 (451–3,605)**	**2,720 (1,151–4,142)**	**705 (34–1,741)**	**<0.001**
Allograft biopsy <1 year after Tx, *n* (%)	**24 (23.5)**	**5 (8.2)**	**19 (46.3)**	**<0.001**
Allograft biopsy >1 year after Tx, *n* (%)	**78 (76.5)**	**56 (91.8)**	**22 (53.7)**	**<0.001**
Living donor, *n* (%)	26 (25.5)	19 (31.1)	7 (17.1)	0.081
Cold ischemia time (h:min), median (IQR)	12:21 (5:27–17:09)	11:02 (2:36–17:00)	13:35 (7:36–17:31)	0.198
Previous transplants, *n* (%)	**20 (19.6)**	**7 (11.5)**	**13 (31.7)**	**0.019**
HLA class I and II mismatch (HLA-A, -B, -DR), median (IQR)	**3 (2–4)**	**3 (2–4)**	**2 (0–3)**	**0.016**
HLA class I mismatch (HLA-A, -B), median (IQR)	**2 (1–2)**	**2 (1–3)**	**1 (0–2)**	**0.020**
HLA class II mismatch (HLA-DR), median (IQR)	1 (0–1)	1 (0–2)	1 (0–1)	0.050
ABO-incompatible Tx, *n* (%)	0 (0)	0 (0)	0 (0)	–
Current PRA ≥ 5%, *n* (%)	22 (21.6)	13 (21.3)	9 (22.0)	0.939
Current PRA ≥ 20%, *n* (%)	13 (12.7)	7 (11.5)	6 (14.7)	0.639
Anti-HLA–DSA, *n* (%)	61 (59.8)	61 (100.0)	–	–
Anti-HLA–DSA class I, *n* (%)	14 (13.7)	14 (23.0)	–	–
Anti-HLA–DSA class II, *n* (%)	35 (34.3)	35 (57.4)	–	–
Anti-HLA–DSA class I and II, *n* (%)	12 (11.8)	12 (19.7)	–	–
Peak MFI of DSA, median (IQR)	8,500 (3,150–17,650)	8,500 (3,150–17,650)	–	–
Sum of MFI, median (IQR)	9,800 (3,300–21,650)	9,800 (3,300–21,650)	–	–
Immunosuppression at the time of biopsy				
Steroids, *n* (%)	101 (99)	61 (100)	40 (97.6)	0.402
Cyclosporine A, *n* (%)	16 (15.7)	12 (19.7)	4 (9.8)	0.177
Tacrolimus, *n* (%)	76 (74.5)	47 (77.0)	29 (70.7)	0.473
MMF or MPA, *n* (%)	80 (78.4)	45 (73.8)	35 (85.4)	0.163
Belatacept, *n* (%)	**4 (3.9)**	**0 (0)**	**4 (9.8)**	**0.024**
Everolimus, *n* (%)	**12 (11.8)**	**3 (4.9)**	**9 (22.0)**	**0.012**
Sirolimus, *n* (%)	2 (2.0)	1 (1.6)	1 (2.4)	1.000
Azathioprine, *n* (%)	2 (2.0)	1 (1.6)	1 (2.4)	1.000

ABMR, antibody-mediated rejection; anti-HLA, anti–human leukocyte antigen; DSA, donor-specific antibody; IQR, interquartile range; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolic acid; PRA, panel-reactive antibodies, Tx, transplantation.
Bold values are significant values ($p < 0.05$).

Administration of IVIG Alone Was as Effective as Administration of the Combination of PS/IA With IVIG for the Treatment of ABMR

Among 102 renal allograft recipients with evidence of ABMR, 84 patients were initially treated with PS, IA, or both and subsequent application of IVIG. As shown in **Figure 3A**, we observed no difference in renal allograft survival rates between ABMR treatment with plasmapheresis or immunoadsorption ($p = 0.44$). The remaining 13 recipients (**Table 1**) were treated with IVIG alone without any application of PS or IA. Surprisingly, renal allograft survival rates were similar among patients receiving IVIG alone and those receiving the combination of PS, IA, or both followed by IVIG ($p = 0.18$, **Figure 3A**).

Adjunctive Immunosuppressive Therapy Did Not Achieve Better Allograft Survival Than Standard ABMR Treatment With PS/IA and IVIG Alone

For 38 of 102 allograft recipients, standard treatment with PS/IA and IVIG was followed by adjunctive immunosuppressive therapy consisting of rituximab ($n = 10$), bortezomib ($n = 11$), thymoglobulin ($n = 9$), or eculizumab ($n = 3$) (**Table 1**). We found that the application of additional immunosuppressive therapy, such as bortezomib, rituximab, thymoglobulin, or eculizumab, did not achieve better renal allograft survival rates than did standard treatment with PS/IA and IVIG alone ($p = 0.83$, **Figure 3B**). Moreover, analyses of the subgroups determined by DSA status found no differences in allograft survival rates between patients receiving additional

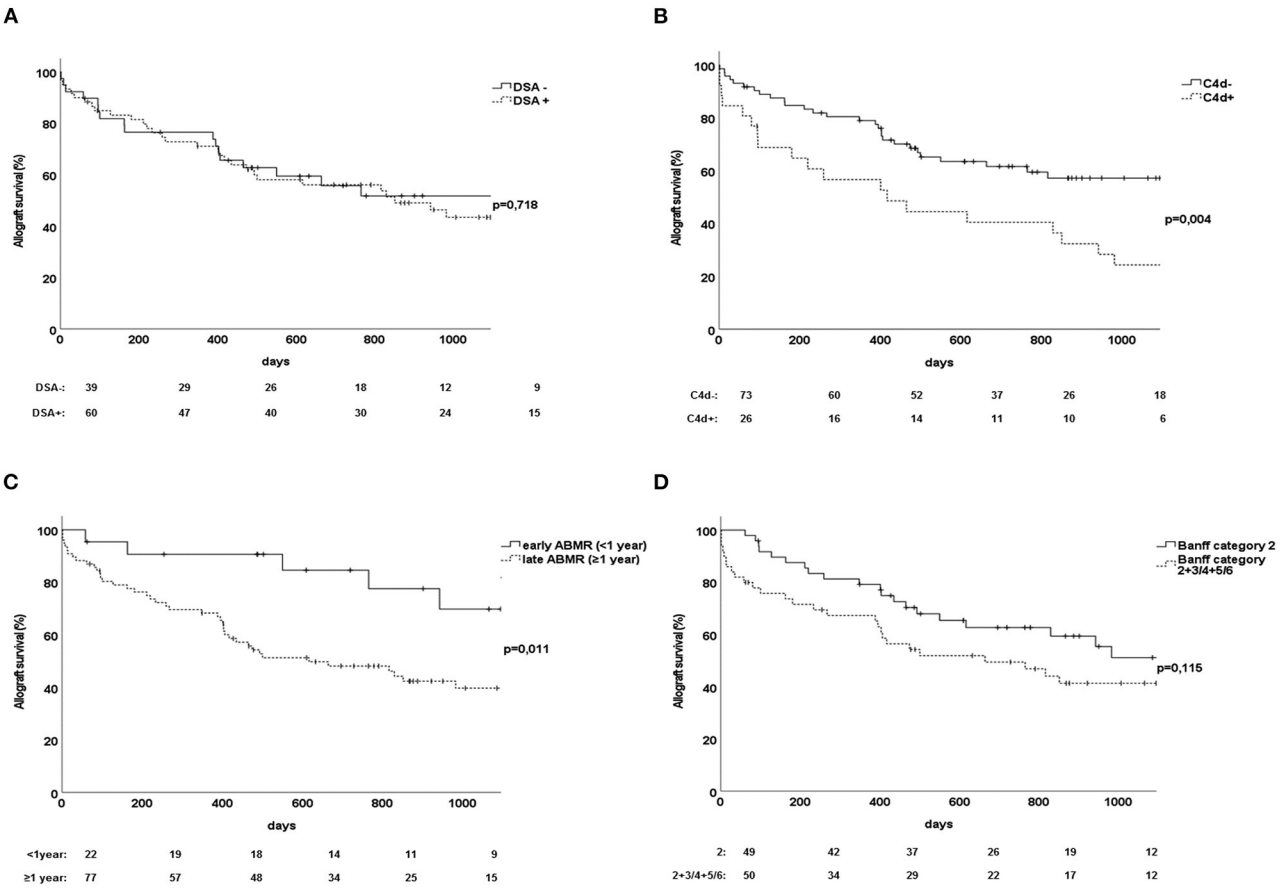

FIGURE 2 | Association of various factors related to accelerated allograft loss due to ABMR among 102 transplant recipients. **(A)** Influence of DSA status of ABMR on renal allograft survival. **(B)** Influence on renal allograft survival of C4d deposits among patients with biopsy-proven ABMR. **(C)** Allograft survival in relation to ABMR detected within the first year after transplant. **(D)** Allograft survival among patients with ABMR and Banff category 2 only compared to Banff category 2 in combination with other pathologic lesions (Banff category 3 or 4 and/or 5 or 6). ABMR, antibody-mediated rejection; DSA, donor-specific antibody.

immunosuppressive therapy and those receiving standard therapy (data not shown).

Increased Maintenance Immunosuppression Exerted a Beneficial Effect on Allograft Survival Among Recipients With DSA-Negative ABMR

Renal allograft recipients exhibiting histologic features of biopsy-proven persisting ABMR received increased maintenance immunosuppression, long-term therapy with IVIG for more than 1 year, or both. Increased immunosuppression was defined as a change in the maintenance immunosuppressive regimen or as a switch from dual to triple immunosuppressive therapy. An acceleration of the dosage of maintenance immunosuppressive drugs was not considered for the analysis. Maintenance immunosuppressive therapy was intensified for 28 patients in the study cohort. These 28 recipients exhibited higher rates of allograft survival than did the remaining 71 patients ($p = 0.01$, **Figure 4A**). In addition, univariate analysis showed that intensification of maintenance immunosuppression exerted a protective effect on allograft survival among recipients with ABMR (HR, 0.37; $p = 0.02$; **Table 3**). However, the results could not be confirmed by multivariate Cox regression analysis (**Table 3**).

With regard to the DSA status of ABMR, we observed significantly better allograft survival after increased maintenance immunosuppression for recipients with DSA-negative ABMR at the 3-year follow-up ($p = 0.01$, **Figure 4B**). Moreover, both univariate and multivariate analysis detected a positive effect on renal allograft survival when persistent DSA-negative ABMR was treated with increased maintenance immunosuppression (**Table 4**). In contrast, intensification of maintenance immunosuppression did not influence allograft survival among patients with DSA-positive ABMR ($p = 0.3$, **Figure 4B**).

Long-Term Therapy With IVIG Improved Allograft Survival Among Patients With DSA-Positive ABMR

Long-term application of IVIG for more than 1 year was a treatment option for recipients with ABMR as detected by

TABLE 3 | Results of univariate and multivariate analyses identifying risk factors and protective factors for allograft failure among 102 renal allograft recipients with ABMR.

	HR $n = 102$	CI (95%)	*p*-value
Univariate analysis			
Risk factors			
DSA^+	1.116	0.615–2.023	0.719
Previous transplants	0.986	0.462–2.134	0.986
$C4d^+$	**2.285**	**1.275–4.098**	**0.006**
ABMR ≥ 1 year	**3.116**	**1.231–7.887**	**0.016**
Banff category 2	0.681	0.383–1.211	0.191
Banff category 2 + 3/4	0.922	0.467–1.820	0.816
Banff category 2 + 5/6	1.445	0.715–2.921	0.305
Banff category 2 + 3/4 + 5/6	2.238	0.881–5.685	0.090
Recipient age at the time of biopsy ≥ 50 years	0.986	0.555–1.751	0.962
Acute ABMR	0.914	0.519–1.612	0.757
Acute+chronic-active ABMR	1.332	0.712–2.490	0.369
Chronic-active ABMR	0.837	0.426–1.645	0.606
Therapy regimen			
Add-on therapies	0.937	0.525–1.672	0.825
Intensification of maintenance immunosuppression	**0.372**	**0.167–0.831**	**0.016**
Long-term IVIG	0.637	0.348–1.169	0.145
Multivariate analysis			
$C4d^+$	**2.522**	**1.405–4.526**	**0.002**
ABMR ≥ 1 year	**2.604**	**0.982–6.901**	**0.054**
Intensification of maintenance immunosuppression	0.470	0.202–1.091	0.079

ABMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific antibody; HR, hazard ratio; IVIG, intravenous immune globulin.
Bold values are significant values ($p < 0.05$).

a follow-up biopsy performed after the administration of the standard combination therapy of PS/IA with IVIG. Long-term therapy with IVIG was administered to a total of 34 recipients. However, allograft survival rates did not differ between recipients who received IVIG fewer than 3 times and recipients treated with long-term IVIG therapy ($p = 0.14$, **Figure 5A**).

After differentiation for DSA status, allograft survival was significantly better among patients with DSA-positive ABMR treated with long-term IVIG than among those treated with short-term IVIG ($p = 0.02$, **Figure 5B**). Excluding patients with prognostic favorable early ABMR confirmed the advantage of long-term compared to short-term IVIG treatment for late DSA-positive ABMR ($p = 0.006$, **Supplementary Figure 1**). The protective effect of repetitive administration of IVIG for more than one year as detected by univariate analysis was also detected by multivariate Cox regression analysis for allograft survival (**Table 4**). Long-term application of IVIG had no effect on allograft survival among patients with DSA-negative ABMR (**Figure 5B**). It should be mentioned that the subgroup of patients with DSA-positive ABMR contained significantly more recipients who were treated with repetitive IVIG applications than did the subgroup of patients with DSA-negative ABMR (**Table 1**).

DISCUSSION

The results of this study showed that a diagnosis of ABMR later than the first year after transplant and C4d positivity as detected by renal allograft biopsy are important indicators of the risk of allograft loss among ABMR patients. Our stepwise treatment analysis showed that the use of adjunctive immunosuppressive therapy, such as rituximab, bortezomib, eculizumab, or thymoglobulin, exerts no additional benefit on

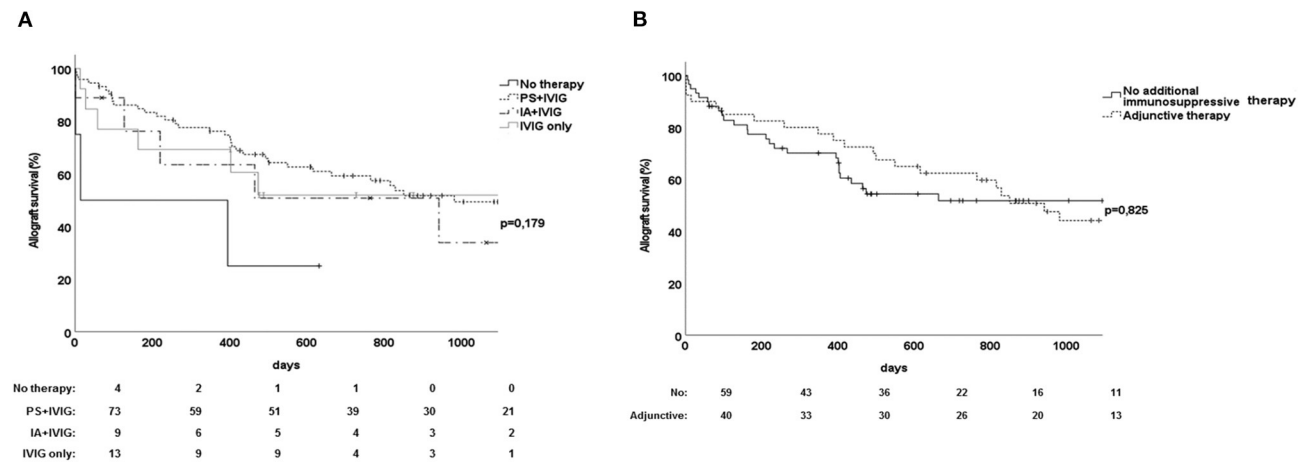

FIGURE 3 | Effect of various treatment approaches on renal allograft survival among 102 recipients with antibody-mediated rejection. **(A)** Comparison between the use of plasmapheresis plus IVIG vs. immunoadsorption with IVIG and IVIG alone. **(B)** Effect on renal allograft survival of the administration of adjunctive immunosuppressive drugs in addition to standard therapy among recipients with ABMR. Adjunctive immunosuppressive drugs were rituximab ($n = 10$), bortezomib ($n = 11$), thymoglobulin ($n = 9$), or eculizumab ($n = 3$). ABMR, antibody-mediated rejection; IA, immunoadsorption; IVIG, intravenous immune globulin; PS, plasmapheresis.

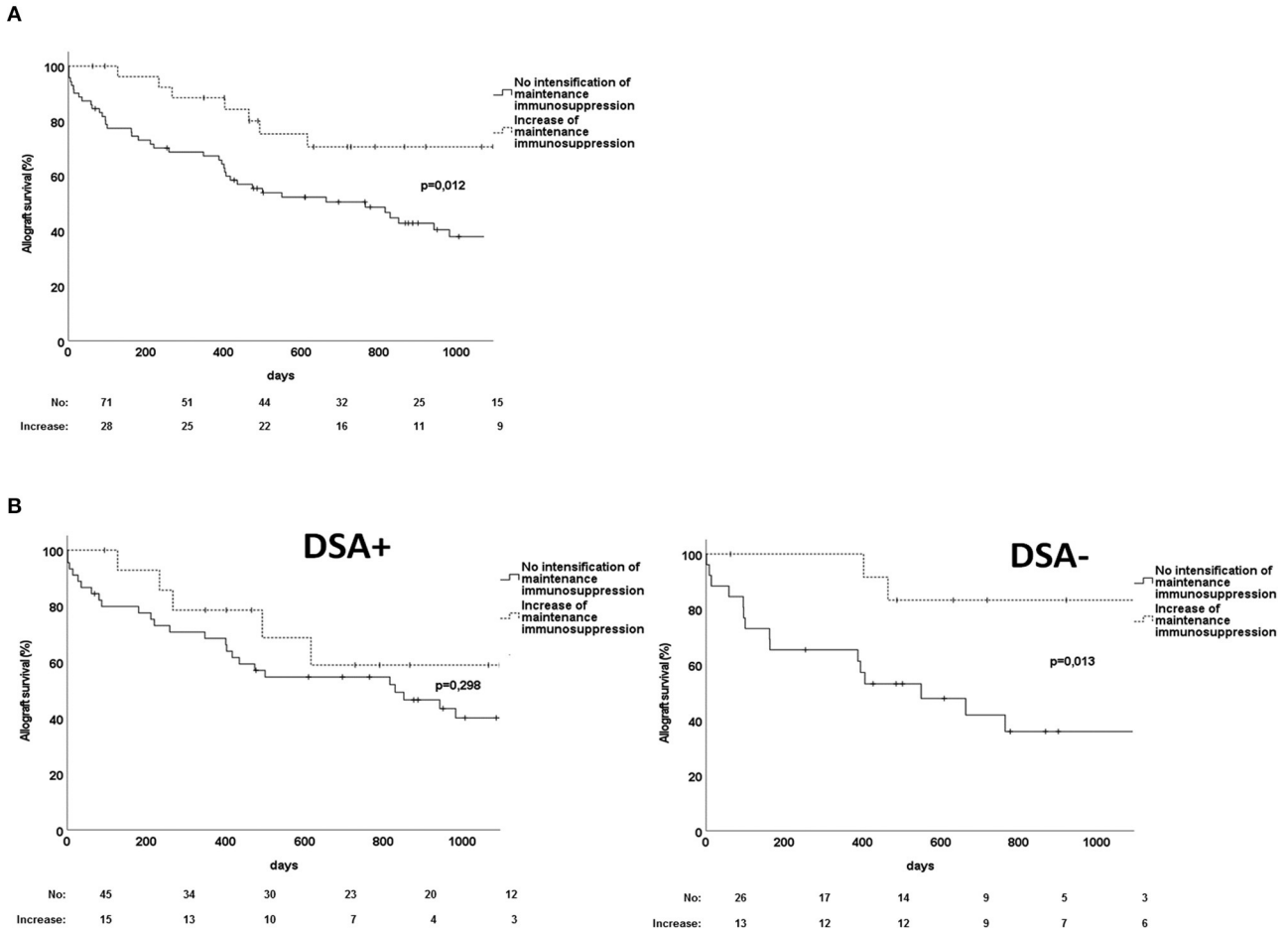

FIGURE 4 | Effect of the increase of maintenance immunosuppression on renal allograft survival among recipients with persistent antibody-mediated rejection. **(A)** Comparison of allograft survival between recipients who were treated with intensified maintenance immunosuppression vs. recipients without increase of maintenance immunosuppression. **(B)** Comparison of the effect of intensified maintenance immunosuppression on allograft survival between recipients with DSA-positive vs. DSA-negative ABMR. ABMR, antibody-mediated rejection; DSA, donor-specific antibody.

graft survival than does treatment with PS/IA and IVIG alone. Our analysis of additional therapeutic effects showed that long-term application of IVIG is more favorable for patients with DSA-positive ABMR, whereas intensification of maintenance immunosuppression therapy is more effective for recipients with DSA-negative ABMR.

We assessed the effect of several ABMR-associated factors on long-term renal allograft survival after the diagnosis of ABMR. Allograft survival rates were similar between DSA-negative ABMR and DSA-positive ABMR. Like us, Crespo et al. and Sablik et al. described a lack of association between DSA status and allograft survival in the presence of ABMR (11, 13). However, our results disagree with those of Senev et al., who suggested that the risk of allograft failure was significant lower for patients with DSA-negative ABMR than for those with DSA-positive ABMR (12). Senev et al. found that DSA-negative ABMR had a transient histologic picture and was less likely to become chronic (12). The discrepancy in the results may be attributed to differences in study design. Senev et al. analyzed protocol biopsies; they included predominantly patients with active ABMR without chronicity and evaluated allograft survival early after transplant (12). In contrast, we included allograft recipients with a biopsy for cause early and late after transplant and followed them for at least 3 years after the detection of ABMR and found that most of them exhibited concomitant histologic features of active and chronic ABMR.

We also found that the timepoint of ABMR diagnosis is a crucial factor determining allograft outcome. Our observation is concordant with those of others showing that the late appearance of ABMR after transplant is a significant risk factor for rapid allograft loss (23, 24).

We found that C4d status as detected by biopsy plays a relevant role in allograft outcome after the occurrence of ABMR. Accordingly, C4d-negative ABMR seems to be an advantageous factor as compared with C4d-positive ABMR. These results are in line with those of a number of previous reports

TABLE 4 | Results of univariate and multivariate analyses identifying risk factors for allograft failure and assessing treatment effects of increased maintenance immunosuppression and long-term therapy with IVIG on allograft survival in the subgroup of 61 recipients with DSA-positive ABMR and 41 recipients with DSA-negative ABMR.

	DSA$^+$ ABMR ***n* = 61**			**DSA$^-$ ABMR** ***n* = 41**		
	HR	**CI (95%)**	***p*-value**	**HR**	**CI (95%)**	***p*-value**
Univariate analysis						
Risk factors						
Previous transplants	0.87	0.3–2.9	0.815	1.14	0.4–3.3	0.811
C4d$^+$	**3.35**	**1.6–6.9**	**0.001**	1.3	0.5–3.7	0.627
ABMR ≥ 1 year	3.53	0.5–26.1	0.216	**3.57**	**1.2–11.0**	**0.026**
Banff category 2	0.61	0.3–1.3	0.180	0.57	0.2–1.6	0.291
Banff category 2 + 3/4	1.07	0.4–2.6	0.886	1.05	0.4–2.9	0.925
Banff category 2 + 5/6	1.36	0.6–3.3	0.509	1.61	0.5–5.0	0.412
Banff category 2 + 3/4 + 5/6	3.22	1.0–10.8	0.059	1.68	0.4–7.4	0.495
Recipient age at the time of biopsy ≥ 50	1.15	0.6–2.4	0.714	0.78	0.3–2.1	0.622
Acute ABMR	0.87	0.4–1.8	0.699	1.03	0.4–2.7	0.953
Acute+chronic-active ABMR	1.23	0.6–2.7	0.605	1.2	0.4–3.4	0.737
Chronic-active ABMR	0.97	0.5–2.1	0.949	0.7	0.2–3.1	0.632
Therapy regimen						
Add-on therapies	1.09	0.5–2.2	0.823	0.67	0.2–1.9	0.452
Intensification of maintenance immunosuppression	0.6	0.2–1.6	0.304	**0.19**	**0.04–0.8**	**0.027**
Long-term IVIG	**0.41**	**0.2–0.9**	**0.021**	1.34	0.5–3.8	0.581
Multivariate analysis						
ABMR ≥ 1 year	.	.	.	2.97	1.0–9.2	0.059
Intensification of maintenance immunosuppression	.	.	.	**0.22**	**0.05–1.0**	**0.048**
C4d$^+$	**3.37**	**1.6–6.9**	**0.001**	.	.	.
Long-term IVIG	**0.41**	**0.2–0.9**	**0.021**	.	.	.

ABMR, antibody-mediated rejection; CI, confidence interval; DSA, donor-specific antibody; HR, hazard ratio; IVIG, intravenous immune globulin.
Bold values are significant values ($p < 0.05$).

acknowledging C4d-positive ABMR as more severe and as associated with a shortened allograft half-life and microvascular inflammation (25–28).

Furthermore, we found that histologic signs of ABMR in combination with T cell–mediated rejection are not linked with poorer allograft survival. Our results contradict those of Matignon et al., who found that the presence of histologic features of T cell–mediated rejection in addition to C4d-positive ABMR is a risk factor for premature allograft failure (29). These discordant results can be partly explained by our inclusion of C4d-positive and -negative ABMR and the relatively small number of patients with C4d-positive ABMR in our cohort. Additionally, we observed a slight trend toward a higher portion of female recipients in the group of patients with DSA-negative-ABMR compared to patients having ABMR with evidence of DSA. This increase may be related to reactivation of memory B cells to non-HLA antigens to which multiparous females have been previously exposed during pregnancy (16).

In the second step, we determined the effect of various ABMR treatment approaches on allograft survival. As expected, treatment of ABMR with PS/IA in combination with IVIG is superior to no treatment. Although treatment concepts for ABMR vary widely, most centers use a combination of PS and IVIG for treating ABMR. In addition, the Transplantation Society working group recommend PS followed by IVIG as the standard of care for removing circulating DSAs (30). However, this recommendation is based on the results of a few randomized controlled trials showing the effectiveness of PS and IVIG for treating ABMR (31–33). Surprisingly, allograft outcome after treatment with the combination of PS/IA with IVIG was not better than after the administration of IVIG alone. In the early 1990s, the immunomodulatory effects of IVIG on T and B cells were recognized. IVIG can initiate apoptosis of B cells and can modulate B-cell signaling (34). The research groups of Peraldi et al. and Jordan et al. published the first reports showing that treatment with high-dose IVIG led to improvement in renal allograft survival after 5 years' follow-up (35). Cooper et al. and Stegall et al. investigated the effect of high-dose IVIG on the production of DSAs and found a modest decrease in DSAs (36). However, the results of Lefaucheur et al. contradict our results. Treating ABMR with high-dose IVIG alone was inferior to treatment with regimens using a combination of IVIG with PS and rituximab (37). Even so, studies comparing the use of IVIG alone with standard therapy combining PS and IVIG are rare, and the question of whether the administration of IVIG alone is as

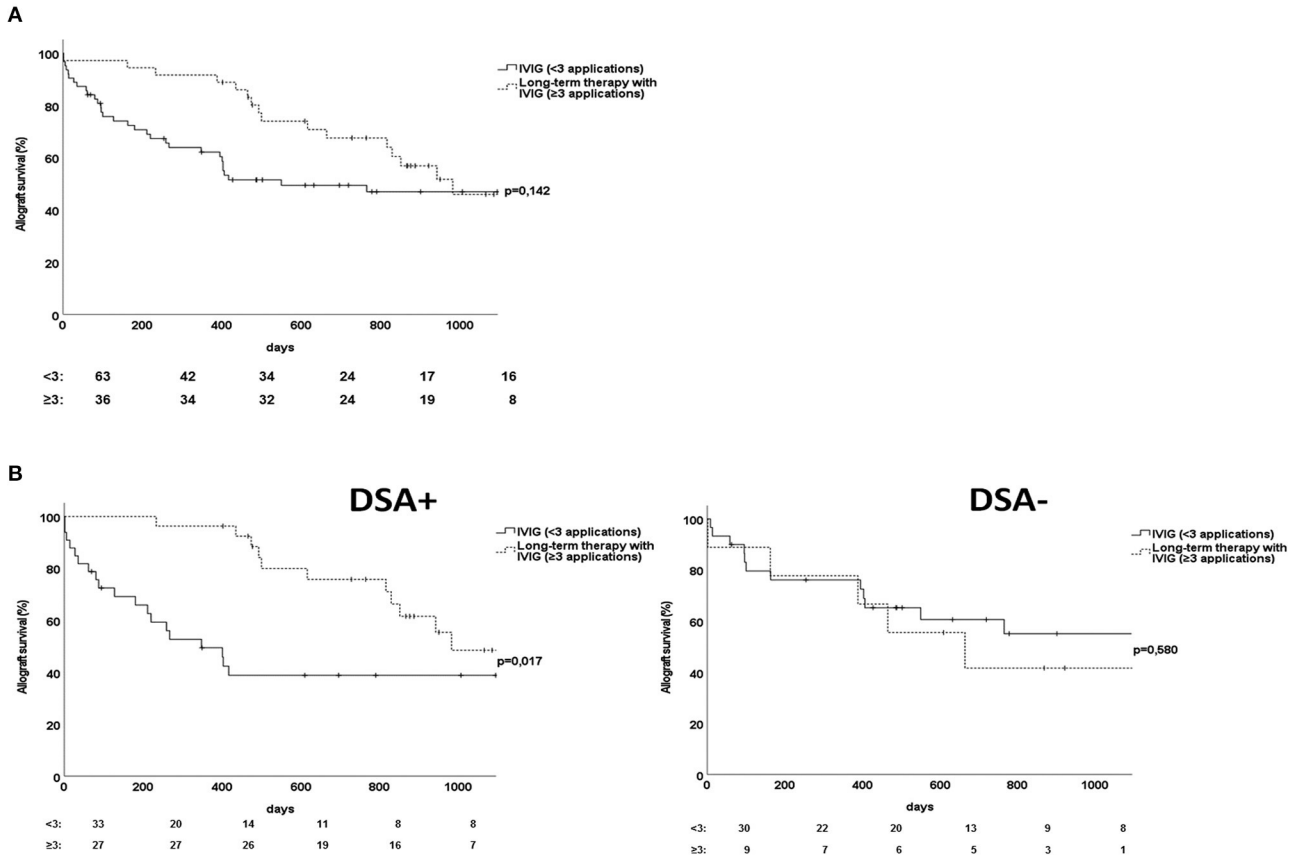

FIGURE 5 | Effect of long-term therapy with IVIG on renal allograft survival of recipients with persistent antibody-mediated rejection. **(A)** Comparison of allograft survival between recipients who were treated with repetitive applications of IVIG over more than 1 year vs. recipients without long-term therapy with IVIG. **(B)** Comparison of the effect of long-term therapy with IVIG on allograft survival between recipients with DSA-positive vs. DSA-negative ABMR. ABMR, antibody-mediated rejection; DSA, donor-specific antibody; IVIG, intravenous immune globulin.

effective as standard treatment for ABMR should be addressed in future trials.

Several centers have used adjunctive treatment strategies for ABMR, predominantly rituximab in combination with plasmapheresis and IVIG. Our study found that adjunctive therapy strategies for ABMR exerted no beneficial effect on allograft survival. We also noted no beneficial effect of additional treatment with rituximab for the entire cohort or for the subgroups with DSA-positive or DSA-negative ABMR. However, the findings about adjunctive therapy of ABMR with rituximab are controversial. The first controlled trial, performed by Lechaufeur et al., found that survival rates were better when rituximab was added to IVIG and PS (37). Subsequent studies of the effect of additional treatment with rituximab were disappointing. In line with our findings, a phase III, multicenter double-blind study by Sautenet et al. found that rituximab had no favorable effect on allograft survival among patients with ABMR and that serious opportunistic infections occurred more often among patients treated with rituximab (38). Similarly, Wan et al. performed a systematic review evaluating the effect of additional treatment with rituximab and found no significant difference between rituximab-treated recipients and recipients receiving standard of care with IVIG and PS (18). The Spanish multicenter, prospective, double-blind TRITON trial performed by Moreso et al. found that, among patients with chronic ABMR, combined therapy with rituximab did not achieve any improvement in allograft outcome (39). The poor effect of rituximab treatment on ABMR reported in the studies might be attributed the fact that the anti-CD20-antibody is not able to reach plasma cells as the main source of DSA because plasma cells are not expressing CD20 on their surface. Pineiro and colleagues performed a study involving a cohort of 62 patients with chronic active ABMR and found that allograft survival was not significantly affected by rituximab treatment compared to treatment with standard therapy (40). Our study did not differentiate between patients with acute or chronic ABMR, but most of our patients exhibited histologic signs of acute and chronic active ABMR simultaneously.

Besides additional therapy with rituximab, in this study we alternatively used other therapies, including bortezomib, eculizumab, or thymoglobulin. Again, allograft survival rates did not differ significantly between the various treatment strategies

for ABMR. With respect to the additional treatment of acute ABMR with bortezomib, several case reports and case series have demonstrated a lower risk of allograft loss after such treatment (41–43). However, in their single-center double-blind BORTEJECT trial, which enrolled 44 renal allograft recipients with late ABMR, Eskandary et al. found similar response rates between a bortezomib-treated group and a group given placebo (44). These recent findings are consistent with our results.

One alternative treatment approach is eculizumab, which targets the complement pathway as a key effector pathway of the ABMR process. A small nonblinded retrospective study by Kulkarni et al. found that eculizumab therapy did not counteract the decrease in eGFR among patients with chronic ABMR associated with *de novo* DSA (45). The ABMR rate in the first 3 months after transplant was significantly lower among patients treated with eculizumab than among historical control subjects treated with PS only (46, 47). However, this effect had disappeared at the follow-up visit 1 to 2 years after transplant. We found that therapy with eculizumab did not significantly affect the allograft survival rates among patients with ABMR, although the results are difficult to interpret because of small patient numbers.

We saw that adjunctive therapy with thymoglobulin failed to achieve a significant improvement in allograft survival rates among some recipients with ABMR. Cihan et al. found a significant amelioration of allograft function in 4 of 9 pediatric kidney transplant recipients with chronic ABMR, but larger studies are lacking (48).

One of the main findings of our analysis of secondary therapies for persistent ABMR was the positive effect of increased maintenance immunosuppression on allograft survival among recipients with ABMR. In most cases, double immunosuppressive therapy consisting of a calcineurin inhibitor and a steroid was supplemented by MMF/MPA. Supporting our data is the finding that immunosuppression with MMF is associated with a decrease in allograft failure that can be partly attributed to the reduction of rejection rates among recipients treated with MMF (49). Moreover, as had been shown by Briggs et al., switching immunosuppressive therapy from cyclosporine to tacrolimus reduces the risk of acute rejection (50). The use of a combination of sirolimus and tacrolimus is known to exert a weaker immunosuppressive effect than that of tacrolimus combined with MMF and to contribute to poorer allograft survival (51). The Transplantation Society working group also came to the consensus that optimization of baseline immunosuppressive therapy is indicated for patients with chronic active ABMR (1).

In particular, the subgroup of recipients in whom DSA-negative ABMR developed benefited from intensification of maintenance immunosuppression. We can speculate that the observed positive effect on allograft outcome is linked to the immunomodulatory effects of intensified maintenance immunosuppression in reducing unidentified triggers of DSA-negative ABMR.

In DSA-positive ABMR, long-term treatment with IVIG exhibited a sustained positive treatment effect compared to short-term IVIG treatment. Therapy with IVIG has been shown to suppress the production of anti-HLA antibodies and to stop the evolution of acute and chronic ABMR (52). Nevertheless, no clinical studies to date have shown that the repetitive administration of IVIG has an advantageous effect on allograft survival after the occurrence of ABMR. A retrospective analysis by Sablik et al. (53) found that allograft function recovered after the administration of IVIG to kidney transplant recipients with ABMR. Long-term therapy with IVIG may induce immunomodulatory effects on DSA production and slow the progression of ABMR (36). However, these results should be interpreted with caution, because significantly more patients with DSA-positive ABMR were treated with IVIG repetitively as a secondary therapy for ABMR. This potential bias may have influenced our observations. Additional clinical trials are necessary to clarify the effect of long-term IVIG therapy on allograft survival and allograft function among patients with ABMR.

We are aware that this study has several limitations. The main limitation is the retrospective study design, which complicates comparisons of our results with those of previous prospective double-blind clinical trials investigating the effect of various therapeutic approaches for ABMR on renal allograft survival. Another limitation is the fact that DSA-negative patients were significantly older than DSA-positive patients, and the subgroup analyses had to take this fact into account. In addition, a significantly higher portion of recipients with DSA-negative ABMR received a second transplant, and early ABMR was diagnosed more frequently in this subgroup. Long-term therapy with IVIG was administered significantly more frequently to DSA-positive recipients than to DSA-negative recipients.

Thus, our study is characterized by potential selection bias because of the retrospective study design that may have affected the differences between the two subgroups in their responses to intensification of immunosuppression and long-term application of IVIG. It should also be noted that only indication biopsies were performed at our center. Otherwise, in assessing the effectiveness of various primary and secondary therapies for ABMR we did not discriminate between early and late ABMR or between the histologic phenotypes of ABMR according to the Banff classification. We focused on the relevance of DSA status to the differences in therapeutic responsiveness of ABMR. Several recipients exhibited morphologic signs of acute ABMR in parallel with chronic-active ABMR or ABMR mixed with T cell–mediated rejection. Of note, 46% of all transplant recipients with DSA-negative ABMR exhibited early ABMR within the first year after transplant; this selection bias regarding a positive ABMR outcome may have skewed these results. Our study did not evaluate new promising treatment options for ABMR, such as tocilizumab, a humanized monoclonal antibody targeting the interleukin-6 receptor (54, 55).

To summarize, our retrospective cohort study showed that the prognosis for patients with ABMR is poor regardless of DSA status; it also confirmed that prognostic factors of ABMR, such as timepoint of diagnosis and C4d status, are clinically relevant. Interestingly, we observed that long-term administration of IVIG to recipients of DSA-positive allografts with preferentially

late ABMR exerts a positive effect, whereas intensification of maintenance immunosuppression therapy is more effective for recipients with DSA-negative ABMR. Additional studies of long-term treatment for ABMR are needed and should consider DSA status in addition to histologic signs of ABMR.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Board of the University Hospital Duisburg-Essen, Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MK, JF-K, and UE contributed to conception and design of the study, performed the statistical analysis, and wrote the first draft of the manuscript. MK organized the database. JHB was responsible for banff grading of renal pathology. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.816555/full#supplementary-material

Supplementary Figure 1 | Comparison of allograft survival between recipients who were treated with repetitive applications of IVIG over more than 1 year vs. recipients without long-term therapy with IVIG in the subgroup of patients having late ABMR and positive DSA status.

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B Cell Composition Is Altered After Kidney Transplantation and Transitional B Cells Correlate With SARS-CoV-2 Vaccination Response

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Background: The COVID-19 pandemic has major implications on kidney transplant recipients (KTRs) since they show increased mortality due to impaired immune responses to SARS-CoV-2 infection and a reduced efficacy of SARS-CoV-2 vaccination. Surprisingly, dialysis patients have shown superior seroconversion rates after vaccination compared to KTRs. Therefore, we investigated peripheral blood B cell (BC) composition before and after kidney transplantation (KT) and aimed to screen the BC compartment to explain impaired antibody generation.

Methods: A total of 105 patients were recruited, and multicolor flow cytometric phenotyping of peripheral venous blood BC subpopulations was performed before and 1 year after KT. Complete follow-up was available for 71 individuals. Anti-SARS-CoV-2 antibodies were collected retrospectively and were available for 40 subjects, who had received two doses of an mRNA-based vaccine (BNT162b2 or mRNA-1273).

Results: Overall, relative BC frequencies within lymphocytes decreased, and their absolute counts trended in the same direction 1 year after KT as compared to CKD G5 patients. Frequencies and absolute numbers of naïve BCs remained stable. Frequencies of double negative BCs, a heterogeneous subpopulation of antigen experienced BCs lacking CD27 expression, were increased after KT, yet their absolute counts were similar at both time points. Transitional BCs (TrBCs) and plasmablasts were significantly reduced after KT in absolute and relative terms. Memory BCs were affected differently since class-switched and IgM-only subsets decreased after KT, but unswitched and IgD-only memory BCs remained unchanged. $CD86^+$ and $CD5^+$ expression on BCs was downregulated after KT. Correlational analysis revealed that TrBCs were the only subset to correlate with titer levels after SARS-CoV-2 vaccination. Responders showed higher TrBCs, both absolute and relative, than non-responders.

Conclusion: Together, after 1 year, KTRs showed persistent and profound compositional changes within the BC compartment. Low TrBCs, 1 year after KT, may

account for the low serological response to SARS-CoV-2 vaccination in KTRs compared to dialysis patients. Our findings need confirmation in further studies as they may guide vaccination strategies.

Keywords: kidney transplantation, CKD G5, COVID-19, SARS-CoV-2 vaccination, B cells

INTRODUCTION

Kidney transplantation (KT) is the preferred form of kidney replacement therapy due to superior survival and quality of life compared to dialysis (1).

Solid organ transplantation, however, necessitates strong immunosuppression to avoid graft rejection. Immunosuppressive regimens focus on T-cells and inhibition of acute cellular rejection (2), which has greatly improved early graft survival in the last decades (3). B cells (BCs) have long been overlooked or regarded as "bad guys" due to production of—often detrimental—alloantibodies. In recent years, BCs have been recognized to serve various other important antibody-independent functions in the context of transplantation (4, 5). In that manner, BC-depleting induction therapy has led to more acute cellular rejection episodes (6), and distinct BC subpopulations have been linked to improved graft survival (7, 8). These findings underline the importance of understanding BC heterogeneity and dynamics of BC subsets in KT.

Comparability of studies monitoring BCs after transplantation is hampered by the lack of a uniform classification of human BCs and varying degrees of BC subset resolution. The following major subpopulations can be differentiated in blood using surface antibodies (9): Transitional (Tr) BCs are immature precursors from the bone marrow and switch to naïve BCs in the periphery. Naïve (i.e., antigen inexperienced) BCs, the most abundant peripheral BC subset, circulate in the blood in search of their specific antigen. Upon antigen recognition, they may differentiate into plasmablasts (PBs), plasma cells (PCs), or memory BCs (mBCs). PBs are short-lived and may migrate to the bone marrow to differentiate into long-lived PCs (10). PBs and PCs secrete highly specific antibodies, which, depending on the antigen, may provide protection against a pathogen, or, in the context of KT, mediate graft rejection. mBCs, the humoral backbone of immune memory, surveil the periphery and quickly mount strong humoral responses upon reencountering of their cognate antigen. Surface expression of IgM and IgD allows further identification of mBCs subpopulations, namely IgM-only (IgM^+, IgD^-), IgD-only (IgM^-, IgD^+), unswitched [IgM^+, IgD^+; often regarded as circulating marginal-zone BCs (11)] and switched (IgM^-, IgD^-) mBCs. Finally, double negative (DN) BCs, identified by the lack of surface IgD and CD27 expression, are suggested to comprise early activated memory BCs and extrafollicularly activated precursors of PBs (12).

Immunosuppression in transplantation is a balancing act, with rejection on one side and infection on the other side (13, 14). Infectious diseases contribute significantly to death in kidney transplant recipients (KTRs) (15–17). To avoid preventable deaths from infections, vaccination prior to transplantation is advised, as vaccination efficacy may be impaired in the post-transplantation setting (18–21).

The COVID-19 pandemic has put KTRs at particular risk. First, more severe and fatal SARS-CoV-2 infections are reported in this population, with an estimated mortality of around 20% of hospitalized KTRs with COVID-19 (22). Second, humoral and cellular immunity after mRNA-based SARS-CoV-2 vaccination and natural infection are markedly reduced in KTRs compared to the general population (23–26). Interestingly, dialysis patients display superior serological responses to mRNA-based vaccination compared to KTRs, almost paralleling results in healthy individuals (24, 25). This difference between dialysis patients and KTRs may be in part explained by the immunosuppressive regimen. However, as other vaccinations have yielded humoral responses despite immunosuppression (19), there might be other factors involved.

Our aim was to explore differences in the BC compartment between the CKD G5 (end-stage kidney disease) setting and the post-transplantation situation. This study adds to the current knowledge by including previously unstudied BC subsets, such as DN BCs and mBC subpopulations, and by depicting profound compositional changes within BCs 1 year after KT. Furthermore, we provide a potential link between pre-vaccination TrBCs and humoral response to SARS-CoV-2 vaccination in KTRs.

MATERIALS AND METHODS

Study Design and Population

One-hundred-and-five CKD G5 patients were recruited prospectively and transplanted at the Department of General, Visceral, and Transplant Surgery at the Medical University of Graz between 2016 and 2020. All patients were (a) above 18 years old, (b) did not receive immunosuppressive therapy at the time of KT, and (c) received a cadaveric organ. Peripheral blood was collected prior to transplantation (T1) and 1 year after transplantation (T2) for flow cytometric BC characterization and analyses after obtaining informed consent according to the Declaration of Helsinki. Only those with complete follow up and intact allograft at T2 were included. Before KT, patients underwent one hemodialysis session to ensure optimal conditions, and peripheral blood for T1 was taken prior to this session.

The study protocol was approved by the Institutional Review Board of the Medical University of Graz, Austria (28-514ex15/16), and the study was registered as #DRKS00026238 in the German Register of Clinical Studies.

Vaccination Cohort and Anti-SARS-CoV-2 Antibody Testing

We aimed to investigate if BCs and BC subpopulations correlate with humoral response to an mRNA-based SARS-CoV-2 vaccine. Therefore, out of 71 KTRs, we included those who were available for follow-up, had received two doses of mRNA-1273 (Moderna) or BNT162b2 (Pfizer/BioNTech), had a functioning graft at the time of vaccination, and had finished their last visit before vaccination. Loss of graft function was defined as return to dialysis. In case of symptomatic or asymptomatic COVID-19, detected by either positive RT-PCR or positive serology for anti-SARS-CoV2 nucleocapsid antibody, at any time prior, during, or after vaccination, the patient was excluded.

The time of antibody testing was defined as T3. SARS-CoV-2 specific antibodies against spike protein were measured with LIAISON TrimericS IgG Assay (DiaSorin, Saluggia, Italy), Elecsys Anti-SARS-CoV-2 S (Roche, Basel, Switzerland), Alinity I SARS-CoV-2 IgG (Abbott Laboratories, Chicago, IL, USA) or COV2T (Siemens Healthineers, Erlangen, Germany), depending on where the blood sample was drawn. To allow for comparability between the different test platforms, respective units were converted to BAU/mL using conversion factors, as provided by the manufacturers. Individuals were defined as "responders" in case of detectable antibody levels (i.e., above detection limit) or "non-responders" without detectable antibodies.

PBMC Isolation and Flow Cytometry Analysis

At both visits, peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized whole blood samples (BD vacutainer tubes containing lithium heparin, Becton Dickinson, Franklin Lakes, NJ, USA). Whole blood was diluted 1:1 with phosphate-buffered saline (PBS) and layered into a tube prefilled with Lymphoprep density gradient media (Stemcell Technologies, Vancouver, Canada). Density gradient centrifugation was performed (20 min, 800 × g at room temperature), and the PBMC layer was collected and washed with PBS. Viability and cell number were measured by the use of an automated dual fluorescence cell counter (LUNA-FL, Logos Biosystems, Anyang, South Korea) prior to multi-parameter staining of 1×10^6 cells per FACS panel. Additionally, 0.5×10^6 cells served as an unstained control. Surface panel staining was performed using BD Lyse/Fix buffer (Becton Dickinson) according to the manufacturer's instructions. All antibodies were purchased from Becton Dickinson (for details refer to **Supplementary Table 2**).

Additionally, 50 µl of fresh whole blood was stained with anti-CD45 APC-H7 antibodies (Becton Dickinson), and 123 count eBeads (Thermo Fisher Scientific, Waltham, MA, USA) were added for the analysis of absolute numbers of leucocyte subpopulations. All samples were acquired on a four-laser BD FACS Fortessa SORP instrument (Becton Dickinson), and data were analyzed using the FlowJo software (Becton Dickinson). UltraComp eBeads (Thermo Fisher Scientific) were used for compensation, and FMO controls were applied for appropriate gating of BC subtypes. The gating strategy is depicted in **Supplementary Table 3** and **Supplementary Figure 1**.

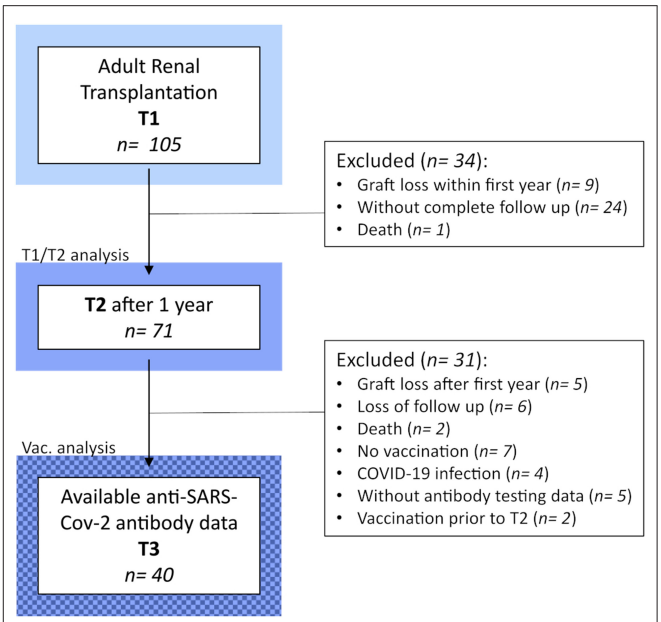

FIGURE 1 | Detailed flow chart of the study design and populations.

Statistical Analysis

All statistical analyses were performed with Statistical Package for Social Sciences (SPSS v26, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Graphs were drawn using GraphPad Prism 8.0.1. Normality was assessed by Kolmogorov-Smirnov test. Normally distributed data are shown as mean [standard error of the mean (SEM)], non-normal data as median [interquartile range (IQR)], and categorical data as absolute values and relative frequencies (%). Differences between two independent groups were calculated with *t*-tests, Mann-Whitney U-tests, χ^2-tests, or Fisher's exact tests, as appropriate. Paired groups were compared using dependent *t*-test or Wilcoxon signed-rank test, depending on the tested variables' distributions. Spearman's rank-based correlation coefficient was used to assess correlations. *P*-values below 0.05 were defined as significant. Formal adjustment for multiple testing was not done.

RESULTS

Baseline Characteristics of the Study Population

Complete follow up, including flow cytometric immune phenotyping at T1 and T2, was available for 71 KTRs (**Figure 1**). Clinical and demographic characteristics of our T1/T2 cohort are shown in **Table 1**. The median age was 56 years, and one-third (33.8%) were female. Sixteen patients (22.5%) had diabetes at the time of KT. Participants were mainly Caucasians (94.4%), and only three individuals (4.2%) were transplanted pre-emptively.

TABLE 1 | Baseline characteristics of study population.

Variable	T1/T2 cohort ($n = 71$)
Age (years)	56 (48 – 64)
Female sex	24 (33.8)
Body-mass index (kg/m²)	26.4 (23.3 – 29.7)
Type 2 diabetes	16 (22.5)
Ethnicity	
Caucasian	67 (94.4)
Asian	1 (1.4)
Other	3 (4.2)
Dialysis prior KT	68 (95.8)
PD	13 (18.3)
HD	55 (77.5)
Dialysis vintage (months)	28 (20 – 43)
Renal disease	
Diabetic	13 (18.3)
Hypertensive	5 (7)
Glomerular	15 (21.1)
Polycystic kidney disease	12 (16.9)
Other/Unknown	26 (36.6)
Immunosuppression	
Induction (BX/ATG)	65/7 (91.5/9.9)
TAC	70 (98.6)
CyA	1 (1.4)
MMF/MPA	70 (98.6)
AZA	1 (1.4)
CS	71 (100)
PRAs > 0% prior KT	5 (7)
Anti-infectives at T2	
TMP/SMX	0 (0)
Valganciclovir	4 (5.6)
Rejection within 1st year of KT	13 (18.3)
Cellular/humoral Rejection	12/1 (92.3/7.7)
BANFF1B	2 (16.7)
BANFF2A	10 (83.3)

Characteristics of T1/T2 Cohort. Continuous variables are expressed as median (± IQR) and categorical variables as frequency (%). KTR, kidney transplant recipient; KT, kidney transplantation; PD, peritoneal dialysis; HD, hemodialysis; BX, basiliximab; ATG, anti-thymocyte globuline; TAC, tacrolimus; CyA, Cyclosporine A; MMF/MPA, mycophenolate mofetil/mycophenolic acid; AZA, azathioprine; CS, corticosteroids; PRAs, panel-reactive antibodies; TMP/SMX, trimethoprim/sulfamethoxazole.

Of those on dialysis prior to transplantation, hemodialysis was four times more prevalent than peritoneal dialysis (80.9% vs. 19.1%), and median dialysis vintage was 28 months. Glomerular (21.1%) and diabetic kidney disease (18.3%) were the most common causes of CKD G5, followed by polycystic (16.9%) and hypertensive kidney disease (7%). In 36.6% of cases, another cause of CKD was present, or a diagnosis could not be made. Immunosuppressive treatment was prescribed as recommended by the 2009 KDIGO guidelines (18). Briefly, patients with a low pre-transplant risk of rejection received basiliximab (BX; 91.5%), and those with a high risk of rejection were treated with recombinant anti-thymocyte globulin (ATG; 9.9%) as induction therapy. High immunological risk was defined according to the 2009 KDIGO guidelines (18). Of note, pre-transplant panel reactive antibodies (PRAs) over 0% were found in only five individuals One patient's induction therapy had to be switched from ATG to BX due to an allergic reaction. Almost all KTRs were started with triple immunosuppression consisting of corticosteroids (CS; 100%), tacrolimus (TAC; 98.6%), and mycophenolic acid (MPA) or mycophenolate mofetil (MMF) (98.6%), with the exception of one patient, who was started on cyclosporine A (CyA; 1.4%) instead of TAC; and one who received azathioprine (AZA; 1.4%) instead of MMF/MPA. Of note, no KTR received rituximab, and, in those included in the T1/T2 analysis, no rejections were observed within four months of T2.

Leucocytes Increase After KT, Whereas Lymphocytes Remain Stable

Immune cell phenotyping in peripheral blood was performed before KT (T1), which reflects CKD G5 status, and 1 year after KT (T2). A significant increase in leucocytes was observed in patients (**Figure 2A**) at T2, which was driven by monocyte and granulocyte expansion (**Figures 2B**,**C**, respectively). Interestingly, BC numbers showed a marked trend for reduction at T2, missing statistical significance (**Figure 2E**), whereas total lymphocyte counts remained stable (**Figure 2D**).

BC Subpopulations Show Profound Changes 1 Year After KT

BC subpopulations were monitored, and changes in relative BC frequencies are depicted in **Figure 3**. Naïve BCs were the major constituent of peripheral BCs, and they were found at similar frequencies at both time points (**Figure 3A**). TrBCs showed a highly significant reduction in patients 1 year after KT (**Figure 3B**), whereas DN BCs were significantly upregulated at T2 (**Figure 3C**). PBs, albeit found at very low frequencies already at T1, were even further reduced after 1 year (**Figure 3D**). In the mBC compartment, we found significantly decreased IgM-only mBCs in patients 1 year after KT (**Figure 3F**), whereas class-switched mBCs trended toward lower frequencies at T2 (**Figure 3G**). Unswitched and IgD-only mBCs remained stable (**Figures 3E,H**, respectively).

In absolute terms, naïve BCs trended toward a decrease after the first year of KT (**Supplementary Figure 2A**). TrBC and PB counts were significantly lower at T2 (**Supplementary Figures 2B,D**, respectively). In contrast to the significant increase of DN BCs with regards to relative frequencies, their absolute numbers were similar at both time points (**Supplementary Figure 2C**). Quantification of mBC counts revealed findings consistent with relative mBC frequencies. More specifically, pos*t*-transplantation IgM-only mBCs (**Supplementary Figure 2F**) and class-switched mBCs (**Supplementary Figure 2G**) were significantly reduced, and IgD-only (**Supplementary Figure 2E**) and unswitched mBCs (**Supplementary Figure 2H**) remained stable.

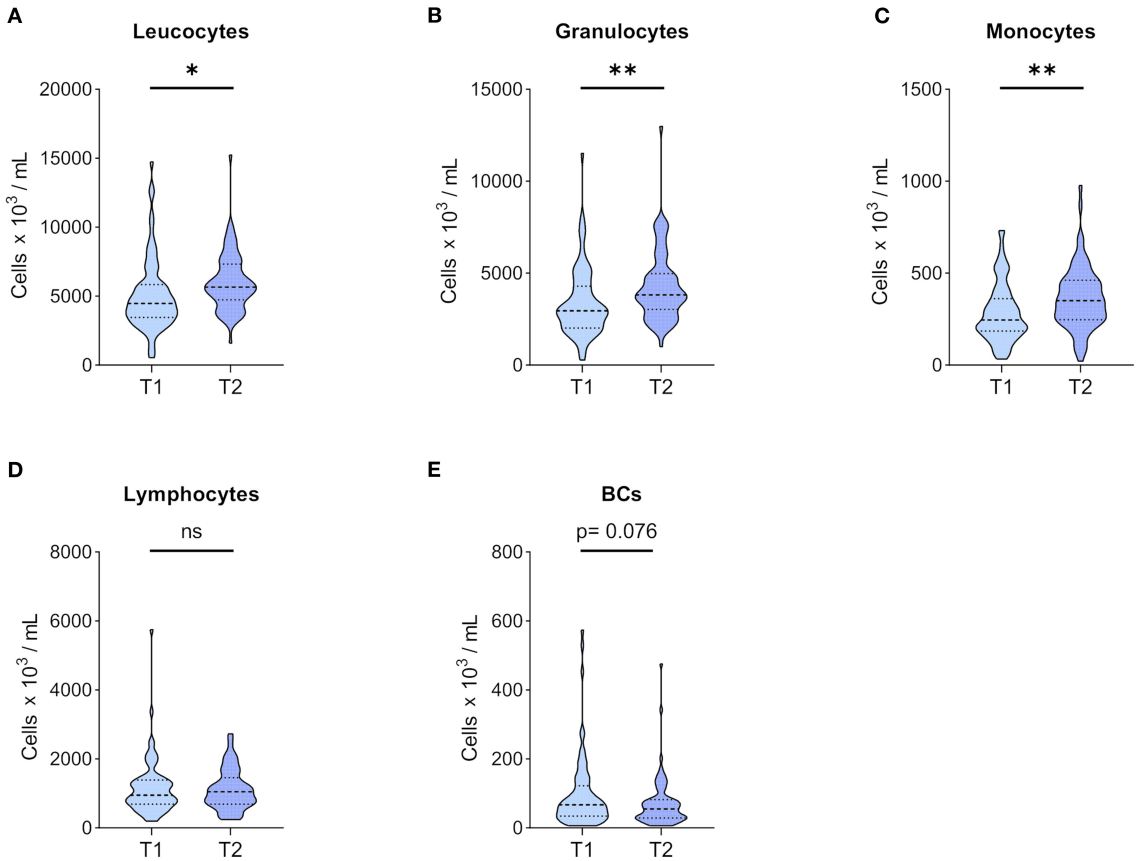

FIGURE 2 | Leucocytes and major leucocyte subpopulation counts. Whole blood of 71 patients was drawn before KT (T1) and 1 year after KT (T2). After staining for CD45, major CD45$^+$ leucocyte subpopulations were differentiated according to forward and side scatter using flow cytometry, and absolute numbers were obtained using 123count eBeads (Thermo Fisher Scientific). Violin plots of absolute numbers of **(A)** leucocytes, **(B)** granulocytes, **(C)** monocytes, **(D)** lymphocytes, and **(E)** BCs are shown at T1 and T2. The heavy dashed line indicates the median, and the light dashed lines mark the IQR. The shapes of the colored areas show the data distributions. Differences between both time points were calculated with Wilcoxon signed-rank test (*$p < 0.05$; **$p < 0.01$).

Activated BCs and Tolerogenic CD27$^-$ CD5$^+$ BCs Are Decreased After KT

Next, we were interested in how CD86 expression, an activation marker on BCs, is affected by the immunosuppressive treatment in the post-transplantation setting compared to T1. We found CD86$^+$ BCs at a significantly lower frequency at T2 than T1 (**Figure 4A**), which was paralleled by a significant reduction of absolute CD86$^+$ BC numbers (**Figure 4B**). CD5$^+$ BCs have gained attention in recent years in the context of transplantation and organ tolerance (27). Therefore, we investigated CD27$^-$ CD5$^+$ BCs in our cohort and found a significant reduction in their numbers (**Figure 4D**) and frequencies (**Figure 4C**).

MMF Dosage Negatively Impacts on PB Abundance at T2

Inosine-5′-monophosphate-dehydrogenase is expressed on all lymphocytes, and inhibition via MPA, or its prodrug MMF, targets T and B cells alike (28). MMF was highly prevalent in our cohort (91.5% at T2), yet dosages differed between patients depending on tolerability or infectious complications. Therefore, we investigated, whether MMF dosing affects absolute and relative BCs 1 year after KT. Frequencies of naive BCs positively correlated, and frequencies of DN BCs and PBs negatively correlated with MMF dosage (**Figure 5A**). In absolute terms, only PBs were negatively correlated with MMF dose (**Figure 5B**).

Characteristics of the Vaccination Cohort

To identify potential biomarkers in the BC compartment for a positive serological response following two doses of an mRNA-based vaccine (mRNA-1273 or BNT162b2), administered 4 weeks apart, we retrospectively collected anti-SARS-CoV-2 antibody testing data from our study population. After excluding subjects with previous COVID-19, and those who completed T2 after vaccination, we were able to perform a comparative analysis in 40 patients (**Figure 1**). Importantly, these individuals displayed very similar dynamics from T1 to T2 in the leucocyte counts and in the BC compartment to the whole study population (**Supplementary Figures 3–6**). They were also similar with regards to the demographic and clinical characteristics at T1 (**Supplementary Table 4**).

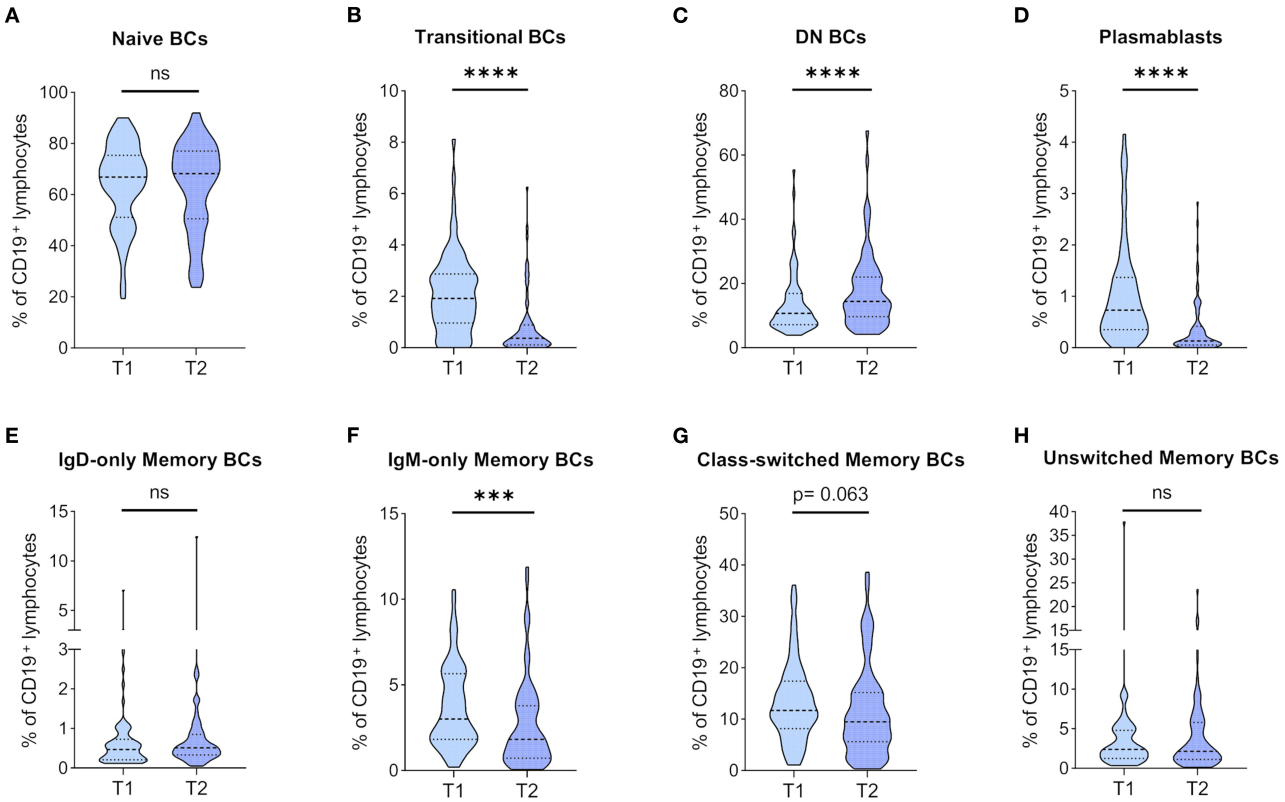

FIGURE 3 | Dynamics of BC subpopulation frequencies before and 1 year after kidney transplantation. PBMCs were analyzed from 71 patients before (T1) and 1 year after KT (T2). Frequencies of **(A)** naïve BCs, **(B)** transitional BCs, **(C)** double-negative (DN) BCs, **(D)** plasmablasts, **(E)** IgD-only memory BCs, **(F)** IgM-only memory BCs, **(G)** class-switched memory BCs, and **(H)** unswitched memory BCs in $CD19^+$ lymphocytes are shown as violin plots. Medians are indicated by heavy dashed lines and IQR by light dashed lines, respectively. The distribution of data points is visualized by the plot shape. Wilcoxon signed-rank test was used for comparison between T1 and T2 (***$p < 0.001$; ****$p < 0.0001$).

We identified 20 patients (50%), who mounted a detectable serological response to vaccination ("responders") and 20 "non-responders" without any detectable anti-SARS-CoV-2 antibodies after two vaccine doses. Anti-SARS-CoV-2 antibody levels were low with a median of 21.75 BAU/mL in responders. **Table 2** compares responders to non-responders with regards to their clinical and demographic characteristics at the time of antibody testing (T3).

One-third ($n = 13$) received BNT162b2, and two-thirds ($n = 27$) were given mRNA-1273. In responders, immunization with mRNA-1273 was more frequent than in non-responders (80% vs. 55%). Immunosuppression was very similar to T2, and no significant differences were found in immunosuppressive treatment between the two groups. Importantly, daily MMF/MPA dose was also comparable between responders and non-responders. Furthermore, there were no acute rejections within 6 months prior to T3. The median time difference between T2 and the first dose was 106.5 weeks and not significantly different between both groups. The median time interval from second dose to T3 was 14.5 weeks, and there was also no apparent difference between responders and non-responders. Both groups were at a similar age at the time of T3, and type 2 diabetes was also equally prevalent. There was a trend for longer dialysis vintage prior to T1 in responders (31.5 vs. 26 months), and both preemptively transplanted individuals were found in the non-responder group. Markers of kidney function at T3 did not differ between groups (**Table 2**).

TrBCs Correlate With the Anti-SARS-CoV-2 Antibody Response

Correlational analyses of anti-SARS-CoV-2 antibody levels with absolute BC numbers and relative BC frequencies at T2 revealed significant positive correlations of antibodies with relative TrBC frequency of $CD19^+$ BCs (**Figure 6A**) and absolute TrBCs (**Figure 6C**). Furthermore, when we compared TrBCs in responders to non-responders, we found that TrBC absolute counts, as well as relative TrBC frequency were significantly higher at T2 in those who were able to mount a humoral response to vaccination (**Figures 6B**,**D**, respectively).

DISCUSSION

BCs have gained attention in the field of transplantation in recent years and even more so during the COVID-19 pandemic, as they

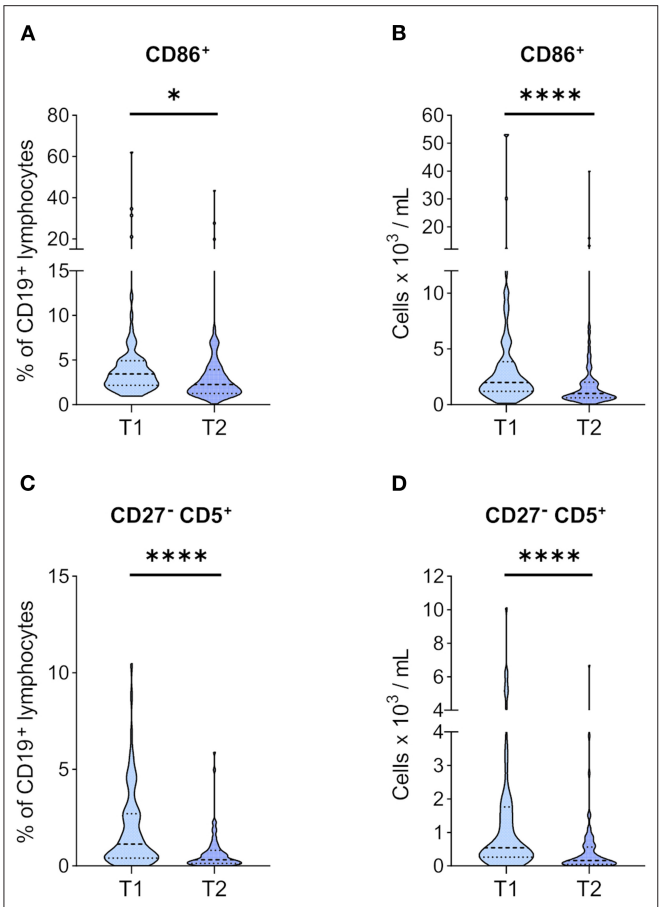

FIGURE 4 | Markers of activation and tolerance in BCs before and 1 year after KT. PBMCs of 71 KTRs before (T1) and 1 year after KT (T2) were stained for CD86, CD27, and CD5. Frequencies of **(A)** $CD86^+$ and **(C)** $CD27^-$ $CD5^+$ in $CD19^+$ lymphocytes are given. Absolute numbers per milliliter of **(B)** $CD86^+$ and **(D)** $CD27^-$ $CD5^+$ were calculated from their relative frequency and total BC counts. Median and IQR are marked by heavy and light dashed lines, respectively. The plots shapes indicate the distributions of data points. Comparisons between T1 and T2 were calculated using Wilcoxon-signed rank test (*$p < 0.05$; ****$p < 0.0001$).

are at the center of two fundamental problems in solid organ transplantation, namely rejection and infection. Accordingly, numerous studies have monitored BCs after transplantation in search of potential biomarkers for graft rejection (7, 8) and tolerance (29), respectively. On the other hand, few have tried to find a connection between BCs and vaccination success in KT (30), even though the problem of low serological response after transplantation is well known (19) and has become imminent during the COVID-19 pandemic (31).

In this study, we evaluated BCs and BC subpopulations before and 1 year after KT in 71 subjects. These two time points reflect different abnormal immunological steady-states, namely CKD G5 (T1) and stable KT (T2). Accordingly, profound compositional and absolute changes in the BC compartment were detected. Furthermore, we examined whether BCs in stable KTRs are connected to serological response to SARS-CoV-2 vaccination and reported a potential link between pre-vaccination TrBCs and humoral response in KT.

Absolute BCs, detected by CD19 positivity, were not significantly regulated in our hands, which is in line with Svachova et al. (32), who showed that after an initial increase, BCs dropped to pre-transplantation numbers after 1 year, and Schlößer et al. (33), who reported stable levels throughout. Nevertheless, the total BC compartment is reduced compared to healthy individuals (34), supporting the notion that BC numbers are already lower in CKD G5 (35). Immunosuppressive induction and maintenance therapy was very homogenous in our cohort (**Table 1**). Therefore, we could not assess potential differences in BC composition after KT due to varying immunosuppressive regimen. However, others have shown that after 1 year, KTRs display similar BC pools regardless of ATG or BX induction (32). Furthermore, *in vitro* data suggests that TAC has limited direct effects on BCs (36), but affects humoral immune response in a T-cell mediated manner (37, 38). MMF/MPA, on the contrary, inhibit proliferation of activated lymphocytes, as they depend on rapid *de novo* purine production (28). We observed a dose dependent reduction of PB frequencies and counts by MMF/MPA, but no effect on total BC abundance in correlational analysis. Preferential reduction of PBs as a consequence of MMF/MPA treatment has been reported in SLE as well (39, 40). Additionally, MMF/MPA therapy in KT is a risk factor for failed seroconversion after influenza vaccination (41), and, most recently, SARS-CoV-2 vaccination (24). In summary, these data support the argument that MMF/MPA inhibits antigenic activation and clonal expansion of BCs, but spares non-proliferating subtypes.

Previous studies have shown that there is a compositional switch in KTRs toward a more "differentiated" circulating BC pool (34), and experimental evidence suggests that immature BCs may be more susceptible to immunosuppressive drugs than mature phenotypes (42). In agreement with those findings, we also observed stable numbers of naïve BCs and a significant reduction of TrBCs. However, whereas others have shown stable or increasing mBCs (32, 34), we found reduced IgM-only and switched mBCs and constant levels of IgD-only and unswitched mBCs. These differences may be explained by varying degrees of mBC subset characterization using IgM and IgD. While most describe switched (IgM^- IgD^-) and unswitched (IgM^+ IgD^+) mBCs, we report minor subsets, IgM-only (IgM^+ IgD^-) and IgD-only (IgM^- IgD^+) mBCs, in KTRs. IgM-only mBCs are widely regarded to originate from germinal centers and to serve as a substrate for switched mBCs (43), but IgD-only mBCs' role and function in health and disease remain largely unknown (44). Evidence hinting toward autoreactivity makes IgD-only mBCs an interesting subset to study in transplantation (45). Thus, our results suggest that not only major BC subpopulations are differently affected by immunosuppression, but also minor subsets.

DN BCs showed an increase in frequency, albeit absolute numbers were similar. DN BCs have been recently recognized as important players in autoimmunity and infectious diseases alike (9, 46), but to our knowledge, their numbers and frequency in

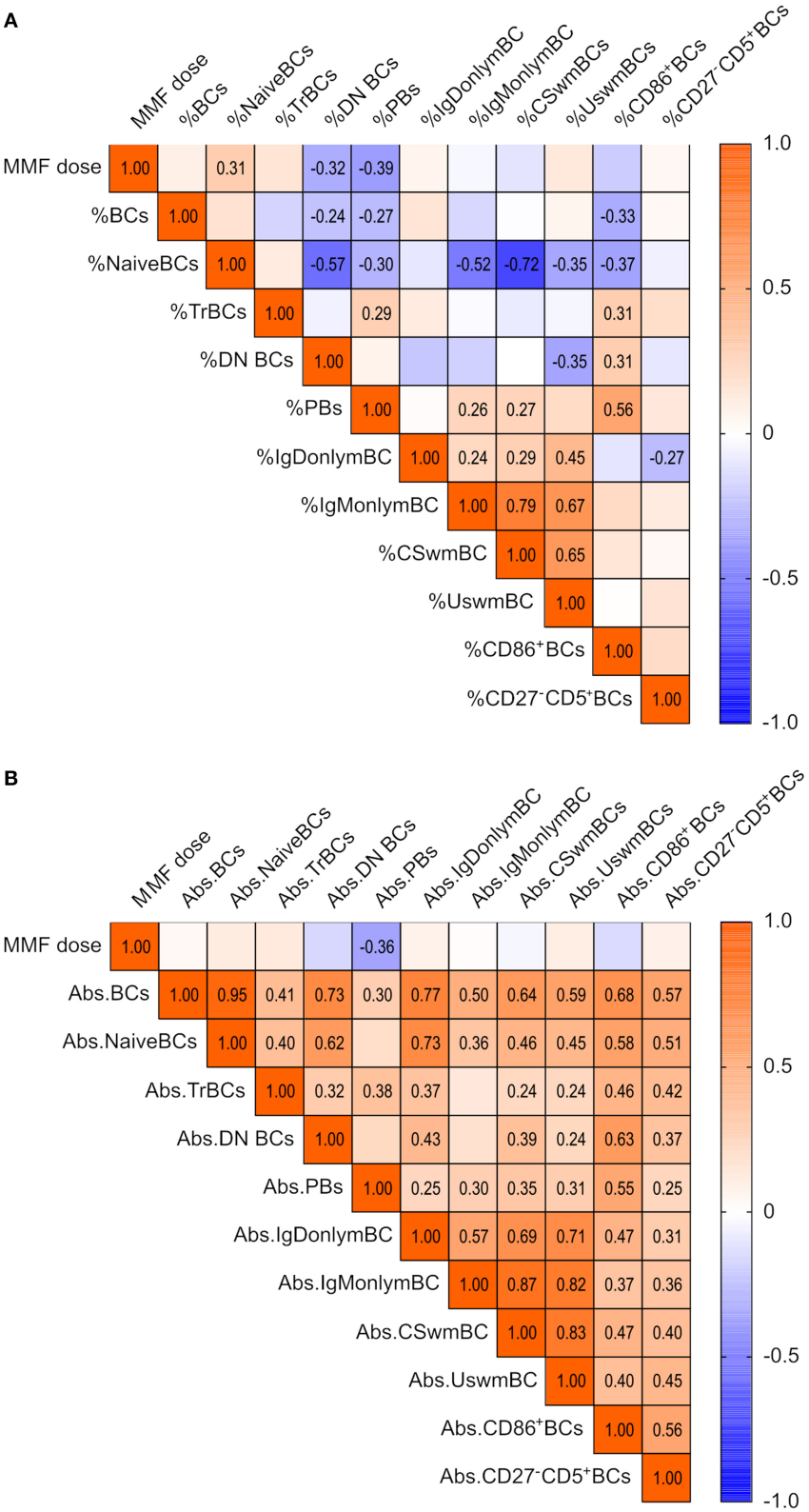

FIGURE 5 | Impact of MMF dose on blood BC pool 1 year after KT in 71 KTRs. Spearman correlation matrix of MMF dose and **(A)** frequencies of BC subpopulations and **(B)** absolute counts of BCs at T2. Correlation coefficients are given for significant correlations only.

TABLE 2 | Characteristics of vaccination cohort at T3.

Variable	Vaccination subcohort ($n = 40$)	Responder ($n = 20$)	Non-responder ($n = 20$)	p-value
Age (years)	64 (54 – 68)[a]	65 (57 – 67)[a]	63 (52 – 68)[a]	0.588
Female Sex	13 (32.5)	6 (30)	7 (35)	1.000
Body-mass index (kg/m^2)	27.8 (24.8 – 29.6)[b]	27.9 (26.2 – 29.7)[b]	27.6 (24.5 – 30.9)[b]	0.860
Type 2 diabetes	12 (30)	5 (25)	7 (35)	0.731
Dialysis prior KT	38 (95)	20 (100)	18 (90)	0.487
PD	9 (23.7)	6 (30)	3 (16.7)	0.451
HD	29 (76.3)	14 (70)	15 (83.3)	1.000
Dialysis vintage (months)	28 (20 – 42.8)	31.5 (21 – 52.75)	26 (15.25 – 40.5)	0.102
Kidney disease				
Diabetic	10 (25)	4 (20)	6 (30)	0.716
Hypertensive	3 (7.5)	1 (5)	2 (10)	1.000
Glomerular	7 (17.5)	5 (25)	2 (10)	0.407
Polycystic kidney disease	7 (17.5)	5 (25)	2 (10)	0.407
Other/Unknown	13 (32.5)	5 (25)	8 (40)	0.501
Rejection	3 (7.5)	0	3 (15)	0.487
eGFR (mL/min/1.73m^2)	47.5 (36.6 – 58.7)	49.3 (39.5 – 63.9)	46,8 (36.3 – 54.9)	0.301
Creatinine (mg/dL)	1.44 (1.18 – 1.76)	1.42 (1.11 – 1.78)	1.47 (1.2 – 1.73)	0.602
U-ACR (mg/g)	26.5 (7.3 – 45.5)[c]	28 (14 – 98)[c]	20 (4 – 43)[c]	0.583
U-PCR (mg/g)	107 (77 – 137)[d]	120.5 (78.5 – 161)[d]	93 (77 – 130)[d]	0.298
Immunosuppression				
TAC	39 (97.5)	20 (100)	19 (95)	1.000
EVE	3 (7.5)	2 (10)	1 (5)	1.000
MMF/MPA	36 (90)	17 (85)	19 (95)	0.605
<1g/d	17 (47.2)	8 (47.1)	9 (47.4)	1.000
>1g/d	19 (52.8)	9 (52.9)	10 (52.6)	1.000
CS	40 (100)	20 (100)	20 (100)	NA
Vaccination				
mRNA-1273/BNT162b2	27/13 (67.5/32.5)	16/4 (80/20)	11/9 (55/45)	0.176
Anti-SARS-CoV-2 antibody level (BAU/mL)	0.59 (0 – 21.78)	21.75 (5.93 – 83.22)	0	<0.001
Interval 2nd dose—T3 (weeks)	15 (9 – 22)[e]	17 (10.3 – 22)[e]	12 (7.5 – 20.8)[e]	0.336
Interval T2 – 1st dose (weeks)	106.5 (85.5 – 151.5)[f]	94 (81 – 148)[f]	132 (85.5 – 159.5)[f]	0.303

Demographic and clinical characteristics of responders and non-responders to SARS-CoV-2 vaccination. Data are reported as median ± IQR and categorical variables as frequency (%). eGFR, estimated glomerular filtration rate; U-ACR, urinary albumin to creatinine ratio; U-PCR, urinary protein to creatinine ratio; NA, not applicable.
[a]39/19/20 values; [b]31/14/17 values; [c]38/19/19 values; [d]37/18/19 values; [e]39/19/20 values; [f]38/20/18 values.

blood have not been reported in KTRs. DN BCs may be further characterized by CXCR5 and CD21 expression into functionally distinct subsets, DN1 and DN2, as proposed by Sanz et al. (9). As we did not include these markers in our panel, we were not able to differentiate between DN2, precursors of antibody-secreting cells, which are generated through an extrafollicular pathway; and DN1 BCs, i.e., activated mBCs with a follicular origin (12). DN2 BCs have been shown to be expanded in SLE flares (12), found abundantly in nephritic kidneys (47), and, most recently, have been linked to COVID-19 severity (48, 49). Hence, it will be interesting to monitor these subsets during the course of KT and in the context of rejection and infection specifically.

PBs and PCs may be considered as effector BCs due to their ability to produce antigen-specific antibodies. PCs are mainly located in the bone marrow and are therefore not easily accessible in humans, while PBs can be found in peripheral blood, albeit at low numbers (10). We found PBs to be even further reduced in KTRs, contrasting previous findings, where PBs were shown to repopulate to pre-KT numbers after 1 year (32). Interestingly, others have shown reduced antigen-specific PBs in KTRs and dialysis patients after SARS-CoV-2 vaccination, and post-vaccine absolute and relative PBs correlated with antibody levels (50). In our hands, pre-vaccination PBs did not correlate with SARS-CoV-2 antibodies. Taken together, these results suggest impaired generation of new effector BCs, but the size of the already existing PB pool is not predictive of serological response. To clarify this, further data on bone marrow PB and PC kinetics would be of great value.

In contrast to findings from Schlößer et al. (33), who showed that CD86 is downregulated very early after KT, but expression rises back to pre-transplantation levels after 1 year, CD86 was permanently downregulated on BCs in

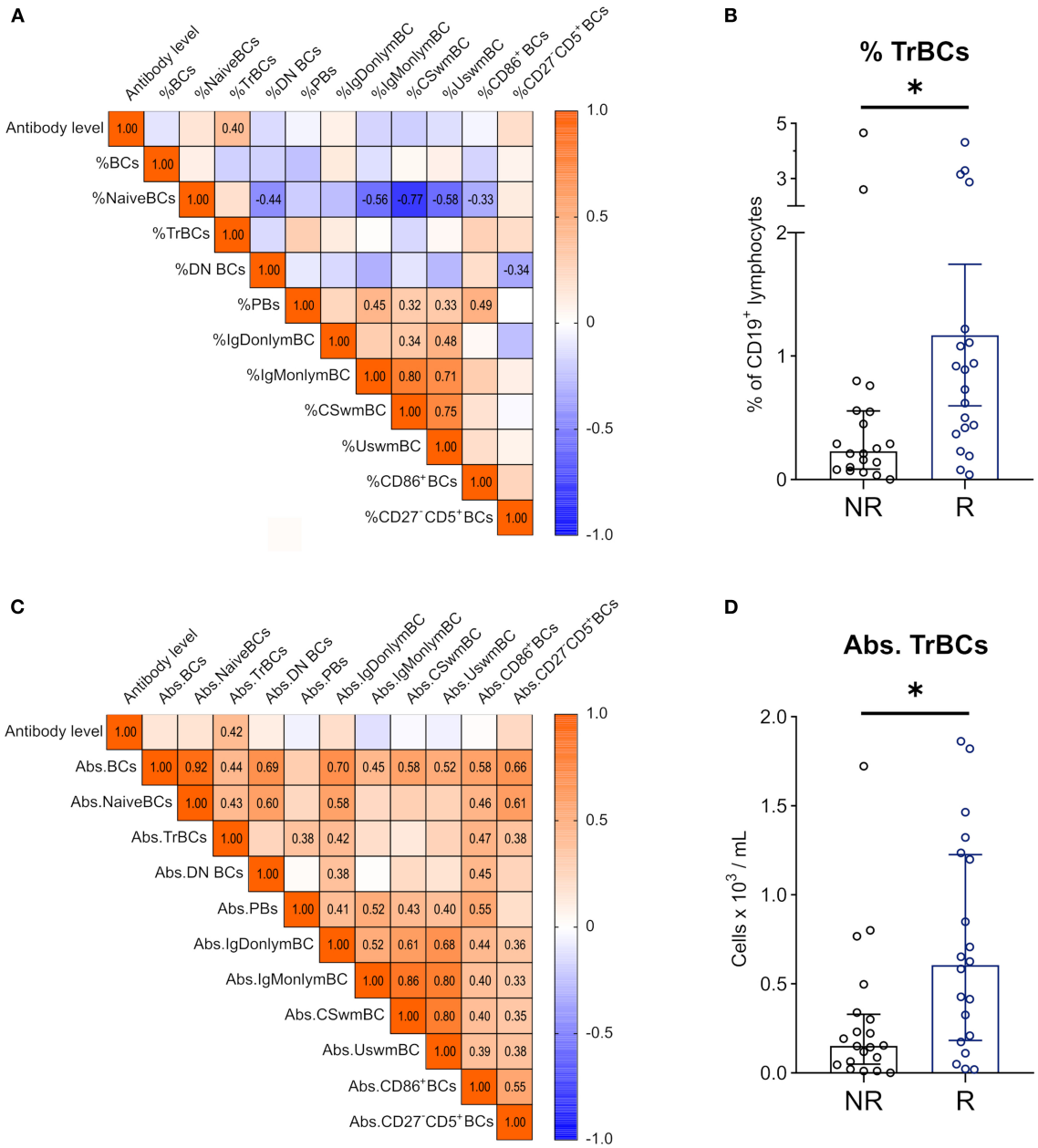

FIGURE 6 | Correlations between anti-SARS-CoV2 antibody levels and BC subpopulations 1 year after KT. Spearman correlation matrix of antibody levels and **(A)** frequencies of BC subpopulations in $CD19^+$ lymphocytes 1 year after KT (T2) and **(C)** absolute counts of BC subpopulations at T2. Numbers are given for significant correlations only. Mann-Whitney-U Test was used to compare relative TrBC frequency **(B)** and absolute TrBC numbers **(D)** between non-responders (NR; $n = 20$, black circles) and responders (R; $n = 20$, blue circles). (*$p < 0.05$).

our cohort. Tolerant KTRs, defined as patients with stable kidney function without immunosuppressive medication, have been reported to display higher $CD86^+$ BCs compared to non-tolerant KTRs (27, 51). With regards to vaccination, Egli et al. (52), found that pre-vaccination BC expression of CD86 and HLA-DR were correlated with subsequent serological response to H1N1 vaccination. We, however, could not find such a link for SARS-CoV-2 vaccination and $CD86^+$ BCs.

In mice, expression of CD5 is used to differentiate between $CD5^+$ B1a and $CD5^-$ B1b cells, whereas the existence and characterization of human B1 cells are still heavily debated (53–56), and CD5 has been found to be expressed on various human BC subsets (57). CD5 is a negative regulator of BC

receptor signaling, and so it is tempting to speculate that higher CD5 expression reduces alloreactive responses in KT (58). Moreover, CD5 promotes the production of IL-10, an inhibitory cytokine, and hallmark for BCs with regulatory functions (Bregs) (59–61). In fact, tolerant KTRs exhibit increased frequencies of blood $CD5^+$ BCs compared to stable immunosuppressed KTRs (27), and decreased $CD5^+$ BCs were associated with rejection in AB0 incompatible KT (62). We observed a significant reduction in $CD27^-$ $CD5^+$ BCs at T2. This contrasts previous findings, where $CD5^+$ BCs, despite some early fluctuations after transplantation, were found at similar numbers before KT and after the first year (63). Again, these differences may be explained by gating discrepancies. Whereas, we focused on $CD5^+$ BCs within the $CD27^-$ negative BC population, others have looked at CD5 expression on BCs in general (62, 63). The finding that $CD27^+$ mBCs downregulate CD5 supports our gating strategy (64).

In accordance with the existing literature, we have also seen a reduction of TrBCs in the post-transplantation setting (32, 65). Low TrBCs have been considered as potential biomarkers for graft rejection (7, 8), and tolerant KTRs display a high frequency of TrBCs (29). Mechanistically, due to their enrichment with Bregs, they may promote tolerance toward the graft (66). Yet, a role of TrBCs in response to vaccination of KTRs has not been described. In our hands, TrBCs significantly correlated with antibody levels after SARS-CoV-2 vaccination in stable KTRs 1 year after transplantation. Moreover, responders to vaccination, characterized as patients with a detectable antibody titer, presented with higher absolute TrBC numbers and higher TrBC frequency in blood than non-responders. In line, Tsang et al. (67) generated a prediction model for serological response to influenza vaccination in healthy individuals, and TrBCs were among the most predictive blood cell populations. However, evidence on pre-vaccination TrBCs in immunocompromised patients is scarce, purely observational, and conflicting. In acute myeloid leukemia patients, a worse response to H1N1 vaccination coincided with higher TrBC frequency than healthy subjects (68). In contrast, TrBCs have also been found abundantly in patients after rituximab therapy, and they responded equally well to H1N1 vaccination as controls with lower TrBCs (69). Nevertheless, patients after rituximab treatment also showed an impaired immune response to SARS-CoV-2 vaccination (70), which might be explained by lower TrBCs, but has not been evaluated so far. Thus, our data warrant further studies to test TrBCs as a potential biomarker for SARS-CoV-2 vaccination success in KTRs and other immunosuppressed patient populations.

The ability to generate specific antibodies to SARS-CoV-2 vaccination indicates intact humoral responsiveness to antigenic stimulation. Accordingly, one may hypothesize that patients with response to SARS-CoV-2 vaccination may also tend to develop allogenic antibodies. Furthermore, it remains to be elucidated whether SARS-CoV-2 vaccination can induce *de novo* formation of donor-specific antibodies (71), as it has been reported for other vaccines (72). Since donor-specific antibodies were not routinely evaluated in our cohort, studies exploring the relationship between allogenic antibodies and SARS-CoV-2 vaccine specific antibodies in transplantation need to be performed in the future.

Our study has several limitations. First, our study was not designed or powered to assess humoral response to SARS-CoV-2 vaccination in a prospective manner. Second, we lack information on peripheral blood leucocytes at the time of vaccination. We cannot rule out changes in the BC compartment from T2 to vaccination, even though immunosuppressive treatment remained similar, and only stable KTRs were assessed. Furthermore, previous reports suggest very little dynamics of BCs several months after KT (34, 73). Third, anti-SARS-CoV-2 antibodies were analyzed on different testing platforms and in different laboratories. Respective values had to be converted to International Standard units for comparison, as recommended by the WHO (74). Though, it is still a matter of debate whether results from different platforms may be used interchangeably (75, 76). We dichotomized the outcome of antibody testing into responders and non-responders, according to the detectability of specific antibodies, with the rationale, that even a minimal response may offer a crude measure of protection. Importantly, there is currently no definitive "protective threshold" for serological response (77).

In conclusion, we have shown a multitude of changes within the BC compartment after KT, including previously not reported subpopulations like DN BCs and mBC subsets. Our results further indicate that pre-vaccination TrBCs in stable KTRs correlate positively with antibody response to SARS-CoV-2 vaccination and are found in higher numbers and frequencies in vaccination responders. Due to low seroconversion rates (23, 24, 78) and emerging evidence of potentially fatal COVID-19 breakthroughs in fully vaccinated transplant recipients (79), it is of major importance to identify those who are unlikely to respond to SARS-CoV-2 vaccination in a timely manner and to emphasize on other means of protection in those individuals. In that manner, the role of TrBCs as pre-vaccination biomarkers for serological response to SARS-CoV-2 and other vaccines in KTRs should be further evaluated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Medical University of Graz, Austria (28-514ex15/16). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS: acquisition, analysis and interpretation of data, drafting manuscript, and approval of manuscript. VP: acquisition,

analysis of data, drafting manuscript, and approval of manuscript. AK: design of study, interpretation of data, reviewing manuscript, and approval of manuscript. KK and AM: interpretation of data, reviewing manuscript, and approval of manuscript. AR, PSc, HS, and PE: design of study, reviewing manuscript, and approval of manuscript. PSt: acquisition of data, reviewing manuscript, and approval of manuscript. BP: design of study, acquisition, analysis and interpretation of data, reviewing manuscript, and approval of manuscript. KE: design of study, acquisition, analysis and interpretation of data, drafting manuscript, and approval of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.818882/full#supplementary-material

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Can Gene Expression Analysis in Zero-Time Biopsies Predict Kidney Transplant Rejection?

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Zero-time biopsies are taken to determine the quality of the donor organ at the time of transplantation. Histological analyses alone have so far not been able to identify parameters that allow the prediction of subsequent rejection episodes or graft survival. This study investigated whether gene expression analyses of zero-time biopsies might support this prediction. Using a well-characterized cohort of 26 zero-time biopsies from renal transplant patients that include 4 living donor (LD) and 22 deceased donor (DD) biopsies that later developed no rejection (Ctrl, n = 7), delayed graft function (DGF, n = 4), cellular (T-cell mediated rejection; TCMR, n = 8), or antibody-mediated rejection (ABMR, n = 7), we analyzed gene expression profiles for different types of subsequent renal transplant complication. To this end, RNA was isolated from formalin-fixed, paraffin-embedded (FFPE) sections and gene expression profiles were quantified. Results were correlated with transplant data and B-cell, and plasma cell infiltration was assessed by immunofluorescence microscopy. Both principal component analysis and clustering analysis of gene expression data revealed marked separation between LDs and DDs. Differential expression analysis identified 185 significant differentially expressed genes (adjusted $p < 0.05$). The expression of 68% of these genes significantly correlated with cold ischemia time (CIT). Furthermore, immunoglobulins were differentially expressed in zero-time biopsies from transplants later developing rejection (TCMR + ABMR) compared to non-rejected (Ctrl + DGF) transplants. In addition, immunoglobulin expression did not correlate with CIT but was increased in transplants with previous acute renal failure (ARF). In conclusion, gene expression profiles in zero-time biopsies derived from LDs are markedly different from those of DDs. Pre-transplant ARF increased immunoglobulin expression, which might be involved in triggering later rejection events. However, these findings must be confirmed in larger cohorts and the role of early immunoglobulin upregulation in zero-biopsies needs further clarification.

Keywords: kidney transplantation, zero-time biopsy, mRNA expression, inflammation, outcome

INTRODUCTION

At the time point of kidney transplantation, many transplant centers routinely collect zero-time biopsies for determining graft quality, as a reference for later biopsies, and for gathering information with the potential to predict the later outcome after transplantation. However, the benefits of this practice are still under debate. Rathore et al. described that zero-time biopsies provide information regarding the general condition of the kidneys of the donors and that interstitial fibrosis and acute tubular injury in living donors (LDs) were significantly associated with allograft dysfunction (1). However, another study revealed that a mild degree of subclinical pathologic findings did not affect graft function after LD kidney transplantation (2). There are also contradictory results and discrepant conclusions in studies that investigated deceased donor (DD) kidneys. While Tavakkoli et al. showed no relationship between histological findings and graft survival in DDs (3), others reported that histological findings predict early graft function (4). Even the use of various standard immunohistological examinations in zero-time biopsies did not reveal useful markers that could indicate graft outcome. In a previous study involving a cohort of living and DDs, we showed that glomerular immune reactivity is a frequent finding in zero-time biopsies. Yet it does not have an impact on graft function not on survival (5). Accordingly, similar studies could not identify immunohistochemical parameters that are predictive of rejection or graft outcome (6).

Given that histological and immunohistochemical analyses have not yet identified parameters that definitely predict subsequent rejection episodes or graft survival, other analytic methods should be considered. Some gene expression analyses have shown that differences in the transcriptome of kidneys of the donors reflect graft function (7). Furthermore, a better understanding of the molecular mechanisms influencing graft outcome might be discovered by analyzing differential gene expression patterns in zero-time biopsies (8).

Here, we tested the hypothesis that expression profiles of transplant-related genes in kidneys of the donors can predict subsequent graft outcomes. To this aim, we examined the gene expression profiles of 26 zero-time biopsies from renal transplant patients who developed no rejection or dysfunction (Ctrl, $n = 7$), delayed graft function (DGF, $n = 4$), T-cell mediated rejection (TCMR, $n = 8$), or antibody-mediated rejection (ABMR, $n = 7$). For expression analysis, RNA was isolated from formalin-fixed, paraffin-embedded (FFPE) sections and quantified with the NanoString Human Organ Transplant panel measuring the expression of nearly 800 genes. Genes with remarkable expression profiles regarding the different outcome groups were further analyzed.

MATERIALS AND METHODS

Renal Tissue Specimens

In this study, zero-time biopsies collected from renal grafts before transplantation between 2015 and 2019 from the Department of Nephrology at the FAU Erlangen-Nuremberg, Germany were included. To identify differences in gene expression in time-zero biopsies, 26 carefully selected FFPE specimens of archived donor kidney biopsies (from the Department of Nephropathology, University Hospital, Erlangen, Germany) were used to evaluate characteristic mRNA expression profiles in zero-time biopsies from patients who later developed DGF ($n = 4$), ABMR ($n = 7$), and TCMR ($n = 8$; 1 LD, 7 DDs) within the first 2.5 years. Zero-time biopsies were taken as a protocol biopsy immediately before implantation of the graft into the recipient. Biopsies from patients with borderline changes or other co-morbidities, such as viral infection, immunoglobulin A (IgA)-nephropathy, or other immune-complex glomerulonephritis, were excluded. Neither patients from the control group nor from the DGF group developed a rejection or a borderline reaction at later stages. Zero-time biopsies from kidneys without signs of renal dysfunction or rejection after 1 year from transplantation served as controls ($n = 7$; 3 LDs, 4 DDs). DGF was defined as impaired renal function necessitating dialysis within the first 10 days post-transplantation and lack of rejection. For the ABMR group, we included zero-time biopsies that later developed ABMR (active type II) with evidence of donor-specific antibodies (DSAs). Zero-time biopsies for the TCMR group were included if cases were developed later on after transplantation acute type IA, IB, or IIA TCMR without signs of ABMR. The study groups, characteristics of patients, and Banff classification are described for donors (**Table 1** and **Supplementary Data 1**) and for recipients (**Supplementary Datas 2**, **3**) (9). The Ethics Committee of the Friedrich-Alexander-University approved the use of archival material, waiving the need for retrospective consent for the use of archived rest material (Re.-No. 4415).

Multiplex mRNA Expression Analysis by NanoString

For expression analysis, RNA was isolated from 15 μm sections using the RNeasy FFPE Kit (Qiagen, Venlo, Netherlands). RNA concentration and purity were measured with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), and isolates with a 260/280 nm absorbance ratio below 1.4 were excluded. All samples had a volume of 25 μl H_2O containing 111–393 ng mRNA and were concentrated using a Savant SPD111 SpeedVac (Thermo Fisher Scientific) at 35°C for 24 min to a volume of 2–3 μl. According to the recommendations of manufacturer, after a hybridization and preparation step, gene expression was analyzed with the NanoString nCounter FLEX Analysis System (NanoString Technologies, Seattle, WA, United States) using the nCounter Banff Human Organ Transplant (B-HOT) panel, containing 760 genes and 10 internal reference genes (10).

Analysis of NanoString Gene Expression Data and Statistics

Analysis of NanoString gene expression raw data (**Supplementary Data 4**) was performed relying on the DESeq2 package v. 1.34.0 (11) within R. v. 4.1.2/Bioconductor v. 3.14 environment (12, 13). Positive/negative controls were excluded from the analysis, and the estimation of size factors was based

on housekeeping genes. Differential expression analysis was performed that includes as factors donor type (living/deceased) and later rejection (yes/no) of the samples. A gene was considered differentially expressed if its (Benjamini–Hochberg) adjusted p was lower than 0.05. Principal component analysis was based on the variance-stabilized transformed counts of the top 100 variable genes. The expression heatmap was generated using standardized variance-stabilized transformed counts relying on the Complex Heatmap package v. 2.10.0. In order to perform functional enrichment analysis, first gene symbols associated with the NanoString nCounter® Human Organ Transplant Panel were mapped to Entrez Gene Ids relying on the biomaRt package

TABLE 1 | Kidney donor characteristics.

	Ctrl	DGF	TCMR	ABMR	Total
Donor n = 26	7	4	8	7	26
Men (%)	2 (29%)	1 (25%)	4 (50%)	3 (43%)	10 (38%)
Age at transplant (years)	49 ± 18	49 ± 9	54 ± 7	52 ± 9	51 ± 12
Body mass index (kg/m^2)	27 ± 3	27 ± 2	29 ± 8	29 ± 3	28 ± 5
Serum creatinine (mg/dl)	1.19 ± 0.96	3.02 ± 2.13	1.36 ± 0.53	2.54 ± 2.13	1.89 ± 1.67
Smoker	0	1	2	1	4
Diabetes	0	1	0	0	1
Hypertension	1	2	3	3	9
CAD	1	1	2	0	4
Sepsis	0	1	3	1	5
ARF	1	4	5	5	15
Deceased (%)	4 (57%)	4 (100%)	7 (88%)	7 (100%)	22 (85%)
Reanimation	2	4	5	3	14
CIT (min)	313 ± 289	795 ± 146	680 ± 280	813 ± 215	635 ± 322

Ctrl, control; DGF, delayed graft function; TCMR, T-cell mediated rejection; ABMR, antibody-mediated rejection; CAD, coronary artery disease; ARF, acute kidney injury; CIT, cold ischemia time. Ranges are stated as mean ± SD.

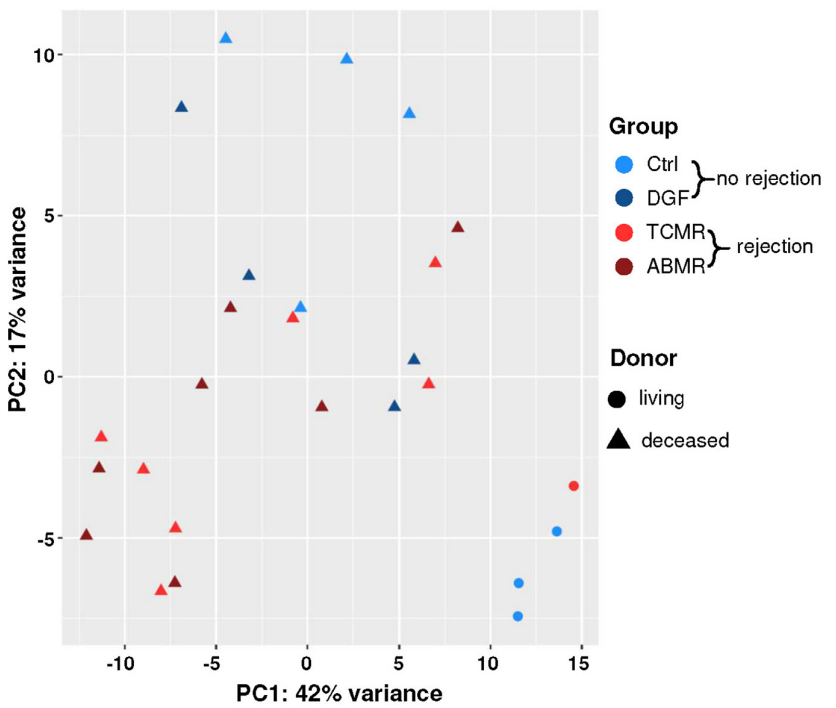

FIGURE 1 | Principal component analysis (PCA) plot of NanoString expression data. The plot of the first two principal components (PC1 and PC2), calculated from the expression data of the 100 genes with the highest variance across all 26 samples. Axis labels report the percentage of total variance explained by each component. Biopsies without later rejection were indicated with blue symbols [Ctrl = light blue; delayed graft function (DGF) = dark blue] and those with later rejection with red symbols [T-cell mediated rejection (TCMR) = bright red, antibody-mediated rejection (ABMR) = dark red]; living donors were denoted using circles and deceased donors using triangles.

v. 2.44.4 (14, 15). Functional enrichment analysis of differentially expressed genes was then performed relying on the enrichGO function of the clusterProfiler package v. 3.16.1 (16) within the R environment v.4.0.3, using Entrez Gene Ids as identifiers. As background for the analysis, the set of uniquely mapped Entrez Gene Ids was employed. Pathways were considered significantly enriched if their associated adjusted value of p was <0.05. Dot plots of the expression of single genes were generated using normalized count data relying on ggplot2 v.3.3.5.

To test for correlation between expression and CIT, Spearman's rank correlation analysis was performed. Wilcoxon rank-sum test was used to assess differences in expression of

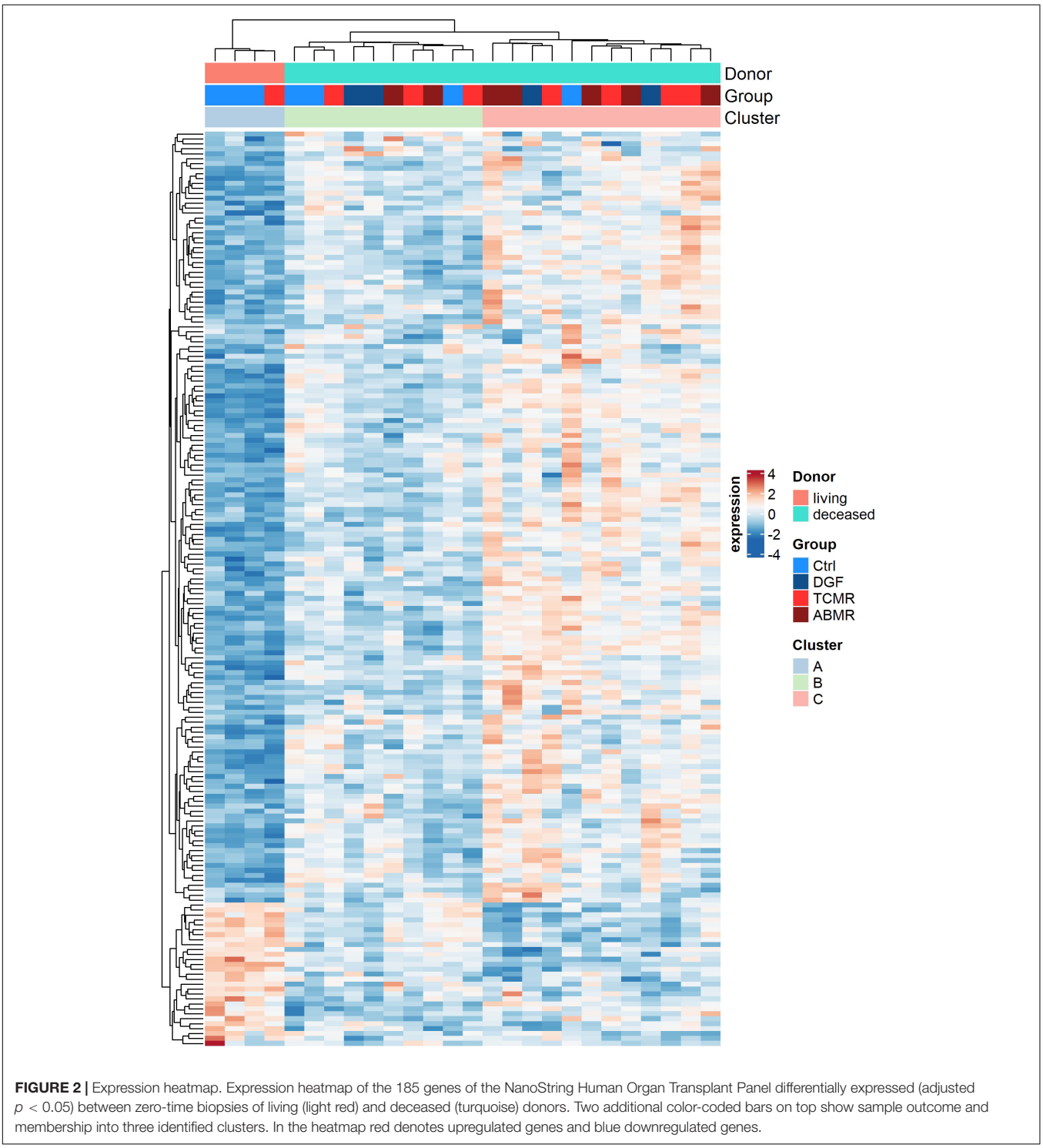

FIGURE 2 | Expression heatmap. Expression heatmap of the 185 genes of the NanoString Human Organ Transplant Panel differentially expressed (adjusted $p < 0.05$) between zero-time biopsies of living (light red) and deceased (turquoise) donors. Two additional color-coded bars on top show sample outcome and membership into three identified clusters. In the heatmap red denotes upregulated genes and blue downregulated genes.

selected genes between ARF and no ARF, an abundance of CD20- and CD138-positive cells between rejection and no rejection group, and to test for differences in age of the donor. A $p < 0.05$ was considered statistically significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$).

Immunofluorescence Double Staining

For immunofluorescence microscopy, 2 μm sections of FFPE kidney biopsies were utilized for the staining procedure. After antigen retrieval in target retrieval solution pH 6 (DAKO Deutschland, Hamburg, Germany) for 2.5 min in a pressure cooker, sections were blocked in 1% bovine serum albumin diluted 1:50 in 50 mM Tris pH 7.4. Sections were then incubated overnight at room temperature with a mouse monoclonal antibody (IgG2a) against human CD20cy (clone L26; M0755 Dako Deutschland) together with a mouse monoclonal antibody (IgG1) against human CD138 (clone B-A38, MSK063, Zytomed Systems GmbH, Berlin, Germany), both diluted in blocking solution. After washing with 50 mM Tris pH 7.4, sections were incubated for 30 min with secondary antibodies: goat anti-mouse IgG1 conjugated with Alexa488 (Dianova GmbH, Hamburg, Germany) and goat anti-mouse IgG2a conjugated with Alexa633 (Life Technologies, Carlsbad, CA, United States). After additional washing, stained slides were covered with VECTASHIELD Vibrance antifade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories, Burlingame, CA, United States) and imaged on the slide-scanner Axio Scan.Z1 (Zeiss, Oberkochen, Germany).

Images of the scanned slides were evaluated with the software QuPath version 0.2.3 (17). Scanned slides were annotated manually for CD20- and CD138-positive cells, while CD138-positive tubular epithelial cells were excluded. Section area was determined by outlining the biopsies using the polygon tool. Finally, the number of CD20- and CD138-positive cells was calculated per section area.

Injury Scores

Histopathological changes were graded according to the Banff classification score of 2013 and 2017 for renal transplant biopsies in the process of routine diagnosis (18, 19). Selected clinical parameters from the time point of biopsy collection were retrospectively investigated. In addition, transplantation relevant parameters, such as cold ischemia time (CIT) and data of the donors (i.e., age, hypertension, serum creatinine, reanimation, sepsis, and acute renal failure (ARF) events before renal transplantation and renal inflammation) were included for correlation analysis with the results of the gene expression analysis.

RESULTS

Gene Expression in Zero-Time Biopsies Is Dependent on Donor Type

Histopathologic examination of the zero-time biopsies revealed no significant differences in kidney grafts with different outcomes (**Supplementary Data 1**). Therefore, we investigated whether differences in gene expression may predict complications, such as DGF, TCMR, or ABMR. The expression profiles of transplantation-related genes were analyzed in 26 zero-time biopsies that later developed DGF, TCMR, ABMR or had normal renal function throughout 1 year after transplantation and showed no signs of rejection (Ctrl) using a NanoString B-HOT panel. Expression analysis revealed that irrespective of the complication group, the greatest differences were observed when comparing donor types. Indeed, principal component analysis of expression data showed a clear separation between biopsies from LDs and those from DDs (**Figure 1**). Furthermore, differential expression analysis including factors "donor" and "later rejection (yes/no)" of the samples, revealed 185 differentially expressed genes between DD and LD biopsies (adjusted $p < 0.05$) (**Figure 2** and **Supplementary Data 5**). Instead, only 11 genes were differentially expressed (adjusted $p < 0.05$) in samples later showing a rejection (TCMR or ABMR) versus samples showing no rejection (Ctrl or DGF).

Genes Differentially Expressed in Zero-Time Biopsies From DD Versus LD Were Enriched in the Pathway "Post-translational Protein Modification"

In order to further characterize the differentially expressed genes, we performed functional enrichment analysis using Gene Ontology Biological process terms and the set of NanoString B-HOT panel genes as background. Interestingly, considering the 185 differentially expressed genes between DD and LD, only "GO: 0043687 post-translational protein modification" was significantly enriched (adjusted $p < 0.05$). Fifteen of the 185 genes were assigned to this pathway (**Table 2**). Among them, HIF1A, TIMP1, C3, PSMB8, and SOCS3 were the genes with the highest fold changes and showed higher expression in DD regardless of the group (**Figure 3**). In addition to being involved in post-translational modifications, these genes are also involved in many other pathways, such as hypoxia-induced processes (GO: 0061418, GO: 0001666, and GO: 0071456; e.g., HIF1A, PSMB8, PSME1, and PSME2), extracellular matrix organization (GO: 0030198, GO: 0043062; TIMP1, TNC, VCAN, and FN1), complement-dependent processes (e.g., GO: 0006956, C3) and pathways related to ubiquitinylation (GO: 0016567; PSMB8, PSME1, PSME2, PSMB10, SOCS3, ASB15, and KLHL13) (**Supplementary Data 6**). Almost all of the 15 genes were also found in the stress response pathway (GO: 0006950). However, none of these other pathways were significantly enriched. Since one of the major differences between DD and LD was the duration of the CIT [median CIT (range) (min); LD: 0 (0–157) vs. DD: 769 (402–1,080)], we assessed the correlation between CIT and the expression levels of the 15 genes of the enriched "post-translational modification" pathway (**Table 2**) and with the top 20 of the 185 differentially expressed genes between DD and LD (**Table 3**). In both cases, the majority of genes showed significant correlations with CIT. Furthermore, 125 (67.6%) of the 185 differentially expressed genes of the comparison of DD

vs. LD were significantly correlated with CIT, indicating that CIT was an important stimulator/regulator of these genes.

The Expression Profiles of DDs Were Associated With the Age of the Donors

Hierarchical clustering of the expression of the 185 differentially expressed genes between DD and LD (**Figure 2**) suggested grouping samples into three clusters: one (cluster A) of LD samples and two (clusters B and C) of DD samples. Most of the genes that were expressed at lower levels in cluster A were expressed more strongly in cluster B and even more strongly in cluster C. With regard to outcome, it is noticeable that more samples belonged to the Ctrl group in cluster B than in cluster C (**Table 4**). In contrast, the proportion of samples with ABMR was

TABLE 2 | Genes differentially expressed between deceased (DD) and living donors (LD) associated with the significantly enriched pathway (adjusted $p = 0.01$) "GO: 0043687 post-translational protein modification".

Gene name	Significance DD vs LD (adj. *p*-value)	DD vs LD (log2 Fold change)	Corr. Coeff. with CIT (Spearman's rank correlation)
HIF1A	7.08e-12	2.06	0.628***
TIMP1	5.02e-08	2.55	0.401*
C3	8.94e-08	3.78	0.420*
PSMB8	5.81e-06	1.09	0.583**
SOCS3	2.66e-05	2.41	0.413*
ASB15	7.77e-05	−2.35	−0.524**
PSME2	1.89e-04	0.71	0.513**
PSMB10	4.44e-04	1.06	0.640***
APOL1	0.004	0.82	0.614***
TNC	0.004	1.84	0.570**
VCAN	0.010	1.62	0.674***
APOE	0.011	−1.12	−0.382
PSME1	0.028	0.38	0.489*
FN1	0.029	1.10	0.327
KLHL13	0.045	0.51	0.141

*CIT, cold ischemia time. *p < 0.05. **p < 0.01. ***p < 0.001.*

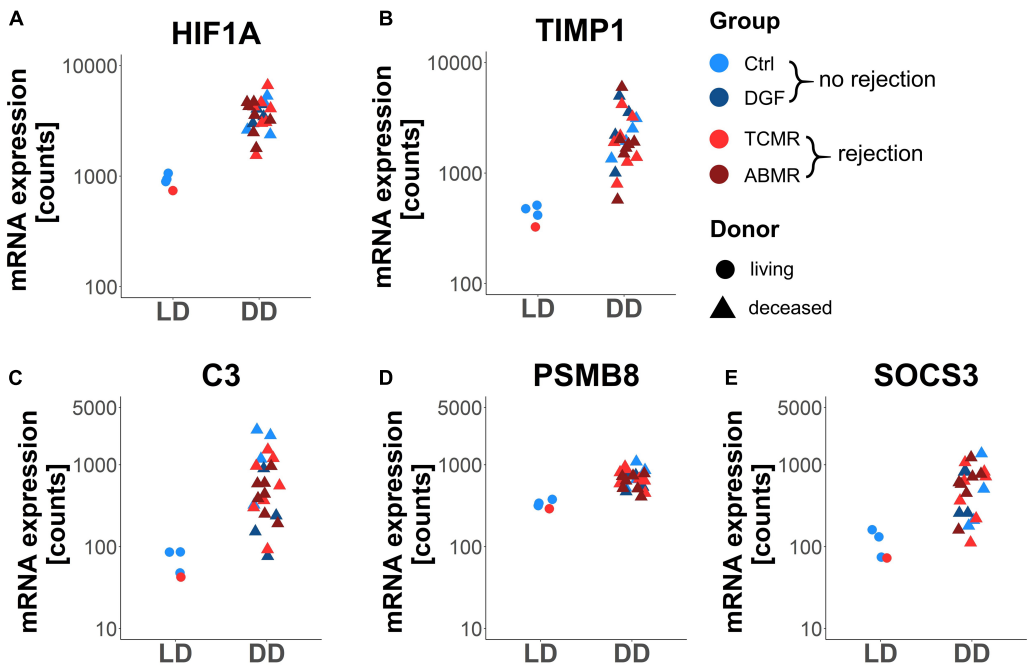

FIGURE 3 | Expression of the top 5 differentially expressed genes between deceased (DD) and living donors (LD) associated with the enriched pathway "post-translational protein modification". Dot plots showing gene expression (normalized counts) of the genes HIF1A **(A)**, TIMP1 **(B)**, C3 **(C)**, PSMB8 **(D)**, and SOCS3 **(E)** in zero-time biopsies of living (LD) and deceased (DD) donors. Adjusted $p < 0.05$ for all shown genes.

TABLE 3 | Correlation of the top 20 differentially expressed genes between deceased (DD) and living donors (LD)with cold ischemia time (CIT).

Gene name	Significance DD vs LD (adj. *p*-value)	DD vs LD (log2 Fold change)	Corr. Coeff. with CIT (Spearman's rank correlation)
SERPINA3	1.56e-20	7.42	0.239
ALDH3A2	4.72e-15	−1.66	−0.544**
OSMR	9.08e-14	2.24	0.604**
S100A9	4.21e-13	4.13	0.395*
HIF1A	7.08e-12	2.11	0.628***
LTF	4.60e-10	4.95	0.267
BCL3	5.15e-10	2.4	0.660***
PLAAT	3.74e-09	2.48	0.483*
RARRES1	4.28e-09	4.13	0.489*
C1QB	5.26e-09	2.25	0.636***
JAK1	5.26e-09	0.668	0.413*
CD163	6.40e-09	2.80	0.462*
IFITM3	2.42e-08	1.92	0.379
C1S	2.52e-08	1.90	0.573**
FPR1	2.52e-08	2.41	0.521**
SOD2	3.13e-08	2.82	0.473*
BCL6	4.86e-08	2.14	0.458*
TIMP1	5.02e-08	2.55	0.401*
STAT3	5.07e-08	1.08	0.439*
S100A8	6.55e-08	4.08	0.284

*CIT, cold ischemia time. *p < 0.05. **p < 0.01. ***p < 0.001.*

TABLE 4 | Distribution of outcome and clinical parameters of samples belonging to the two DD clusters (B, C) of **Figure 2**.

		Deceased donors (DD)	
		Cluster B	**Cluster C**
Outcome	Ctrl	3/10 (30%)	1/12 (8.3%)
	DGF	2/10 (20%)	2/12 (16.7%)
	TCMR	3/10 (30%)	4/12 (33.3%)
	ABMR	2/10 (20%)	5/12 (41.7%)
	Rejection	5/10 (50%)	9/12 (75%)
Clinical parameters	ARF	6/10 (60%)	9/12 (75%)
	Reanimation	8/10 (80%)	6/12 (50%)
	Sepsis	2/10 (20%)	3/12 (25%)
	Hypertension	5/10 (50%)	4/12 (33.3%)
	CIT *[min]	609 (402; 1008)	868 (480; 1080)
	Donor age (**)	61 (36; 76)	36 (25; 65)

*The distribution of donor outcome and categorical clinical parameters are provided in terms of proportion with respect to the total number of samples within each cluster (with the associated percentage within brackets). For quantitative clinical parameters, the median value (with minimum and maximum values within brackets) is reported. Ctrl, control; DGF, delayed graft function; TCMR, T-cell mediated rejection; ABMR, antibody-mediated rejection; ARF, acute renal failure; CIT, cold ischemia time. *p < 0.05; **p < 0.01 (Wilcoxon rank-sum test).*

higher in cluster C (**Table 4**). CIT was significantly lower in DD cluster B compared to cluster C. (**Table 4**). No significant differences were observed between clusters B and C for other clinical parameters that include the occurrence of ARF, sepsis, hypertension, or the need for reanimation (**Table 4**). However, the age of donors in cluster B was significantly higher, with median age of 25 years higher than that of samples in cluster C (**Table 4**).

Immunoglobulin Genes Were Upregulated in Zero-Time Biopsies From Patients Later on Developing Rejection

While still adjusting for donor type (DD vs. LD), differential expression analysis was performed to compare samples developing rejection events later on to samples without rejection (controls and cases that developed DGF). In contrast to the high number of differentially expressed genes between

TABLE 5 | Correlation of the differentially expressed genes between rejection (R) and no-rejection (NR) with cold ischemia time (CIT).

Gene name	Significance R vs NR (adj. *p*-value)	R vs NR (log2 Fold change)	Corr. Coeff. with CIT (Spearman's rank correlation)
IGHG2	6.73e-06	3.01	0.288
IGHG3	6.73E-06	2.98	0.286
IGKC	6.73E-06	3.11	0.284
IGHG4	7.74E-06	2.86	0.297
IGHA1	0.00064	2.36	0.364
IGHG1	0.00064	2.85	0.275
IGLC1	0.00065	1.78	0.364
IGHM	0.016	1.47	0.232
CD24	0.033	−0.60	0.266
SERPINA3	0.036	−1.78	0.239
CIITA	0.048	0.70	0.302

DD and LD, only 11 genes were significantly differentially expressed in zero-time biopsies with later rejection (**Table 5** and **Figure 4**). Interestingly, 8 of these belonged to genes coding for immunoglobulin chains, i.e., four IgG heavy chains (**Figures 4A–D**), one IgA heavy chain (**Figure 4E**), one IgM heavy chain (**Figure 4F**), an immunoglobulin lambda chain (**Figure 4G**), and kappa light chain (**Figure 4H**). With lower fold change CIITA (**Figure 4I**), coding for a protein involved in transactivation of class II major histocompatibility complex (MHCII) was differentially expressed. All immunoglobulin chains and CIITA were more strongly expressed in transplants with later rejection events. In contrast, CD24 (**Figure 4J**), coding for a surface protein expressed on mature granulocytes and B-cells and modulating growth and differentiation signals to these cells, and SERPINEA3 (**Figure 4K**), coding for a serine protease inhibitor, were expressed at a lower level in the rejection group compared to the no-rejection group. None of the genes differentially expressed in rejection vs. no-rejection groups significantly correlated with CIT (**Table 5**). Functional pathway enrichment analysis for the genes differentially expressed in the rejection vs. no-rejection group resulted in significant enrichment of 62 pathways (adjusted $p < 0.05$), with the top ones being: GO: 0006911 phagocytosis, engulfment; GO: 0010324 membrane invagination; GO: 0099024 plasma membrane invagination; GO: 0006958 complement activation, classical pathway; GO: 0008037 cell recognition; GO: 0002455 humoral immune response mediated by immunoglobulin (**Supplementary Data 7**).

CD20-Positive B-Cells and Plasma Cells Are More Abundant in Zero-Time Biopsies From Renal Transplants Experiencing Later Rejection Episodes

Since immunoglobulins were expressed by mature B cells and plasma cells, we evaluated the abundance of CD20-positive B-cells and CD138-positive plasma cells in FFPE sections of the zero-time biopsies using immunofluorescence microscopy. While in zero-time biopsies from Ctrl and DGF, CD20- and CD138-positive cells were detected only sporadically (**Figures 5A,B**), in TCMR, and in ABMR, these cells often occurred locally clustered (**Figures 5C,D**). Consequently, the number of CD20- (**Figure 5E**) and CD138-positive cells (**Figure 5F**) per section area was significantly higher in samples from transplants with later rejection events. In addition, the expression of immunoglobulins correlated well with numbers of CD20- and CD138-positive cells (**Figure 5G**). The highest correlation with the number of CD20-positive B-cells was found for the expression of IGLC1 (**Figure 5H**) and with the number of CD138-positive cells for the expression of IGHM (**Figure 5I**).

Genes Associated With Later Rejection Were More Strongly Expressed in Kidneys of Donors With Pre-transplant ARF

Since genes differentially expressed in rejection vs. no-rejection groups did not correlate with CIT, we investigated whether gene expression was correlated with other pre-transplant parameters. No correlation could be detected between gene expression levels and the need for reanimation or with the occurrence of sepsis in the donors (data not shown). In contrast, gene expression levels of 5 differentially expressed genes (i.e., IGHG1, IGHG3, IGHA1, CD24, and IGLC1) were significantly associated with the occurrence of ARF prior to the explanation of the organs of donors and three more genes (i.e., IGHG2, IGHG4, and IGKC) showed a strong tendency toward this association (**Figure 6**).

DISCUSSION

After kidney transplantation, impaired kidney function due to DGF or rejection events, such as TCMR and ABMR, occurs on regular basis. At worst, this can lead to loss of the transplant. Graft loss due to these complications partly depends on factors related to the recipient but possibly also on the organs of donors. Suri et al. estimated that characteristics of donors account for 35–45% of the variability of early graft function (20). In the past, various studies could not produce consistent results on the predictive value of histological changes that were already present in the organs of donors at the time of transplantation with regard to later complications or graft loss (1–4). In our small collective of zero-time biopsies, analysis of histological changes using Banff classification failed to detect changes that can be used

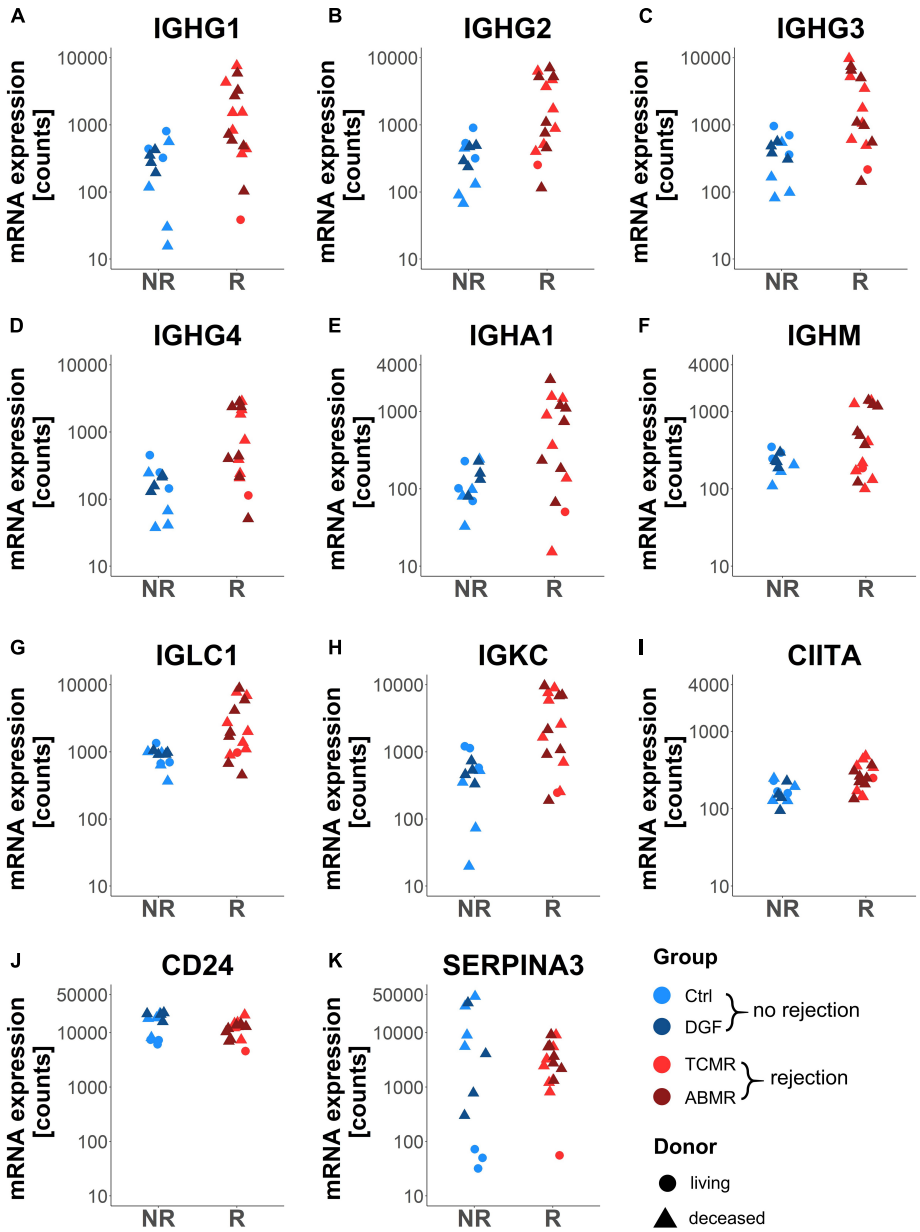

FIGURE 4 | Dot plots of genes differentially expressed in zero-time biopsies of patients later developing rejection (R) versus no rejection (NR). Dot plots showing gene expression of the genes IGHG1-4 **(A–D)**, IGHA1 **(E)**, IGHM **(F)**, IGLC1 **(G)**, IGKC **(H)**, CIITA **(I)**, CD24 **(J)**, and SERPINA3 **(K)** in zero-time biopsies. Adjusted $p < 0.05$ for all shown genes.

as predictors for the later outcomes. The investigation of mRNA expression profiles, with the aid of multiplex analyses, such as microarrays or NanoString analyses, enables the recording of a huge number of parameters simultaneously. While most existing studies compare the gene expression profiles of healthy grafts with rejection biopsies (9, 21–25), there are so far only a few that examine zero-time biopsies using either few pre-selected genes (8, 26) or multiplex arrays (7, 27–29). Earlier gene expression studies using zero-time biopsies focused on differences between LDs and DDs (29), gene expression profiles in biopsies with histological changes (27) or DGF (7, 28). Although we included only a low number of zero-time biopsies derived from LDs in our study, we could confirm earlier findings showing a differential gene expression pattern comparing DD vs. LD (29). The high number of 185 differentially regulated genes when comparing DD with LD indicates that the donor type significantly influences expression. The extent to which these differentially regulated genes influence the subsequent outcome cannot be determined on the basis of our study. Surprisingly, only one pathway was significantly enriched, suggesting that conditions in DD were

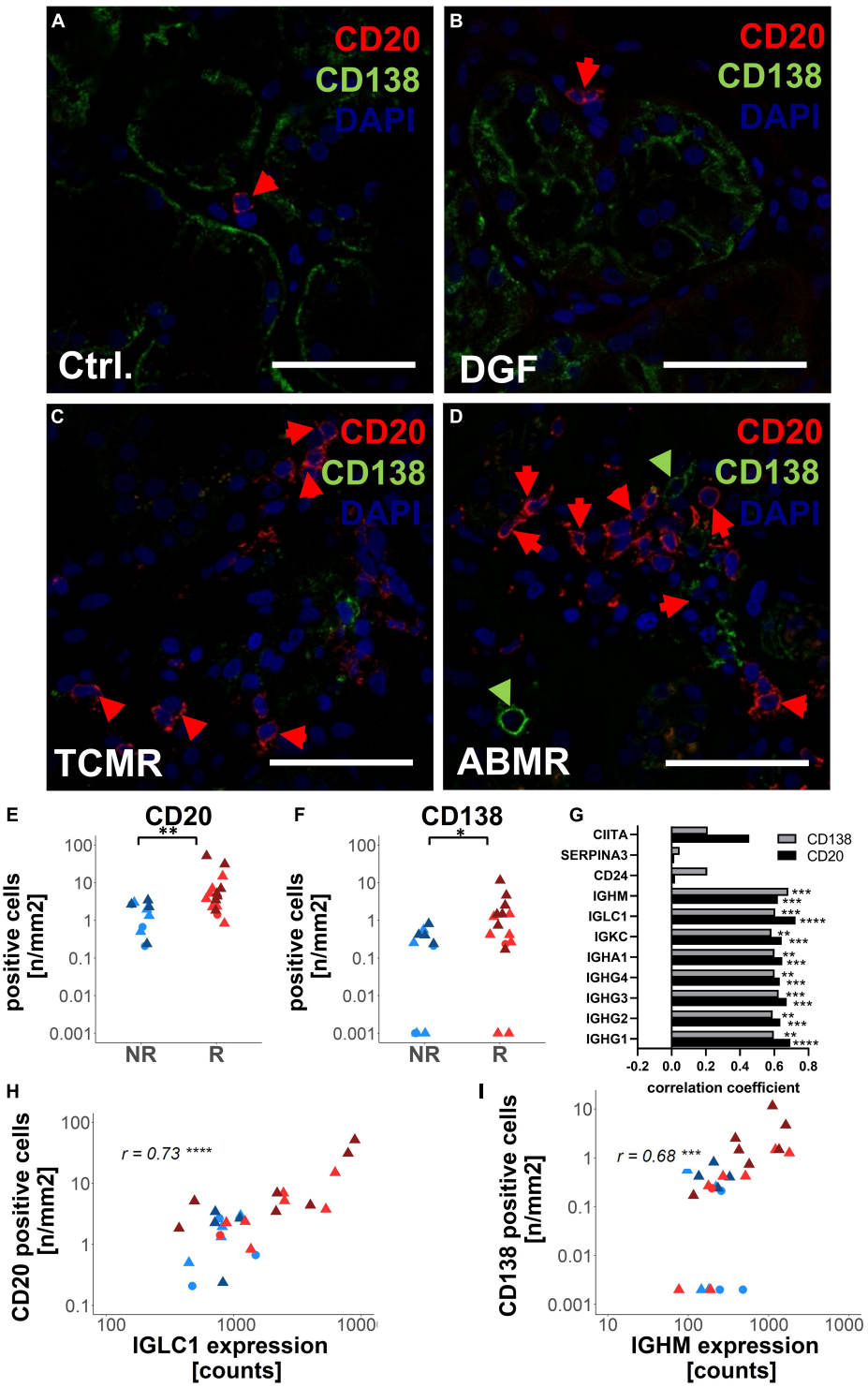

FIGURE 5 | Analysis of antibody-producing cells in zero-time biopsies with the different later outcomes. CD20-positive B-cells and CD138-positive plasma cells were analyzed using immunofluorescence microscopy in zero-time biopsies of Ctrl **(A)**, delayed graft function (DGF) **(B)**, T-cell mediated rejection (TCMR) **(C)**, and antibody-mediated rejection (ABMR) **(D)**. Examples of CD20-positive cells were marked by red arrows and CD138-positive cells by green arrows. The numbers of CD20-positive cells **(E)** and CD138-positive **(F)** per biopsy area were shown in biopsies that later on developed rejection (R) or no rejection (NR). Correlation between the expression levels of differentially expressed genes between rejection and no rejection was shown **(G)**. Histograms for correlation of the number of CD20-positive cells with the gene expression level of IGLC1 **(H)** and numbers of CD138-positive cells with gene expression levels of IGHM **(I)** in zero-time biopsies were shown. Scale bar represents 50 μm; r: Spearman's rank correlation coefficient; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

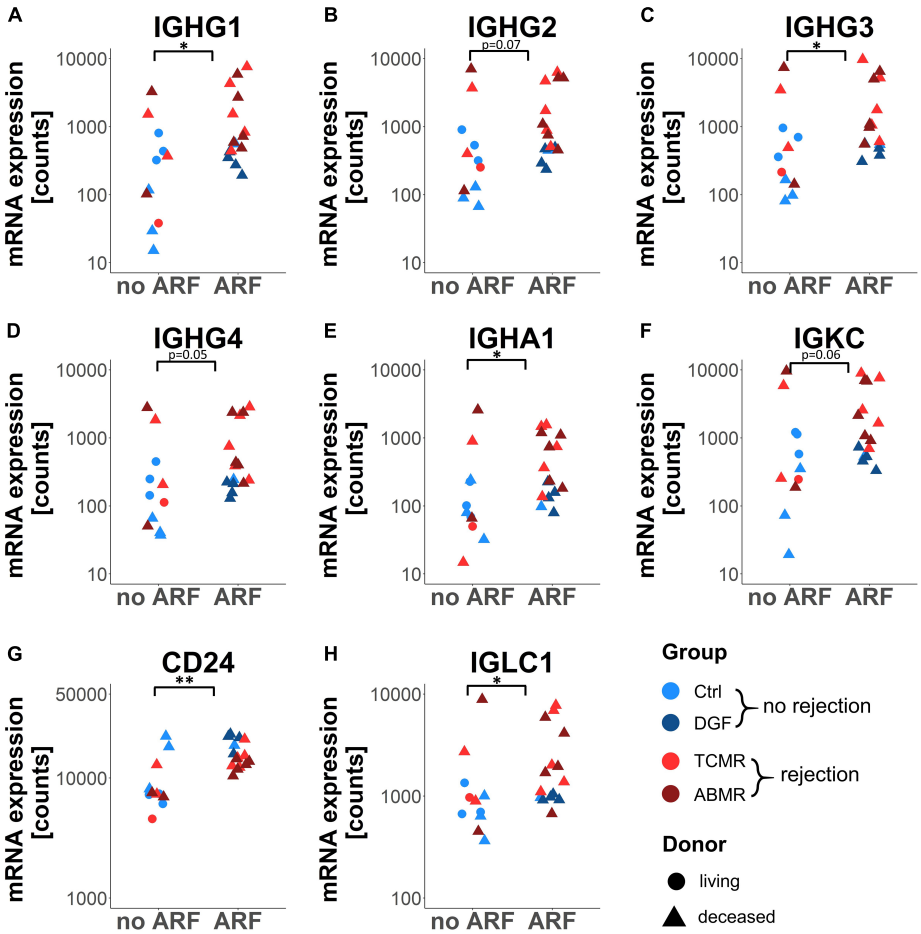

FIGURE 6 | Dot plot of eight selected genes differentially expressed between rejection and no rejection grouped according to the presence of acute renal failure (ARF). Dot plots showing gene expression of the genes IGHG1–4 **(A–D)**, IGHA1 **(E)**, IGKC **(F)**, CD24 **(G)**, and IGLC1 **(H)** in zero-time biopsies. *$p < 0.05$; **$p < 0.01$.

complex and induced no specific pathway. Several studies on kidney transplantation could demonstrate that graft survival was improved in recipients receiving a transplant from an LD (30, 31). One major difference between LD and DD is the lack of CIT in LD. Our data clearly showed that the expression level of the majority of the genes differentially regulated in DD vs. LD correlated with the duration of CIT. The importance of the hypoxic trigger is supported by overexpression of HIF1A in DD, which is the gene with the 5th highest significance level, even if this transcription factor is mainly controlled by oxygen-dependent stabilization on protein level. HIF1A stabilization occurs by post-translational protein modification, in which the only pathway enriched in DD compared to LD is involved and which includes several proteasomal proteins. Earlier studies reported that prolonged CIT is a known risk factor for allograft loss (32). However, in our study, we searched for gene expression changes in zero-time biopsies that may predict a later complication. In contrast to previous studies, we specifically investigated zero-time biopsies from transplants that later developed either DGF, TCMR, or ABMR, since the number of transplants that developed DGF or rejection events was very low in consecutive cohorts under investigation in earlier studies (7). The comparison of all 4 different groups showed no significantly changed expression patterns for the respective complication groups DGF, TCMR, and ABMR, which may be also due to the low sample numbers per group. Within the DD samples, two different clusters could be identified showing different levels of upregulation compared to the LD group. Although one cluster contained more controls and the other more ABMR, the two clusters did not differ significantly in terms of outcome and clinical parameters. However, there was a significant difference in the age of donor. Since most of the genes monitored on the NanoString B-HOT panel are related to the immune system, the observed age-dependent differences might reflect the changes described as immunosenescence, which can affect graft outcome (33). However, in our pilot study, assignment to one of the two gene expression clusters B and C did not allow a clear prediction regarding the subsequent outcomes. Since we observed that the gene expression profile in DGF was similar to Ctrl and expression profiles in TCMR resembled those of ABMR, we decided to compare biopsies with and without future rejection episodes to assess whether gene

expression in zero-time biopsies can predict future rejection. Only 11 genes were found to be differentially expressed between rejection and no rejection samples, 8 of which coded for immunoglobulin chains. Functional pathway enrichment analysis of these genes highlighted the enrichment of more than 60 pathways that are mainly associated with antibody-mediated responses, such as activation of the classical complement pathway or the antibody-mediated humoral immune response. Using immunofluorescence microscopy, we demonstrated that this increased expression of immunoglobulins in zero-time biopsies later developing rejection was due to an increased presence of CD20-positive B-cells and CD138-positive plasma cells. B-cell rich infiltrates in allograft biopsies were not associated with worse outcomes in types I and II acute rejection, but a possible contribution of B-cells to allograft rejection could not be excluded in a previous study (34). Another study reported the protective effects of intra-graft CD20-positive cells in cell-mediated rejection (35). Especially transitional B-cells, a subgroup of CD20-positive cells expressing $CD24^{high}$, were found to play a protective role (36). Interestingly, in our gene expression analysis, CD24 was one of the differentially regulated genes in the rejection group and exhibited a lower expression compared to the no rejection group, thus indicating that protective B-cell subpopulations might be less abundant. However, a meta-analysis of the effects of CD20-positive B-cell infiltration during allograft rejection revealed an increased risk of graft loss (37). In principle, immunoglobulins play a role in complement-mediated immune response and ABMR (38). In ABMR, donor-specific antibodies attack the graft. However, in zero-time biopsies, only donor B- and plasma cells can be detected in the kidney, which would only attack the kidney if autoantibodies were built. Therefore, the meaning of increased immunoglobulin expression remains unclear. Studies investigating gene expression using microarray analysis on biopsy samples with established rejection also observed significantly increased upregulation of immunoglobulins (39). Interestingly, the expression of immunoglobulin was not triggered by hypoxia but associated with prior ARF. Clinical studies reported that the adjusted relative risk for DGF was increased by severity of ARF in the pre-transplantation of the kidneys of donors (40) but was not associated with long-term graft failure (41). However, ARF in pre-transplant kidneys is a potential trigger for the expression of rejection-promoting genes. However, other inducers not yet known cannot be excluded.

In our study, transplant zero-time biopsies that later developed DGF showed comparable gene expression to controls or even a slight downregulation of transplantation-relevant genes, confirming similar observations reported by Hauser et al. (28). However, microarray analysis followed by unsupervised analysis clustered zero-time biopsies into 3 different groups, one of which with a significantly higher incidence of subsequent DGF (7). This indicates that differences in gene expression may also play a role in the development of DGF, but may not have been included in our NanoString array, which is limited to 770 genes. This study is limited by the small number of biopsies examined per subsequent transplant complication. The differences observed in gene expression between rejection and no rejection are relatively minor and the type of rejection in this study cannot be clearly predicted. The restriction to 770 pre-selected genes carries the risk that other genes, which are important for later rejection, will not be detected. Furthermore, the ratio of transplants from LDs to DDs differed between groups and was highest in the control group, while the ABMR and DGF groups exclusively contained DDs. However, also adjusting for donor type, significantly differentially expressed genes between rejection and no rejection group could be identified.

In conclusion, this pilot study clearly showed that gene expression analysis can be performed using standard protocol zero-time biopsies without the need to take an extra biopsy for this purpose. We suggest that the expression of inflammation-associated genes in the transplant, already determined at the time of donation, at least in part influences the transplant outcome, which is already determined at the time of donation. Furthermore, the upregulation of immunoglobulin genes in zero-time biopsies may indicate an increased risk for subsequent rejection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EV collected and analyzed the data, performed the experiments, and wrote the manuscript. SS analyzed the data and performed the experiments. MB-H collected and analyzed the data and edited the manuscript. KA collected data and edited the manuscript. EB edited the manuscript. JM-D and KH collected the clinical data. FF and MA analyzed the expression data and wrote the manuscript. CD initiated the study, collected and analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.793744/full#supplementary-material

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Archetypal Analysis of Injury in Kidney Transplant Biopsies Identifies Two Classes of Early AKI

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All transplanted kidneys are subjected to some degree of injury as a result of the donation-implantation process and various post-transplant stresses such as rejection. Because transplants are frequently biopsied, they present an opportunity to explore the full spectrum of kidney response-to-wounding from all causes. Defining parenchymal damage in transplanted organs is important for clinical management because it determines function and survival. In this study, we classified the scenarios associated with parenchymal injury in genome-wide microarray results from 1,526 kidney transplant indication biopsies collected during the INTERCOMEX study. We defined injury groups by using archetypal analysis (AA) of scores for gene sets and classifiers previously identified in various injury states. Six groups and their characteristics were defined in this population: No injury, minor injury, two classes of acute kidney injury ("AKI," AKI1, and AKI2), chronic kidney disease (CKD), and CKD combined with AKI. We compared the two classes of AKI, namely, AKI1 and AKI2. AKI1 had a poor function and increased parenchymal dedifferentiation but minimal response-to-injury and inflammation, instead having increased expression of PARD3, a gene previously characterized as being related to epithelial polarity and adherens junctions. In contrast, AKI2 had a poor function and increased response-to-injury, significant inflammation, and increased macrophage activity. In random forest analysis, the most important predictors of function (estimated glomerular filtration rate) and graft loss were injury-based molecular scores, not rejection scores. AKI1 and AKI2 differed in 3-year graft survival, with better survival in the AKI2 group. Thus, injury archetype analysis of injury-induced gene expression shows new heterogeneity in kidney response-to-wounding, revealing AKI1, a class of early transplants with a poor function but minimal inflammation or response to injury, a deviant response characterized as PC3, and an increased risk of failure. Given the

relationship between parenchymal injury and kidney survival, further characterization of the injury phenotypes in kidney transplants will be important for an improved understanding that could have implications for understanding native kidney diseases (ClinicalTrials.gov #NCT01299168).

Keywords: biopsy, kidney transplantation, injury, archetypes, gene expression

INTRODUCTION

Injury is universal in kidney transplants because of donation-implantation, presenting an opportunity to study the molecular characteristics associated with parenchymal damage. It is usually classified as acute kidney injury (AKI) or chronic kidney disease (CKD), but injury at the molecular level covers a wide spectrum of phenotypes. The emergence of the Molecular Microscope® Diagnostic System (1–5) for identifying rejection allows us to focus on understanding the injury component of gene expression independent of rejection. We recently analyzed injury-related features of kidney transplant biopsies as a spectrum, rather than dichotomizing between AKI and CKD (6). We performed principal component analysis (PCA) on microarray results from 1,526 indication biopsies, based on their expression of transcript sets and classifier scores associated with AKI- or CKD-related histology features, depressed estimated glomerular filtration rate (eGFR), and proteinuria. The resulting PC1 reflected no injury vs. injury, while PC2 reflected early AKI vs. late CKD. PC3 distinguished inflamed injury, including T cell-mediated rejection (TCMR; negative PC3) from uninflamed injury (positive PC3). High PC3 was increased in early AKI and CKD, and correlated with increased expression of an epithelial polarity and adherens junctions gene, PARD3, that is increased in AKI and CKD but decreased in TCMR.

This study aimed to describe the clinical classes of biopsies corresponding to these injury PC scores and understand the functional status and risk of progression, particularly in kidneys with no rejection. We used archetypal analysis (AA) to develop a classification of biopsies based on parenchymal injury features previously used for PCA (6). We studied genome-wide transcript expression measured by microarrays from 1,526 indication kidney transplant biopsies from the INTERCOMEX study (ClinicalTrials.gov #NCT01299168), following AA strategies previously used to categorize rejection-related phenotypes (3). We examined the relationships between injury archetype groups and time post-transplant, eGFR, proteinuria, rejection, histology lesions, and graft survival. Having previously defined each biopsy in terms of its molecular rejection status, our goal was to understand each kidney in terms of its parenchymal integrity and injury-induced phenotype—its response-to-wounding. This would allow all biopsies to be described both in terms of their rejection state and their injury state, and would give a complete molecular phenotype that relates to prognosis.

Abbreviations: AA, archetypal analysis; ABMR, antibody-mediated rejection; AKI, acute kidney injury; ATAGC, Alberta Transplant Applied Genomics Centre; ci>1Prob, classifier for fibrosis; CKD, chronic kidney disease; ct>1Prob, classifier for atrophy; DGF, delayed graft function; EABMR, early-stage ABMR; eGFR, estimated GFR; FABMR, fully-developed ABMR; IGTs, immunoglobulin transcripts; IRRAT, AKI transcripts; IRITD3, injury-repair-induced transcripts day 3; IRITD5, injury-repair-induced transcripts day 5; KT1, kidney parenchymal transcripts 1; KT2, kidney parenchymal transcripts 2; LABMR, late-stage ABMR; lowGFRProb, classifier for probability of eGFR $\leq$ 30; MCATs, mast cell transcripts; MMDx, Molecular Microscope® Diagnostic System; PBTs, pathogenesis-based transcript set; PCA, principal component analysis; PC1, principal component 1; PC2, principal component 2; PC3, principal component 3; ProtProb, classifier for probability of proteinuria; RF, random forest; TCMR, T cell-mediated rejection; Time post-transplant, time of biopsy post-transplant.

MATERIALS AND METHODS

The population and some methods were previously published (6).

Statistics

All analyses were done using the R programming language (7). Because classifier and archetype scores are frequently skewed, non-parametric tests were used where applicable, for example, Spearman's test for correlations and Wilcoxon's signed-rank test for comparing medians.

Study Population

As published (6), the 1,526 biopsies for clinical indications included in this study were obtained prospectively from established international centers (listed in **Supplementary Table 1**) with consent under local Institutional Review Board (IRB)-approved protocols (ClinicalTrials.gov NCT01299168). A portion (mean 3 mm) of one core was immediately stabilized in RNA*later*® and shipped to the Alberta Transplant Applied Genomics Centre (http://atagc.med.ualberta.ca) for processing. Gene expression was measured on Affymetrix PrimeView arrays unless the biopsy was inadequate for analysis [e.g., too small or RNA degraded: ∼4% of biopsies (5)]; 1,745 biopsies had enough RNA quality to run on microarray chips. Of these, we used 1,679 that had been assigned histological diagnoses.

Previous analyses (8) have indicated that biopsies with high medulla content sometimes have slightly altered molecular characteristics. For this reason, we removed the 153 biopsies we estimated to have <10% cortex [by measuring expression of the glomerulus-specific gene podocin (8)], leaving 1,526 biopsies for all analyses shown in this study. No additional inclusion/exclusion criteria were used. CEL files are available on the Gene Expression Omnibus website (GSE124203).

Demographics and histological findings have been described previously for the set of 1,679 biopsies (2). Demographics of the 1,526 indication biopsies from 1,280 patients are shown in **Supplementary Table 2** and histology and DSA in **Supplementary Table 3**.

Histology/Clinical Data

Proteinuria and delayed graft function (DGF) were defined as per the centers' standard-of-care, as were histological diagnoses following Banff guidelines (9, 10). Since there is no "AKI" category defined by histology, we took all biopsies not diagnosed with any specific disease or condition ("no major abnormalities") and classified them as "clinical AKI" if ≤6 weeks post-transplant, or "normal" if >6 weeks post-transplant.

Inputs for Injury AA

The molecular injury scores used as inputs have been published (6). Further details of the pathogenesis-based transcript sets (PBTs) for U219 arrays are provided at https://www.ualberta.ca/medicine/institutes-centres-groups/atagc/research/gene-lists.

Four classifiers were used to generate input scores for AA: $ci{>}1_{Prob}$ (ci-lesion score > 1 vs. ≤ 1) (11); $ct{>}1_{Prob}$ (ct-lesion score > 1 vs. ≤ 1) (11); $lowGFR_{Prob}$ (eGFR ≤30 vs. >30) (12); and $Prot_{Prob}$ (proteinuria positive vs. negative) (12). In all cases, 12 different classifier methods were trained [see (2) for details], and the median test set scores from the 12 used as the final estimate. All scores were based on the left-out sets in 10-fold cross-validation, that is, all scores were predicted from training set models that had no information whatsoever concerning the left-out test sets they were predicting. Our previous publication (3) used the same algorithmic methods but was based on the full set of 1,679 biopsies. For reasons explained above, all classifiers were rerun using the smaller 1,526 population for this study. All classifiers were implemented with functions from the R "caret" library (13).

We used eight PBTs as input and to interpret the results: six increased in injury, namely, damage-associated molecular pattern transcripts (DAMPs) (14, 15), AKI transcripts (IRRATs) (16), injury-repair-induced transcripts day 3 (IRITD3s) and injury-repair-induced transcripts day 5 (IRITD5s) (17), immunoglobulin transcripts (IGTs) (18), and mast cell transcripts (MCATs) (19); plus two parenchymal transcript sets characteristic of well-differentiated kidney tissue, namely, KT1 (which exclude solute carriers) and KT2 (solute carriers) (20) that are decreased in injury. A PBT score is calculated as the geometric mean of the fold change of all probe sets in the PBT vs. the mean expression of those probe sets in a defined control population, that is, the mean fold change across all probe sets. We use four nephrectomy samples as our controls.

Injury AA

Our use of AA for rejection has been published (3). AA (21) finds a small number of hypothetical archetypes that represent extreme "phenotypes" within a data set. The number of archetype clusters chosen is largely subjective. We examined models using between two and seven clusters and chose six clusters based on what we believe produced the most informative and interpretable categorization. Each sample is assigned scores for each of the six clusters, which sum to 1.0. By convention, each sample is assigned to a group ("cluster") based on the highest of its scores.

Visualization of Archetype Cluster Distributions

The output from the AA was six archetype scores for each of the 1,526 biopsies. This cannot easily be visualized without dimensionality reduction. It is conventional to use PCA to assign the biopsies in two- or three-dimensional space, and then color the biopsy symbols using the archetype cluster assignment [e.g., (22, 23)]. We follow this convention using the same 1,526 × 12 data matrix as input for both the PCA and AA.

Moving-Average Plots

For all plots showing moving averages, the data were first sorted by ascending order of the x-axis variable, for example, time post-transplant. The mean of the x and y variables of the (ordered) biopsies 1-400 were then calculated and plotted. The sliding window was then incremented to biopsies 2-401, the means recalculated and plotted, and so on. Generally, these data had a great deal of scatter, and the sliding window approach was used to see general trends in the data, as are regression lines in standard linear regression.

Survival Analysis

Three-year post-biopsy survival was analyzed using one randomly selected biopsy per patient. We used random forests (RFs), as implemented in the "randomForestSRC" package (24). RF is less sensitive to multicollinearity problems than is Cox regression and is also able to model interaction effects between predictors to some extent. Ten thousand trees were grown for each analysis using the nsplit = 1 parameter.

RESULTS

Injury AA

Figure 1 shows the PCA distribution of biopsies described previously (6): **Figure 1A**, PC2 vs. 1 and **Figure 1B**, PC2 vs. 3. PC1 represents all molecular injury vs. uninjured tissue. PC2 separates AKI (negative PC2) from CKD (positive PC2). PC3 is a new dimension that separates uninflamed injury with high expression of some unusual epithelial transcripts such as PARD3 (positive PC3) from inflamed injury (negative PC3).

In **Figure 1**, biopsies are colored by their membership in the six injury archetype groups. As described below, these are completely different from the rejection archetype groups (3). The large symbols numbered 1–6 indicate the location of the actual archetype (theoretical idealized phenotype). Assignment of biopsies to archetype groups should be thought of as the "most likely" cluster based on their location in multivariate molecular space, rather than as a definitive classification system. As such, the injury archetypes are interpretable as "clinical scenarios." Based on the results outlined below, we assigned provisional descriptive labels based on clinical and molecular characteristics to each of the six injury clusters, namely, no injury, minor injury, AKI1, AKI2, CKD, and CKD/AKI. We introduced the names here for clarity.

Notably, the AKI1 differs from AKI2 in all three PC dimensions, namely, less PC1, is shifted more positively in PC2, but strongly positive PC3.

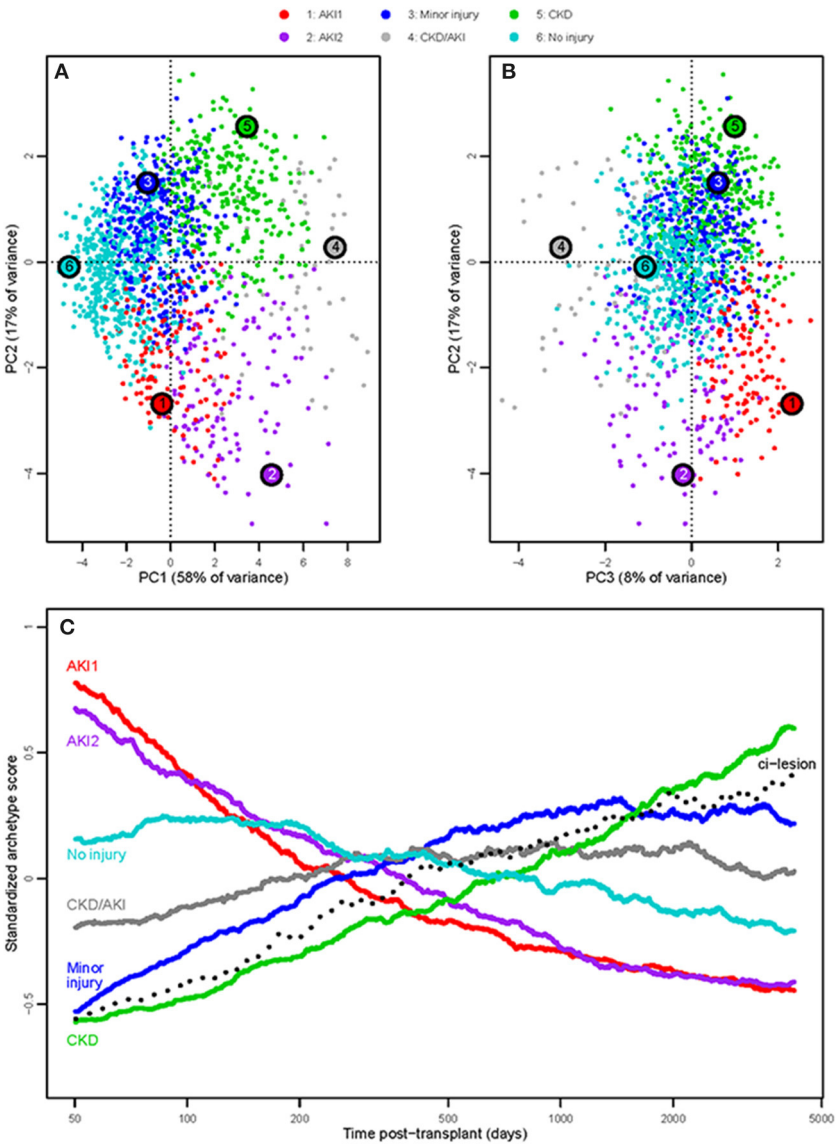

FIGURE 1 | Injury-based PCA, colored by injury archetype cluster. **(A)** PC2 vs. PC1 scores for each biopsy; **(B)** PC2 vs. PC3 scores; **(C)** moving averages of standardized injury archetype scores (window size = 400 biopsies). As there are large differences in mean scores between archetypes, all scores were standardized to a mean of 0.0 before plotting. The y-axis is in standard deviation units. Biopsies sorted by ascending time of biopsy post-transplant.

Distribution of the Injury AA Scores Over Time Post-transplant

Figure 1C shows the moving averages of the six archetype scores vs. time post-transplant.

The AKI1 and AKI2 scores were highest immediately post-transplant then declined steadily. CKD scores rose steadily, similar to the rise in histological atrophy-scoring scores reflecting the cumulative burden of injury (donation-implantation and later injuries). The minor injury score rose with time, somewhat like CKD, but plateaued late. Histological fibrosis, shown here as a dotted line, rose steadily with time as previously reported, paralleling the CKD score (4).

The no injury score peaked at 3–7 months (as AKI changes recovered), then declined slowly over time as expected in an indication biopsy population as CKD increased.

Clinical Features of the Injury AA Groups

The clinical characteristics of the six archetype groups are summarized in **Table 1**. The no injury group is the most normal (least amount of injury) and can serve as a control.

Groups AKI1 (N = 127) and AKI2 (N = 130) were both relatively early (median 40 and 51 days post-transplant). The CKD group (N = 310) was the latest post-transplant (2,102 days). The no injury group was earlier on average than minor injury (365 vs. 1,070 days).

TABLE 1 | Summary of clinical features of the injury archetype groups.

		AKI1	AKI2	Minor injury	CKD/AKI	CKD	No injury
	N	127	130	377	55	310	527
Clinical variables for all biopsies	Median time of biopsy post-transplant (mean)	***40 (181)***	51 (157)	1,070 (1,875)	867 (1,670)	**2,102 (2,674)**	365 (1,088)
	Mean eGFR (cc/min/m^2)	***22***	27	52	32	35	**53**
	Mean donor age (median)	**50 (54)**	46 (48)	***41 (42)***	46 (48)	45 (46)	43 (44)
	% of column with donor age >50 years	**63%**	43%	***32%***	45%	41%	33%
	Deceased donor (%)	**89%**[a]	65%	67%	73%	73%	***60%***
Clinical variables for biopsies with no molecular rejection	Median time of biopsy post-transplant (mean)	32 (177)	***19 (81)***	1,050 (1,929)	1,221 (1,412)	**2,510 (2,890)**	309 (1,089)
	Mean eGFR (cc/min/m^2)	***21***	26	**54**	48	35	52
	Mean donor age (median)	**50 (54)**	50 (52)	***43 (43)***	44 (40)	47 (50)	44 (45)
	% of column with donor age >50 years	**62%**	59%	***33%***	40%	51%	36%
	Deceased donor (%)	89%[b]	74%	70%	**100%**	75%	***59%***

The highest in each row is bolded and shaded. The lowest in each row is bolded and italicized.

[a] *Fisher's exact test between AKI1 vs. AKI2 $p < 0.001$.*

[b] *Fisher's exact test between AKI1 vs. AKI2 $p = 0.02$.*

Both AKI1 and AKI2 had low eGFR (22 and 27, respectively). The small CKD/AKI group ($N = 55$) had a low mean eGFR (32). The no injury group ($N = 527$) had a relatively normal eGFR (53), as did the minor injury group (52).

The lowest donor age and % donors >50 was in the no injury and minor injury groups. More AKI1 biopsies were from donors >50 years of age (63 vs. 43%), but this difference disappeared when only the biopsies without rejection were considered; both groups had many kidneys from older donors. Aging may be operating in both AKI groups as expected; older donor kidney results in more early dysfunction.

The AKI1 group had the most deceased donors and the no injury group had the least.

AKI1 was also strongly associated with deceased donors compared to AKI2 and the other groups, particularly the no injury group.

Thus, injury groups assigned exclusively by the molecules and machine learning have strong clinical associations. Most of these results were similar when all rejection was excluded and are thus relatively independent of the rejection states of these biopsies.

Transcript Set Scores in the Injury AA Groups

In **Table 2**, the injury archetype groups AKI1 and AKI2 had many of the features expected in molecular AKI compared to the no injury, such as recent injury transcripts, dedifferentiation, macrophage transcripts, and injury PC1 (the degree of global injury). However, AKI1 differed markedly in many scores from AKI2.

Compared to no injury, the AKI1 and AKI2 groups had increased expression of recent injury-induced transcript sets, but AKI1 was always lower than AKI2.

Transcript sets increased in atrophy-fibrosis (reflecting plasma cell, mast cell, and B cell infiltration) were very low in AKI1 compared to AKI2 and all other groups, even the no injury group.

Dedifferentiation, loss of normal kidney parenchymal transcripts (KT1), was less in AK1 than in AKI2.

Macrophage transcripts were increased in AKI1 and AKI2 but were less in AKI1 than AKI2.

Compared to AKI2, AKI1 had lower injury PC1 scores and less negative injury PC2 scores, indicating less response-to-wounding in AKI1 than AKI2. But AKI1 had a strong positive injury PC3 score, whereas AKI2 had a negative PC3 score, indicating a deviant response in AKI1 vs. AKI2.

AKI1 biopsies did have injury-induced features when compared to no rejection biopsies, specifically a response-to-wounding. However, these injury features in AKI2 were more distinct.

The CKD and CKD/AKI groups had elevated expression of a recent injury and atrophy-fibrosis-related transcripts, and CKD/AKI had the highest expression of macrophage transcripts and the greatest loss of parenchymal transcripts (KT1). PC1 was highest in CKD/AKI and PC2 was highest in CKD.

These results were similar when all rejection was excluded (**Supplementary Table 4**).

Molecular Rejection in the Injury Archetype Groups

AKI1 had the fewest molecular TCMR diagnoses of any group (1/127, <1%), even less than in the no injury group (**Table 3**). Only 14% of AKI1 had any rejection, mostly early-stage antibody-mediated rejection (EABMR). In contrast, the AKI2 biopsies had 39% TCMR and 57% rejection overall. The small group 4

TABLE 2 | Mean scores for AKI and CKD-related pathogenesis-based transcript sets (PBTs) in injury archetype groups ($N = 1,526$).

Biological processes	Mean transcript set and classifier score[a] in biopsies grouped by highest archetype score	AKI1	AKI2	Minor injury	CKD/AKI	CKD	No injury
		(*N* = 127)	(*N* = 130)	(*N* = 377)	(*N* = 55)	(*N* = 310)	(*N* = 527)
PBTs increased by recent injury	AKI transcripts (IRRATs)	1.70	**2.43**[c]	1.16	2.26	1.72	***0.95***
	IRITD3	1.09	1.20[c]	1.02	**1.21**	1.12	***0.96***
	IRITD5	1.28	1.58[c]	1.26	**1.72**	1.42	***1.15***
PBTs increased in atrophy-fibrosis	IGTs	***0.87***	1.37[c]	2.59	**6.47**	3.56	1.38
	MCATs	***1.10***	1.47[c]	2.80	**5.36**	5.26	1.46
	BATs[b]	***1.03***	1.17[c]	1.16	**1.67**	1.27	1.07
Parenchymal transcript PBTs decreased by injury	KT1	0.85[c]	0.68	0.90	***0.47***	0.78	**0.93**
Macrophage infiltration PBTs	QCMATs[b]	1.26	2.24[c]	1.40	**2.44**	1.50	***1.19***
	AMATs[b]	1.37	2.43[c]	1.52	**2.69**	1.74	***1.24***
	Injury PC1	−0.07	2.6[c]	−0.61	**5.68**	2.47	***−2.26***
	Injury PC2	*−1.81*	***−2.35***[c]	0.56	−0.21	**1.29**	−0.12
	Injury PC3	**1.26**[c]	−0.39	0.21	***−1.98***	0.49	−0.44

The highest in each row is bolded and shaded. The lowest in each row is bolded and italicized.

[a] *The gene sets were derived in human cell lines, human transplants, and mouse models to reflect biological processes relevant to rejection and injury.*

[b] *These were the transcript sets or classifiers not used in the Injury AA analysis.*

[c] *t-test of AKI1 vs. AKI2 $p < 0.001$.*

AMAT, alternative macrophage associated transcripts 1; BATs, B cell-associated transcripts; DAMPs, damage-associated molecular pattern transcripts; IGTs, immunoglobulin transcripts; IRITD3, injury-repair-induced transcripts day 3; IRITD5, injury-repair-induced transcripts day 5; IRRAT, AKI transcripts; KT1, kidney parenchymal transcripts 1; KT2, kidney parenchymal transcripts 2; MCATs, mast cell transcripts; QCMAT, quantitative constitutive macrophage-associated transcripts.

CKD/AKI ($N = 55$) had 87% rejection. Rejection was present in about half of minor injury and CKD biopsies, and 18% of biopsies with no injury, mostly EABMR.

Histological diagnoses in the injury archetype groups are presented in **Table 4**. In general, the pattern was in agreement with the molecular rejection groups. There were few diagnoses of rejection in the AKI1 group, with most AKI1 assessed as relatively normal or mild atrophy-fibrosis [this is consistent with our previous finding that molecular AKI changes that correlate with eGFR loss are not consistently detectable by histology (16)].

Relationship of Injury and Rejection AA Scores to eGFR

In **Figure 2A**, we used RFs to examine the relative importance of molecular injury archetype scores and rejection archetype scores (3) for predicting disturbed function (eGFR ≤ 30). Injury scores were strongly predictive of poor function, while rejection scores were not.

We also used random forests to assess the relative importance of molecular rejection and injury AA scores in terms of predicting graft failure within 3 years of biopsy (**Figure 2B**). Injury scores were more important than rejection scores alone (error rate 0.35, data not shown). These findings using injury archetypes are consistent with our previous analyses using other injury measurements (12).

The actuarial survival curves for the six injury archetype groups are shown in **Figure 2C** (3-year death censored graft survival after biopsy). The poorest survival was in CKD and CKD/AKI, but survival was also poor in AKI1, even though this group had less rejection. The best survival was in the no injury followed by the minor injury group.

Analysis of Early (≤6 Weeks) Non-rejecting Biopsies

We selected all early biopsies ≤6 weeks post-transplant that had also been designated "no rejection" by the rejection archetype model (3) ($N = 171$ of 201 early biopsies shown in **Table 5**), permitting us to study a pure set of early biopsies with no rejection.

By injury archetype assignments, these 171 biopsies included 58 AKI1, 41 AKI2, and 64 no injury. Eight were in other injury archetype clusters.

In these biopsies, AKI1 and AKI2 were similar in mean time post-transplant, % donor age>50, renal function, and rates of DGF. AKI1 had a higher fraction of deceased donors (93 vs. 74%).

AKI had a higher expression of the top PC3 gene, PARD1; AKI2 had a higher expression of the top PC1 gene, ANXA2, and of all PBTs increased by recent injury and more loss of parenchymal transcripts.

Interstitial fibrosis was low in both (although higher in AKI1), and interstitial inflammation was less in AKI than AKI2.

The rate of graft loss by 3 years was higher in AKI1 kidneys (26%) than in AKI2 kidneys (8%).

TABLE 3 | Distribution of molecular rejection diagnoses in injury archetype groups.

Rejection archetype groups	Number of biopsies in each injury archetype group (% of column total)						
	AKI1	**AKI2**	**Minor**	**CKD/AKI**	**CKD**	**No-injury**	**Total**
Early-stage ABMR (EABMR)	12	11	73	*1*	39	49	185
Fully-developed ABMR (FABMR)	3	*5*	76	6	43	17	150
Late-stage ABMR (LABMR)	2	7	23	5	30	*8*	*75*
TCMR (TCMR1+TCMR2)	*1*	51	*20*	36	40	23	171
% of column with rejection[a] (number)	***14%* (18)**	57% (74)	51% (192)	**87% (48)**	49% (152)	18% (97)	38% (581)
% of column with no rejection[a] (number)	**84% (109)**	43% (56)	49% (185)	***13%* (7)**	49% (158)	82% (430)	62% (945)
Total	127	130	377	55	310	527	1,526

[a] *The highest % in these rows is bolded and shaded. The lowest is bolded and italicized. The lowest is bolded and italicized.*

TABLE 4 | Distribution of histological diagnoses in the injury archetype groups (grouped by highest injury score) ($N = 1,526$).

Histology diagnosis ($N = 1,526$)			# of biopsies in each injury archetype group (%of column total)						Total
			AKI1	**AKI2**	**Minor**	**CKD/AKI**	**CKD**	**No-injury**	
Total			127	130	377	55	310	527	1,526
# Rejection $N = 708$ (36%)	**ABMR-related**	ABMR	11	14	**114**	***7***	78	65	289
		Transplant Glomerulopathy (TG)	***0***	***0***	**21**	2	17	6	46
		ABMR suspected	2	2	**10**	***1***	7	7	29
		Mixed	***0***	6	**18**	10	15	6	55
	TCMR-related	TCMR	***7***	**32**	21	13	19	**32**	124
		BK	***1***	10	6	6	8	**14**	45
		Borderline	10	14	23	***3***	17	**53**	120
	All rejection-related (% of column)		***31 (24%)***	78 (60%)	**213 (56%)**	42 (76%)	161 (52%)	183 (35%)	708 (46%)
# No rejection $N = 818$ (64%)	No major histologic abnormalities ("Normal")		65	30	73	***3***	41	233	**445**
	Diabetic Nephropathy		1	***0***	7	***0***	**11**	4	23
	Glomerulonephritis		3	***1***	**37**	***1***	27	**37**	106
	IFTA-no other disease		18	12	36	***8***	**52**	49	175
	Other		9	9	11	**1**	18	**21**	69
	All with no rejection (% of column)		96 (76%)	52 (40%)	164 (44%)	***13 (24%)***	149 (48%)	**344 (65%)**	818 (54%)
Histology scores	Interstitial fibrosis (mean ci)		0.97	0.90	1.26	1.90	**2.01**	***0.81***	1.24
	Tubular atrophy (mean ct)		0.82	0.69	1.13	1.78	**1.84**	***0.77***	1.12

The highest value per row is bold with shading; the lowest is bolded and italicized.

The striking differences between early biopsies with AKI1 vs. AKI2 are that AKI1, despite a very low eGFR, has less molecular injury change, less inflammation, less parenchymal dedifferentiation but instead has increased PC3 and increased expression of the top PC3-correlated gene, PARD3.

Relationships Between AA Injury Groups and Graft Survival in Biopsies With No Rejection

Biopsies with no molecular rejection were grouped by AA assignment, and 3-year survival probability was assessed for each group (**Figure 3**) (after removing rejection, the CKD/AKI group was too small for reliable survival estimates).

In all biopsies, the CKD and AKI1 groups had the worst survival probability, while the no injury group had the best prognosis (**Figure 3A**).

When only biopsies within 42 days post-transplant were considered, AKI1 displayed impaired survival probabilities (**Figure 3B**) (there was no CKD).

When biopsies later than 42 days post-transplant were considered, AKI1, AKI2, and CKD all had reduced survival probability (**Figure 3C**).

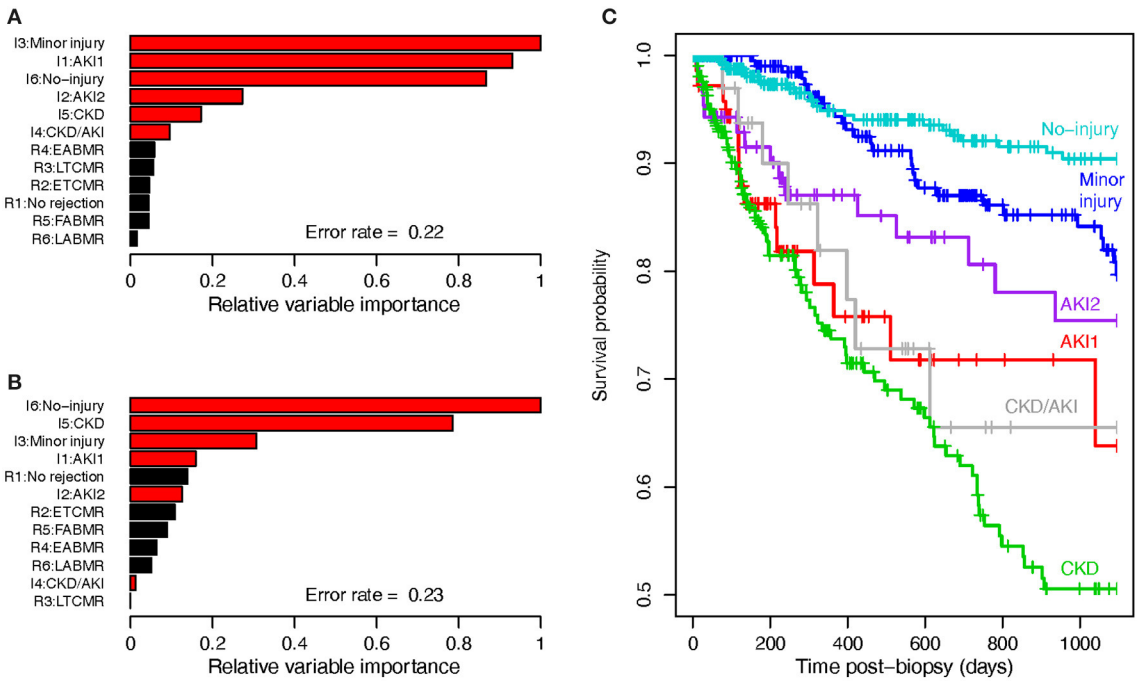

FIGURE 2 | Graft survival analyses. **(A)** Random forests assessing variable importance in predicting low eGFR (eGFR < 30). **(B)** Random forests assessing variable importance in predicting 3-year death-censored graft survival. **(C)** Kaplan-Meier curves showing 3-year post-biopsy actuarial survival in the six AA injury archetype groups.

Thus, AKI1 with no rejection had impaired short-term outcomes despite (or possibly because of) the relative lack of typical AKI changes and inflammation.

DISCUSSION

This study was designed to establish a molecular classification of parenchymal injury-related scenarios that extended beyond simply designating AKI and CKD and define the relationship of these new groups to histology, rejection, function, and survival. Having previously used molecular rejection features measured in genome-wide microarrays to classify rejection (2), we now extended this approach to describe the injury-related classes, and define both the "injury-ness" and the "rejection-ness" features of every biopsy. We explored injury scenarios in 1,526 kidney transplant indication biopsies, taken between 1 day and 33 years post-transplant. We assessed injury using 12 predefined scores, including transcripts induced in AKI and CKD, transcripts lost with kidney injury, and injury-related classifiers reflecting atrophy, fibrosis, low eGFR, and proteinuria as previously used for our injury PCA (6). AA produced six scores per biopsy, and clusters were assigned based on the highest score of the six. The six clusters included two groups with early injury, AKI1 and AKI2, with severe dysfunction and frequent DGF; CKD, CKD/AKI, minor, and no injury. AKI1 and AKI2 differed in that AKI1 had lower expression of the usual AKI-induced genes such as ANXA2, little inflammation, and virtually no TCMR. Compared to AKI2, AKI1 also had lower injury-induced transcripts (e.g., IRRATs), lower macrophage transcripts, and less parenchymal dedifferentiation. However, AKI1 had high PC3 and related gene PARD3. In other words, AKI1 had less evidence of the conventional response-to-wounding than AKI2 despite severely impaired function and instead had an alternative or deviant response, PC3. The best predictors of disturbed function (eGFR $\leq$ 30) and graft loss were injury archetypes, not rejection archetypes. High rates of failure occurred in CKD and AKI1 even when rejection was excluded (4). We conclude that it is important to recognize the diversity in injury classes when interpreting kidney transplant biopsies. Assessing injury phenotypes provides novel insights into changes that are largely silent in histology (16) and profoundly affect function and prognosis.

A comparison of the 127 AKI1 biopsies to the 130 AKI2 biopsies revealed previously unknown heterogeneity in early kidney transplant biopsies, particularly in those from deceased donors, and invited a specific examination of the early biopsies before 6 weeks post-transplant. Removing biopsies with rejection from the early ($\leq$6 week) cohort showed diversity in the phenotype of early kidney transplant dysfunction independent of rejection. AKI1 with no rejection still had severe dysfunction, abundant DGF, but virtually no inflammation.

Although increased AKI1 and AKI2 scores were both common with donor age >50, we remain concerned that preexisting somatic cell senescence mechanisms could be playing a role in AKI1, processes that are not readily assessed in genome-wide biopsy studies. Aging and senescence

TABLE 5 | Distinct phenotype in early biopsies (≤6 weeks post-transplant) with no molecular rejection[a] (*N* = 171).

Variable		Injury archetype			
Mean scores in:		**AKI1 (*N* = 58)**	**AKI2 (*N* = 41)**	**No-injury (*N* = 64)**	**Others (*N* = 8)[d]**
Time of biopsy post-transplant		**17**	**16**	21	28
Fraction donors >50 years of age		**28/48 (58%)**	**20/36 (56%)**	*25/59 (42%)*	5/7 (71%)
Fraction deceased donors (%)		**93%**	**74%**	*46%*	63%
PARD3 (top PC3 increased gene)		**268[c]**	**233**	*177*	178
ANXA2 (top PC1 increased gene)		**1,074[b]**	**1,551**	*810*	1,154
PBTs increased by recent injury	IRRAT	**1.79[b]**	**2.63**	*1.05*	1.52
	IRITD3	**1.10[b]**	**1.24**	*0.98*	1.11
	IRITD5	**1.26[b]**	**1.56**	*1.21*	1.48
PBTs decreased in injury	KT1	**0.85[b]**	**0.71**	0.94	0.81
Histology lesions	Interstitial fibrosis (Banff ci-score)	**0.77**	**0.58**	0.69	*0.25*
	Interstitial infiltrate (Banff i-score)	**0.16[c]**	**0.56**	0.41	*0.00*
	Total inflammation (Banff ti-score)	**0.11[c]**	**0.57**	0.69	*0.00*
Renal function	Creatinine at biopsy median (mmol/L)	**431**	**405**	*165*	186
	eGFR at biopsy median (cc/min/m^2)	**16.0**	**15.5**	45.0	45.0
Fraction failed by 3 years post biopsy (death censored)		**12/46 (26%)[e]**	**3/39 (8%)**	4/59 (7%)	*0/7 (0%)*
Fraction designated delayed graft function (DGF) (%)		**31/58 (53%)**	**19/41 (46%)**	*7/64 (11%)*	1/8 (13%)

[a] *Defined as molecular rejection archetype 1 (no rejection).*
[b] *p-value < 0.001 based on t-test between AKI1 and AKI2.*
[c] *p-value < 0.05 based on t-test between AKI1 and AKI2a.*
[d] *Others include minor, CKD/AKI, and CKD groups.*
[e] *p-value < 0.05 based on Fisher's exact test between AKI1 and AKI2.*

are not necessarily predicted by calendar age, and the possibility remains that aging/senescence processes were more advanced in kidneys that developed AKI1 after donation, almost always from deceased donors. The PC3-related changes in AKI1 such as increased PARD3 remind us that AKI1 is not only deficient in the usual AKI-induced response-to-wounding but also deviates toward other PC3-related characteristics.

Molecular injury measurements are critical in understanding functional disturbance and outcomes because wounding is an intermediate phenotype that integrates the total burden of parenchymal damage from donation-implantation, rejection, recurrent disease, BK, other insults, and advancing biological aging. **Figure 4** represents the potential links between these sources of parenchymal injury and eventual organ failure. The injury phenotypes themselves are a final common pathway to be distinguished from the upstream injury-inducing mechanisms and diseases that are the critical targets of treatment. Perhaps treatments of the injured tissue itself will eventually emerge, possibly targeting some of the key molecules induced by injury. The sources of injury should be avoided or promptly treated if possible, but they are not always identified, and their effects may linger after apparently successful treatment, for example, after successful treatment of TCMR. Thus, injury phenotypes can be misinterpreted as autonomous when the cause of injury is either not detected or no longer operating. Moreover, badly injured tissue may also progress autonomously at some stage, for example, nephron loss may progress autonomously due to podocyte loss (25).

Rejection is a major source of parenchymal injury and has complex relationships with injury phenotypes because TCMR and ABMR have different effects on the parenchyma. TCMR is an interstitial process almost always associated with parenchymal injury. The AKI1 phenotype virtually excludes TCMR. By contrast, EABMR is usually associated with minimal injury because EABMR is a glomerular/microcirculation disease that usually has little initial impact on parenchymal function. In ABMR, nephrons do not usually drop out until the glomeruli deteriorate with double contours (FABMR), beginning the development of CKD. There are two pathways from rejection to parenchymal deterioration: direct, as in TCMR, and indirect through glomerular damage and eventual nephron shutdown, as in ABMR. The latter may be relevant to primary glomerular diseases such as diabetic nephropathy and glomerulonephritis, where nephrons are spared until the glomerular changes are advanced.

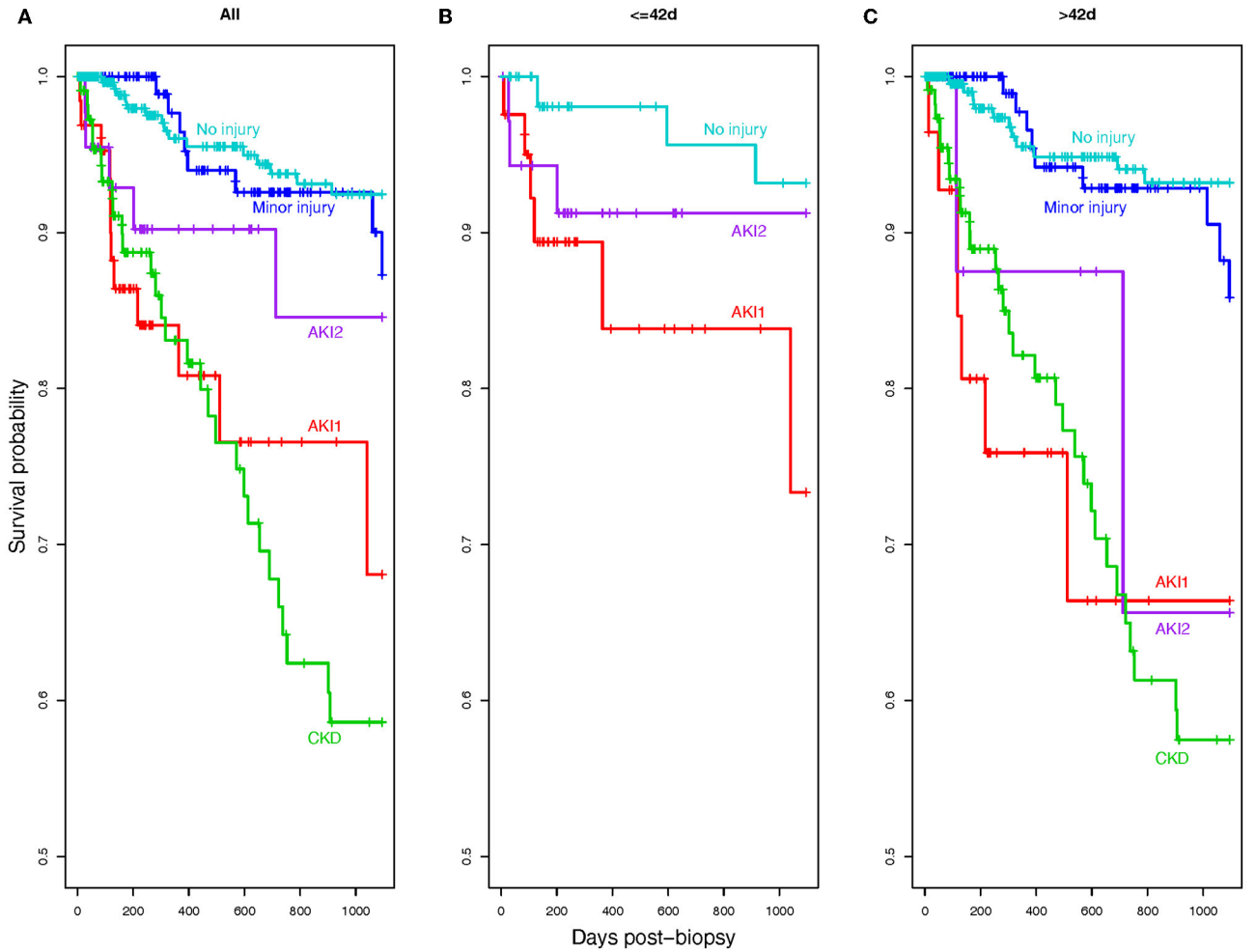

FIGURE 3 | Kaplan-Meier curves showing actuarial 3-year death-censored graft survival in the injury AA groups after excluding all biopsies with rejection. **(A)** Injury AA groups were compared across time post-biopsy. We also examined the differences between, **(B)** short-term (≤42 days post-biopsy), and **(C)** long-term (>42 days post-biopsy) survival probabilities. The CKD/AKI group was removed from these plots due to the very small group size and scarcity of events.

Distinguishing between AKI1 and AKI2 may be useful in the management, given that AKI2 changes seem to predict recovery better than AKI1 changes, but recognizing such heterogeneity could be particularly useful in evaluating injury prevention and treatment strategies. We anticipate that interventions directed at typical AKI-related changes that are prominent in AKI2 may be less successful in AKI1. This heterogeneity within AKI may help us to understand why treatments for AKI have met with little success, as well as distinguish those patients who are less likely to recover.

The strengths of this analysis include the large unselected study population from multiple centers with detailed phenotyping sampled over a wide range of time post-transplant. However, the restriction to indication biopsies in IRB protocols imposes limitations in that we do not know the natural history of the molecular changes in individual kidneys. To some extent, intrastudy comparisons such as AKI1 vs. AKI2, and the use of the no injury group as an internal control offset these limitations and allow us to see the natural history of the population. Also, the risk predictions in the present study exclusively use molecular features, but incorporating major clinical variables such as eGFR and proteinuria and histology atrophy-fibrosis lesions may improve risk predictions (12).

The injury-induced changes in kidney transplants (separated from rejection processes) have lessons for native kidney diseases in general, in that primary diseases drive injury but the injury phenotypes based on the parenchymal state are the final common pathway determining function and prognosis. AKI and CKD are a useful dichotomy for epidemiological analysis (26), but the molecular states are a spectrum based on continuous numbers and reveal new classes that are clinically important such as the uninflamed but high-risk AKI1 group of damaged kidneys, many from older donors. In this sense, the richness of biopsies, data, and phenotypes available in the kidney transplant population provides potentially useful insights for native kidney disease studies.

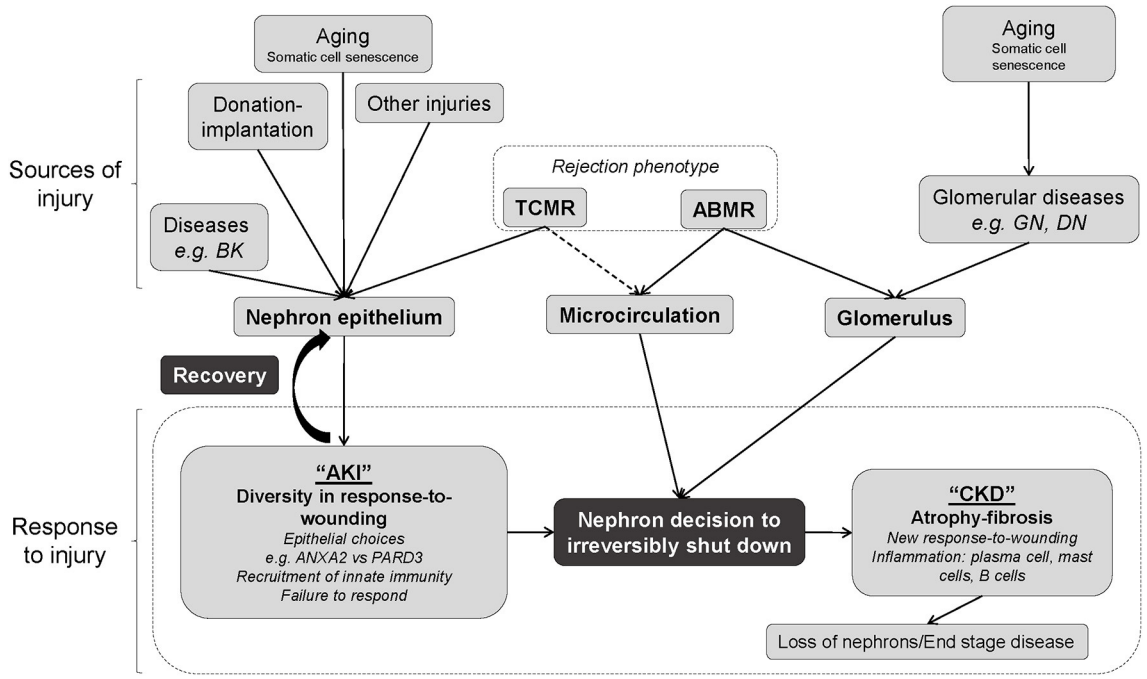

FIGURE 4 | Schematic diagram representing the relationships between sources of injury and response to injury in kidney transplant biopsies based on these analyses. Interplay between sources of injury, pre-existing limitations such as aging, and response to injury by the nephron. There are two routes to irreversible nephron shutdown, namely, the epithelial injury and through glomerulus injury. Epithelial injury should trigger the response-to-wounding, which involves epithelium, matrix, and microcirculation, and evokes innate immunity. Failure to mount a response to wounding and adopting a "PC3"-related response (e.g., PARD3) with minimal inflammation leads to failure to recover. Many sources of injury (separate from and including rejection) interact with the nephron epithelium, producing acute kidney injury (AKI). In this instance, the epithelium can be repaired and the organ can recover, or progress to nephron failure. Alternatively, aging and/or ABMR can contribute to glomerular disease and ABMR can additionally affect the microcirculation, affecting the glomerulus and again causing nephron shutdown, which eventually leads to chronic kidney disease (CKD). If this occurs, a loss of nephrons and end-stage renal disease may occur. Different sources of injury may interact to cause many forms of injury, and injury itself predicts the graft survival while the rejection status does not. Thus, defining the heterogeneity within biopsy injury is an important part of clinical management.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, GSE124203.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Established International Centers (listed in **Supplementary Table 1**) with consent under local IRB-approved protocols (ClinicalTrials.gov NCT01299168). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PH and KM-T collected, analyzed, interpreted the data, and prepared the manuscript. GB, JB, GE, FE, GG, MM, OV, and AP-P interpreted the data and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.817324/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Early Estimated Glomerular Filtration Rate Trajectories After Kidney Transplant Biopsy as a Surrogate Endpoint for Graft Survival in Late Antibody-Mediated Rejection

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Background: Late antibody-mediated rejection (ABMR) after kidney transplantation is a major cause of long-term allograft loss with currently no proven treatment strategy. Design for trials testing treatment for late ABMR poses a major challenge as hard clinical endpoints require large sample sizes. We performed a retrospective cohort study applying commonly used selection criteria to evaluate the slope of the estimated glomerular filtration rate (eGFR) within an early and short timeframe after biopsy as a surrogate of future allograft loss for clinical trials addressing late ABMR.

Methods: Study subjects were identified upon screening of the Vienna transplant biopsy database. Main inclusion criteria were (i) a solitary kidney transplant between 2000 and 2013, (ii) diagnosis of ABMR according to the Banff 2015 scheme at >12 months post-transplantation, (iii) age 15–75 years at ABMR diagnosis, (iv) an eGFR > 25 mL/min/1.73 m^2 at ABMR diagnosis, and (v) a follow-up for at least 36 months after ABMR diagnosis. The primary outcome variable was death-censored graft survival. A mixed effects model with linear splines was used for eGFR slope modeling and association of graft failure and eGFR slope was assessed applying a multivariate competing risk analysis with landmarks set at 12 and 24 months after index biopsy.

Results: A total of 70 allografts from 68 patients were included. An eGFR loss of 1 ml/min/1.73 m^2 per year significantly increased the risk for allograft failure, when eGFR slopes were modeled over 12 months [HR 1.1 (95% CI: 1.01–1.3), $p = 0.020$] or over 24 months [HR 1.3 (95% CI: 1.1–1.4), $p = 0.001$] after diagnosis of ABMR with landmarks set at both time points. Covariables influencing graft loss in all models were histologic evidence of glomerulonephritis concurring with ABMR as well as the administration of anti-thymocyte globulin (ATG) at the time of transplantation.

Conclusion: Our study supports the use of the eGFR slope modeled for at least 12 months after biopsy-proven diagnosis of late ABMR, as a surrogate parameter for future allograft loss. The simultaneous occurrence of glomerulonephritis together with ABMR at index biopsy and the use of ATG at the time of transplantation–likely representing a confounder in pre-sensitized recipients–were strongly associated with worse transplant outcomes.

Keywords: surrogate end point validation, antibody-mediated allograft rejection, landmark analysis, donor-specific anti HLA antibodies, allograft loss, estimated glomerular filtration rate (eGFR), fine and gray model

INTRODUCTION

Late antibody-mediated rejection (ABMR) after kidney transplantation is a major cause of long-term allograft loss and a difficult-to-treat disease entity, since its detection is frequently hampered by a clinically indolent onset, even in the presence of meanwhile often established routine longitudinal donor-specific antibody (DSA) testing and protocol biopsy programs (1, 2). This may result in irreversible chronic glomerular damage and fibrosis consistent with chronic ABMR (cABMR) at the time of diagnosis (3). Furthermore, treatment strategies are limited to date and especially in the presence of cABMR, no therapeutic agent has been shown to interfere with the course of kidney functional decline and allograft loss rates when compared to the standard of care i.e., the optimization of maintenance immunosuppression (2, 4). Recently, promising treatment strategies such as interleukin-6 (IL-6) blockade with monoclonal antibodies tocilizumab or clazakizumab were tested in small clinical trials and currently clazakizumab undergoes intense investigation in the up-to-date largest phase III study ever conducted in patients with ABMR (IMAGINE trial, NCT03744910) (5, 6).

One of the major hurdles for the design of such a trial is the difficulty of defining a valid and feasible endpoint (7). The gold standard of demonstrating improvement in graft survival was shown to be an unrealistic endpoint in trials addressing late ABMR, since in general the overall number of eligible patients with a potentially reversible disease course is low and the timespan from diagnosis until graft loss does often last over many years (8). This would therefore require large sample-sizes, only realizable when embedded within international multicenter trials (9). The definition of adequate surrogate endpoints has therefore been proposed and the use of the slope of estimated glomerular filtration rate (eGFR) is widely accepted as such a measure, next to other upcoming promising novel endpoints (7, 9–12).

Abbreviations: ABMR, antibody-mediated rejection; ATG, anti-thymocyte globulin; Bx, biopsy; CD, cluster of differentiation; CDC, complement-dependent cytotoxicity; CI, confidence interval; CNI, calcineurin inhibitor; DSA, donor-specific antibody; EDTA, ethylenediaminetetraacetic acid; eGFR, estimated glomerular filtration rate; GN, glomerulonephritis; HLA, human leukocyte antigen; IA, immunoadsorption; iBx, index biopsy; IL, interleukin; IQR, interquartile range; IVIG, intravenous immunoglobulin; LME, linear mixed effects; MDRD, modification of diet in renal disease; MFI, mean fluorescence intensity; MMF/MPA, mycophenolate-mofetil/mycophenolic acid; mTORi, inhibitor of mammalian target of rapamycin; PLEX, plasma exchange; PRA, panel-reactive antibodies; PTC, peritubular capillaries; SAB, single antigen bead; TCMR, T cell-mediated rejection; Tx, transplantation; XM, crossmatch.

In this context we investigated whether eGFR slopes within 12–24 months after a biopsy showing ABMR would deliver sufficient information to evaluate its association with graft survival in a clinically meaningful way that might aid at identifying suitable patients to be enrolled into clinical trials. This retrospective study was conducted by screening the Vienna transplant biopsy database for late biopsies formally fulfilling morphologic criteria for ABMR and by applying in- and exclusion criteria commonly used in trials addressing ABMR in order to create a highly granular cohort. Historic sera were re-evaluated for the presence of DSA at the time of biopsy, and we applied appropriate statistical models with purposeful selection of covariables to model eGFR slopes and to assess their impact on graft survival in patients diagnosed with late active ABMR.

MATERIALS AND METHODS

Study Design

This is a retrospective study designed to assess the correlation of the eGFR slope within the first 12–24 months after biopsy-proven late active ABMR with graft survival. We defined pre- and post-biopsy time points with pre-specified deviation windows for kidney function measurements (**Supplementary Table 1**). Our patient cohort was derived by searching our electronic transplant biopsy database (Department of Pathology, Medical University Vienna) for lesions compatible with ABMR and by applying the Banff 2015 criteria (13). Once a biopsy formally fulfilled histomorphologic and immunohistochemical criteria of active or chronic active ABMR following the Banff 2015 scheme, the following inclusion and exclusion criteria were applied:

Inclusion Criteria

- Kidney transplant recipient of a solitary live or deceased donor kidney transplanted between January 1, 2000 and July 31, 2013.
- Kidney transplantation at least 12 months prior to index biopsy.
- Diagnosis of biopsy-proven ABMR according to Banff 2015 criteria on or before July 31, 2014.
- Age 15–75 years at the time of diagnosis of ABMR.
- At least two longitudinal measurements of serum creatinine within the first year prior to or at diagnosis of ABMR, including baseline creatinine at the time

of diagnosis, and at least two measurements of serum creatinine post-diagnosis of ABMR.
- Minimum of 3 years follow-up after diagnosis of ABMR to ascertain allograft status.

Exclusion Criteria

- Recipient of a multi-organ transplant.
- First diagnosis of active ABMR after July 31, 2014.
- eGFR < 25 ml/min/1.73 m^2 at the time of diagnosis of active ABMR to exclude patients with a high likelihood of timely graft loss.

Patients who were transplanted but not followed at our or at an associated center were excluded. Since many biopsies were carried out before the implementation of routine Luminex testing at our center, which was available since 2009, and in order to verify serological presence of anti-HLA donor-specific antibodies (DSA) at the time of index biopsy, we collected frozen historical sera from different in-house sources when available and subjected them to pooled single-antigen bead testing. We defined a final analysis cohort of 70 allografts composed of 55 allografts with antibody-verified ABMR at the time of biopsy and 15 allografts highly suspicious for ABMR, but without definitive proof of DSA at the time of biopsy. We included these 15 biopsies in the analysis based on recent Banff updates with less emphasis on imperative DSA confirmation (13, 14). However, as provided in the **Supplementary Tables 1–5** and **Supplementary Figures 1–4**, the 55 allografts with verified DSA were also analyzed separately to assess consistency of our results.

This study was approved by the institutional ethics committee of the Medical University Vienna (EK1104/2019) and was carried out in compliance with the Good Clinical Practice Guidelines, principles of the Declaration of Helsinki 2008, and the Declaration of Istanbul.

Data Extraction and Laboratory Measurements

Demographic variables and laboratory measurements were collected using the hospital's patient management software and medical records at the transplant outpatient clinic of the Department of Nephrology and Dialysis, Division of Medicine III at the Medical University of Vienna. Estimated glomerular filtration rate (eGFR) was assessed using the seven-variable MDRD equation provided that data on serum albumin and blood urea nitrogen (BUN) were available. Otherwise, we used the four-variable MDRD equation or, for one pediatric patient, the Schwartz equation was used (15, 16). We chose the MDRD formula since the majority of our patients already had reduced eGFR at the time of index biopsy.

Human Leukocyte Antigen Antibody Detection

We used LABscreen Single Antigen assays (One Lambda, A Thermo Fisher Scientific Brand, Canoga Park, CA, United States) for the characterization of anti-HLA reactivity patterns according to the manufacturer. Testing was performed retrospectively on frozen sera or plasma obtained close to or at the time of biopsy. To counteract complement interference, serum samples were treated with 10 mM EDTA (17). Donor-specificity was determined using serological, low- or high-resolution donor/recipient HLA typing methods for HLA-A, -B, -Cw, -DR, -DQ and -DP, whichever was available. A mean fluorescence intensity (MFI) value of 1,000 was used as the threshold for positivity, below 1,000 only clear epitope reactivity patterns on several beads > 500 MFI were counted as positive. The immunodominant DSA was called according to the bead that revealed the highest MFI.

Biopsies

Histomorphologic lesions and immunohistochemistry (C4d) were assessed on formalin-fixed paraffin-embedded sections. We applied the Banff 2015 classification to diagnose ABMR using the following lesion criteria: glomerulitis (g), peritubular capillaritis (ptc), transplant glomerulopathy (cg), and C4d (BI-RC4D; Biomedica, Vienna, Austria). All included biopsies were either for clinical indication (n = 58) or study protocol biopsies performed within the BORTEJECT trial (n = 12) (18).

We also included cases of ABMR with concurrent glomerulonephritis (GN), taking histomorphologic criteria besides endocapillary hypercellularity and/or basal membrane contours for the diagnosis of ABMR into account.

Outcome Analysis

The pre-defined outcome of this study was death-censored graft survival defined as return to permanent dialysis, re-transplantation or transplant nephrectomy, whichever occurred first. Secondary outcomes were overall graft survival and patient death. Patients alive at the date of last known follow-up or at the end of study were right censored. All patients were followed up until July 31, 2017 (end of study).

Statistics

Previously defined patient characteristics at the time of transplantation and at the time of index biopsy were summarized using descriptive statistics. Variables were tested for statistically independent distribution between patients who experienced graft loss during the study period versus patients who did not. For categorical variables, the Chi-Squared test was used while for metric or ordinally scaled variables, the Mann-Whitney U-test was used. For visual inspection of overall- and death-censored graft survival the Kaplan-Meier method was used. When analyzing subgroups, group comparison was done using the Log-rank test.

Change of eGFR across time was modeled as a linear spline by a linear mixed effects (LME) model. Two different LME models were calculated, the first one stretching to 12 months after index biopsy (time point 12) and the second one to 24 months after biopsy (time point 24), to account for different study periods that would be acceptable in therapeutic intervention trials. In both models, the intercept was defined at the time of index biopsy (time point 0) and the slope before biopsy was calculated starting at 12 months before index biopsy (time point -12). Linear splines were used in the LME models, avoiding a step in the curve at the intercept and allowing different slopes before and after biopsy.

Statistical significance of the eGFR slopes was examined by the one-sample *t*-test.

Modeling the Relationship Between Estimated Glomerular Filtration Rate Slope and Time-to-Event Data

The association between change in eGFR and risk of graft failure was assessed with the multivariate competing risk proportional hazard regression model from Fine and Gray, where graft loss represents the event of interest and death the competing risk (19). For all covariables sub-distribution hazards are given. Patients alive with a functioning graft at the date of last known follow-up or at the end of study were censored. The proportional hazards assumption was checked by examining Schoenfeld type residuals. In case of non-proportionality we added a term of the concerned variable multiplied with the logarithm of time for correction.

Two different analyses predicting future graft loss were performed: One beginning at 12 months after index biopsy, using the eGFR slope modeled over -12 to 12 months, and the other model beginning at 24 months after index biopsy, using the eGFR slope modeled over -12 to 24 months. Landmarks were set at the time points 12 and 24 to exclude events that happened already before collection of model information was completed, and since prognostic statements cannot be based on information available in the future (20). The eGFR slope before index biopsy and the eGFR slope after index biopsy were both engaged as covariables. Other clinically meaningful covariables were included into the model such as deceased donor type, use of lymphocyte depleting agent as induction agent (used in pre-sensitized patients at our center), the occurrence of a glomerulonephritis in addition to ABMR within the biopsy, TCMR concurrent with ABMR, C4d-positivity in PTC and triple immunosuppression at index biopsy.

For all analyses SAS 9.4 for Windows (Cary, NC, United States), IBM SPSS Statistics Version 24 (IBM, Armonk, NY, United States) and GraphPad Prism 9.1.1 (GraphPad Software, San Diego, CA, United States) was used. A two-sided p-value of less than 0.05 was considered statistically significant.

RESULTS

Study Flow and Patient Demographics

As depicted in **Figure 1**, we extracted 2,776 biopsies that fulfilled the primary screening criteria for transplantation period, timing of biopsy and center follow-up. Overall, 344 biopsies formally fulfilled the histopathologic criteria for Banff 2015 ABMR and were further assessed for detailed in- and exclusion criteria. A total cohort of 70 allografts from 68 patients was analyzed.

Variables at the Time of Transplantation

Patient baseline demographics at the time of transplantation are provided in **Table 1**. Median recipient age was 46 years (IQR 30–54) and 28 patients (40%) were female. Prior kidney transplantation had occurred in 22 (31%) of patients and median highest CDC-PRA was 10% (IQR 3–46). The median donor age was 48 years (IQR 37–57) and 84% of included patients received a deceased donor transplant.

With respect to differences in baseline variables, patients who experienced graft loss (n = 31, 44%) vs. no graft loss had a tendency toward more frequent prior kidney transplantation (45 vs. 21%, p = 0.027) and were less frequently administered induction therapy with an anti-IL-2 antibody (3 vs. 21%, p = 0.032).

Baseline variables for the antibody-verified cohort (n = 55) are provided in **Supplementary Table 2**. Here we found numerically more frequent prior kidney transplantations in patients who experienced graft loss vs. no graft loss (42 vs. 19%, p = 0.071) and their CDC-PRA was significantly higher [Median highest CDC-PRA 14% (IQR 10–77) vs. 7% (0–22), p = 0.022 and median latest CDC-PRA 4% (0–55) vs. 0% (0–13), p = 0.025].

Variables at the Time of Index Biopsy

As provided in **Table 2**, the median recipient age was 49 years (IQR 36–58) and the median time from transplantation to biopsy was 34 months (IQR 19–75). With respect to biopsy-cause, we found that 83% of patients were biopsied for clinical indication and 17% were biopsied for the presence of DSA without clinical deterioration. When comparing patients experiencing graft loss vs. no graft loss, we found that patients with graft loss were more often biopsied for clinical cause (94 vs. 74%, p = 0.034) and had a lower median baseline eGFR [35 (IQR 28–39) vs. 45 mL (39–54), $p < 0.001$] respectively.

When analyzing anti-HLA reactivities we found that 31% had DSA against HLA class I only, 40% against HLA class II only and 29% against both HLA class I and II (**Table 2**). Median sum of all DSA MFI was 15,726 (IQR 6,044-25,732) and 62% of immunodominant DSA (DSA with the highest MFI) were directed against HLA class II. The median MFI of the immunodominant DSA was 11,733 (IQR 5,505–16,403). DSA-specificities are depicted in **Supplementary Figure 1**. The detection of *de novo* DSA was limited by the relatively small proportion with available pre-Tx DSA status. Seventeen patients had undergone pre-Tx Luminex testing, of which 15 patients were DSA-positive (including three cases where anti-HLA allele-specificity was not recorded) and two patients were DSA-negative. We found that seven of the 14 patients (50%) with known anti-HLA-specificities (12 DSA-positive cases, two DSA-negative cases) had developed *de novo* DSA, of which all were directed against HLA class II (**Table 2**). Specificities of *de novo* DSA are provided in **Supplementary Table 3**. In patients who experienced graft loss vs. no graft loss we found no statistical differences regarding any DSA specificity or strength (**Table 2** and **Supplementary Table 2**).

With respect to immunosuppression, at the time of biopsy 76% of patients were on triple immunosuppression, 23% on dual immunosuppression and 1% was on CNI monotherapy. There were no differences between patients with graft loss vs. without graft loss regarding immunosuppressive medication. Overall, 43 (61%) of patients were treated with any anti-rejection treatment following biopsy. Of the 19 (27%) patients who had received intravenous steroids as anti-rejection treatment, we found a significant difference between patients who experienced graft loss vs. without graft loss (42 vs. 15%, p = 0.013, **Table 2**).

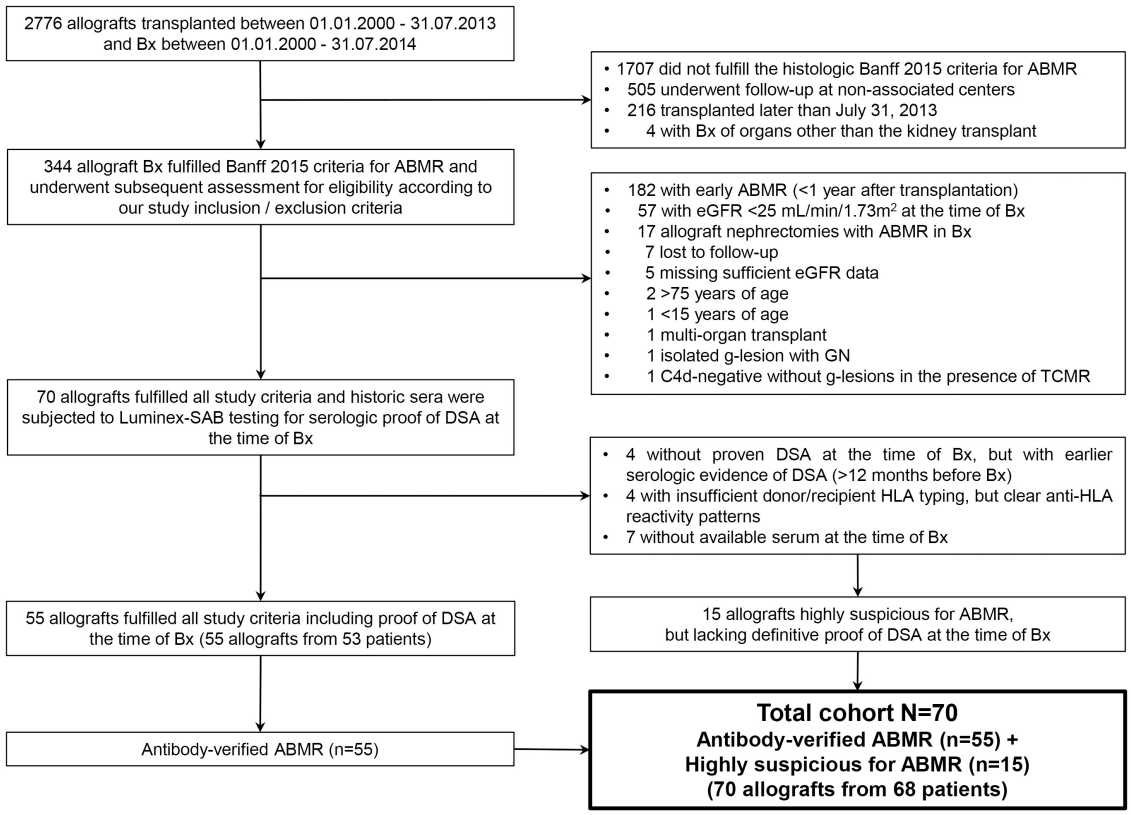

FIGURE 1 | Flowchart of the biopsy and patient selection process. After strictly applying all inclusion and exclusion criteria historical sera were searched for in order to carry out collective SAB testing as some biopsies dated back until the year 2000, long before Luminex testing was available at our center. The total cohort consisted of 70 biopsies from 68 patients (two biopsies came from repeat kidney transplants from the same two patients).

Biopsy Characteristics

Histomorphologic and immunohistochemical results are provided in **Table 3**. The majority of biopsies (71%) fulfilled the Banff 2015 criteria for chronic/active ABMR whereas 29% fulfilled the criteria for acute/active ABMR. With respect to complement activation detected in immunohistochemistry we found that 26 biopsies (37%) had a positive linear C4d staining in PTC. Twenty-nine (41%) of biopsies showed concurrent TCMR, 14 cases presented with concurrent GN (20%) and in four cases (6%) thrombotic microangiopathy was present. In biopsies with transplant glomerulopathy we found that patients who experienced graft loss had a higher median cg score compared to patients without graft loss [cg score 3 (IQR 1–3) vs. 1 (0–3), $p = 0.026$].

Overall, Death-Censored and Patient Survival Since Transplantation and Index Biopsy

The median follow-up time until graft loss, patient death or end of study was 88 months (IQR 61–132) after transplantation and 41 months (27–67) after index Bx (iBx). Unadjusted overall allograft survival at 1, 2, 3, 5, and 10 years after index iBx was 93, 80, 64, 53, and 15%, and 97, 83, 71, 59, and 29% for death-censored allograft survival, respectively. The median time until overall graft loss after transplantation was 132 months and 68 months after iBx, whereas median time until death-censored graft loss after transplantation was 160 months and 76 months after iBx. Patient survival at 1, 2, 3, 5, and 10 years after transplantation was 100, 96, 94, 88, and 55%, while after iBx patient survival was 94, 93, 90, 84, and 55%, respectively. Kaplan-Meier curves for overall allograft and death-censored allograft survival since transplantation and since iBx are provided in **Figures 2A,B**, patient survival since transplantation and iBx is depicted in **Supplementary Figures 2A,B**.

Analysis of Estimated Glomerular Filtration Rate Slopes Before and Early After Index Biopsy

The dynamics of eGFR trajectories spanning from 12 months before iBx until either 12 or 24 months after iBx were inspected applying a linear mixed effects (LME) model. **Figures 3A,B** depicts the single point eGFR measurements as well as the individual slopes and the mean overall eGFR slope from either −12 before to 12 months after iBx (**Figure 3A**) or from −12 before to 24 months after iBx (**Figure 3B**) for the total

TABLE 1 | Variables recorded at transplantation.

Parameter	Total cohort (*n* = 70)	Graft loss (*n* = 31)	No graft loss (*n* = 39)	*p*-value
Variables recorded at transplantation				
Recipient age, years, median (IQR)	46 (30–54)	41 (28–53)	51 (35–56)	0.142
Female sex, *n* (%)	28 (40)	9 (29)	19 (49)	0.095
Primary diagnosis				
Glomerulonephritis, *n* (%)	18 (26)	10 (32)	8 (21)	n.a.
Vascular nephropathy, *n* (%)	2 (3)	0 (0)	2 (5)	n.a.
Diabetes, *n* (%)	3 (4)	0 (0)	3 (8)	n.a.
Polycystic kidney disease, *n* (%)	9 (13)	7 (23)	2 (5)	n.a.
Hypertension, *n* (%)	6 (9)	2 (6)	4 (10)	n.a.
Other, *n* (%)	32 (46)	12 (39)	20 (51)	n.a.
Prior kidney transplant, *n* (%)	22 (31)	14 (45)	8 (21)	0.027
Pre-sensitized (CDC-PRA $\geq$ 40% or DSA), *n* (%)[a]	26 (37)	11 (35)	15 (39)	0.798
CDC-PRA				
Highest,% median (IQR)	10 (3–46)	10 (0–76)	10 (4–30)	0.892
Latest,% median (IQR)	1 (0–17)	1 (0–22)	0 (0–15)	0.767
Preformed anti-HLA DSA [17/70 (24%) were tested pre-Tx], *n* (%)[b]	15 (88)	3 (18)	12 (71)	n.a.
HLA class I, *n* (%)[b]	4 (24)	1 (6)	3 (18)	n.a.
HLA class II, *n* (%)[b]	3 (18)	0 (0)	3 (18)	n.a.
Both classes, *n* (%)[b]	5 (29)	3 (18)	2 (12)	n.a.
Class unknown, *n* (%)[b]	3 (18)	0 (0)	3 (18)	n.a.
Peri-transplant (induction) therapy, *n* (%)[c]	33 (47)	13 (42)	20 (51)	0.436
IA + ATG/anti-IL-2 antibody or ATG/Muromonab-CD3, *n* (%)	24 (34)	12 (39)	12 (31)	0.487
Anti-IL-2 antibody as single induction agent, *n* (%)	9 (13)	1 (3)	8 (21)	0.032
Delayed graft function, *n* (%)	20 (29)	8 (26)	12 (31)	0.648
Donor age, years, median (IQR)	48 (37–57)	51 (40–58)	45 (35–57)	0.242
Deceased donor, *n* (%)	59 (84)	26 (84)	33 (85)	0.932

[a] *Before 2009, pre-sensitized patients were defined as having a CDC-PRA $\geq$ 40%.*
[b] *Refers to percent of patients that underwent Luminex testing before transplantation, which was available since 2009 in our center.*
[c] *Four patients had a positive CDC-XM and underwent peritransplant-XM conversion with immunoadsorption according to our center-protocol.*
ATG, anti-thymocyte globulin; CD3, cluster of differentiation 3; CDC-PRA, complement-dependent cytotoxicity panel-reactive antibodies; DSA, donor-specific antibody; HLA, human leukocyte antigen; IA, immunoadsorption; IQR, interquartile range; IL-2, interleukin-2; n.a., not applicable; Tx, transplantation; XM, crossmatch.

cohort. Linear splines were introduced to avoid a step at the transition from pre-iBx to post-iBx eGFR course. **Supplementary Figures 3A,B** provides both graphs for the antibody-verified cohort. Interestingly, in either observation period, from −12 to 12, and–even more pronounced–from −12 to 24 months post-iBx we observed a moderation of the eGFR decline in the post-iBx course. This observation was confirmed in the antibody-verified cohort. **Table 4** and **Supplementary Table 4** list the values for the eGFR intercept, the slope pre-iBx and the slope post-iBx from both LME models for the total cohort and the antibody-verified cohort accounting for fixed and random effects. Both, the total cohort and the antibody-verified cohort had well comparable eGFR intercepts at iBx. We detected a statistically highly significant overall decline of the eGFR slope in all models, that revealed a slight flattening of the post-iBx slope ($p < 0.001$). For example, the slope in the total cohort changed from pre-iBx −9.2 mL/min/1.73 m^2 (95% CI: −12.1 to −6.2) to post-iBx −5.5 mL/min/1.73 m^2 (95% CI: −7.3 to −3.6) in the 24-month LME model (**Table 4**, $p < 0.001$).

Since our cohort contained a substantial amount (61%, **Table 2**) of patients who had received anti-rejection treatment after iBx showing ABMR, we wanted to assess whether this impacted on eGFR slopes. Therefore, we calculated an LME including anti-rejection treatment as fixed effect. We found no significant interaction of anti-rejection treatment with pre- as well as post-iBx eGFR slopes (**Supplementary Table 5**).

The Impact of Early Estimated Glomerular Filtration Rate Slopes After Index Biopsy on Graft Loss

We applied a multivariate Cox proportional hazards regression model accounting for the competing risk of death to assess the impact of the pre-iBx (−12 months) and early post-iBx (12 or 24 months) eGFR slope on graft loss. To avoid immortal time bias we set a landmark at 12 months when looking at the 12 months post-iBx eGFR slope and at 24 months when evaluating the 24 months post-iBx eGFR slope. **Figure 4** and **Supplementary Figure 4** depict Forest plots of the total cohort and the antibody-verified cohort. We found in both models that the 12 months pre-iBx eGFR slope was not associated with allograft loss, whereas the 12 months and the 24 months post-iBx eGFR slope were significantly associated with graft loss. The hazard ratio (HR) for the 12 months post-iBx slope was 1.1 (95% CI: 1.0–1.3) reflecting a 10% risk increment with each 1 mL/min/1.73 m^2 eGFR decline per year ($p = 0.020$). For the 24 months post-iBx eGFR slope we recorded a 30% risk increment for allograft loss with each 1 mL/min/1.73 m^2

TABLE 2 | Variables recorded at index biopsy.

Parameter	Total cohort (*n* = 70)	Graft loss (*n* = 31)	No graft loss (*n* = 39)	*p*-value
Variables recorded at index biopsy				
Age, median (IQR)	49 (36–58)	47 (35–54)	54 (40–59)	0.113
Time Tx to iBx (months), median (IQR)	34 (19–75)	56 (19–76)	32 (22–65)	0.624
Baseline eGFR (mL/min/1.73 m^2), median (IQR)	40 (33–48)	35 (28–39)	45 (39–54)	<0.001
Index biopsy for clinical cause (vs. DSA +), *n* (%)	58 (83)	29 (94)	29 (74)	0.034
Rise in serum creatinine, *n* (%)	20 (29)	11 (35)	9 (23)	0.254
Onset of or rise in proteinuria, *n* (%)	22 (31)	9 (29)	13 (33)	0.700
Both, *n* (%)	16 (23)	9 (29)	7 (18)	0.273
Anti-HLA DSA at iBx[a]				
HLA class I DSA only, *n* (%)[a]	17 (31)	9 (38)	8 (26)	0.352
HLA class II DSA only, *n* (%)[a]	22 (40)	8 (33)	14 (45)	0.375
HLA class I and II DSA, *n* (%)[a]	16 (29)	7 (29)	9 (29)	0.991
MFI_sum of all detected DSA, median (IQR)[a]	15,726 (6,044–25,732)	15,230 (6,967–34,219)	16,104 (4,416–22,754)	0.585
Immunodominant anti-HLA DSA at iBx[a]				
HLA class I[a]	20 (37)	11 (46)	9 (29)	0.157
HLA class II[a]	34 (62)	12 (50)	22 (71)	0.157
MFI_max, median (IQR)[a]	11,733 (5,505–16,403)	13,684 (6,967–16,780)	9,235 (4,229–16,575)	0.642
De novo anti-HLA DSA [14/70 (20%) with known DSA specificities pre-Tx][a,b]	7 (50)	1 (7)	6 (43)	n.a.
De novo HLA II DSA in pre-Tx DSA + patient, *n* (%)[a,b]	5 (36)	1 (7)	4 (29)	n.a.
De novo HLA II DSA in pre-Tx DSA- patient, *n* (%)[a,b]	2 (14)	0 (0)	2 (14)	n.a.
No *de novo* DSA in pre-Tx DSA + patient, *n* (%)[a,b]	7 (50)	2 (14)	5 (36)	n.a.
Triple immunosuppression, *n* (%)	53 (76)	23 (74)	30 (77)	0.791
Tacrolimus-based, *n* (%)	31 (44)	13 (42)	18 (46)	0.724
Cyclosporine A-based, *n* (%)	18 (26)	10 (32)	8 (21)	0.264
mTORi-based, *n* (%)	4 (6)	0 (0)	4 (10)	0.066
Dual immunosuppression, *n* (%)	16 (23)	8 (26)	8 (21)	0.600
No steroids, *n* (%)	7 (10)	3 (10)	4 (10)	0.936
No MMF/MPA/Azathioprine, *n* (%)	7 (10)	3 (10)	4 (10)	0.936
No CNI/mTORi, *n* (%)	2 (3)	2 (6)	0 (0)	0.108
CNI monotherapy, *n* (%)	1 (1)	0 (0)	1 (3)	0.369
Medication non-adherence reported by patient, *n* (%)	6 (9)	2 (6)	4 (10)	0.572
Anti-rejection treatment following iBx, *n* (%)	43 (61)	21 (68)	22 (56)	0.333
Steroids, *n* (%)	19 (27)	13 (42)	6 (15)	0.013
ATG or IVIG, *n* (%)	2 (3)	1 (3)	1 (3)	0.869
IA or PLEX ± steroids ± IVIG, *n* (%)	11 (16)	6 (19)	5 (13)	0.456
Rituximab + IVIG, *n* (%)	3 (4)	0 (0)	3 (8)	0.114
Bortezomib, *n* (%)	8 (11)	1 (3)	7 (18)	0.054

[a] *Numbers refer to the 55 patients with verified DSA at the time of biopsy provided in* ***Supplementary Table 2*** *(Graft loss: n = 24, no graft loss n = 31), respectively.*
[b] *Refers to percent of patients that underwent Luminex testing before transplantation, which was available since 2009 at our center and where the specificities of pre-Tx DSA were documented. In three patients with verified DSA before transplantation, specificities were not documented.*
ATG, anti-thymocyte globulin; iBx, index biopsy; CNI, calcineurin inhibitor; DSA, donor-specific antibody; HLA, human leukocyte antigen; IA, immunoadsorption; IQR, interquartile range; IVIG, intravenous immunoglobulins; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolic acid; mTORi, inhibitor of mammalian target of rapamycin; n.a., not applicable; PLEX, plasma exchange; Tx, transplantation.

eGFR decline per year (HR: 1.3, 95% CI: 1.1–1.4, $p = 0.001$), respectively. Other variables with significant HRs in both models were the use of a lymphocyte depleting agent at Tx and the finding of a GN concurrent to ABMR. At inspection the covariable concurrent GN revealed violation of the proportional hazards assumption and was therefore corrected for as described in the Methods section. Nevertheless, a high HR of 71.3 (12 months post-iBx) and 96.0 (24 months post-iBx) with corresponding wide 95% CI (6.3–804 and 6.7–1383, $p = 0.001$) remained after correction in both models, possibly reflecting the deleterious effect of this less frequent biopsy-finding on allograft loss, and was therefore kept in the model.

DISCUSSION

We conducted this study to elucidate whether early eGFR slopes, from 12 up to 24 months after biopsy, could serve as a surrogate for future allograft loss in patients diagnosed with late ABMR. The search for a "minimally clinically meaningful difference"

TABLE 3 | Index biopsy results.

Parameter	Total cohort (*n* = 70)	Graft loss (*n* = 31)	No graft loss (*n* = 39)	*p*-value
Index biopsy results				
Microcirculation inflammation (g > 0 ± ptc > 0), *n* (%)	67 (96)	29 (94)	38 (97)	0.425
g score, median (IQR)	2 (1–2)	2 (1–2)	2 (1–2)	0.859
ptc score, median (IQR)	2 (1–2)	2 (1–2)	2 (1–2)	0.342
g + ptc score, median (IQR)	3 (2–4)	3 (2–4)	3 (2–4)	0.859
Transplant glomerulopathy (cg > 0), *n* (%)	53 (76)	26 (84)	27 (69)	0.156
cg score, median (IQR)	2 (1–3)	3 (1–3)	1 (0–3)	0.026
Linear C4d + in PTC, *n* (%)	26 (37)	11 (35)	15 (38)	0.919
C4d score, median (IQR)	0 (0–2)	0 (0–1)	0 (0–2)	0.898
Histologic criteria of acute/active ABMR, *n* (%)	20 (29)	7 (23)	13 (33)	0.323
Histologic criteria of chronic/active ABMR, *n* (%)	50 (71)	24 (77)	26 (67)	0.323
Concurrent TCMR, *n* (%)	29 (41)	14 (45)	15 (38)	0.572
Borderline lesion, *n* (%)	18 (26)	9 (29)	9 (23)	n.a.
IA or IB, *n* (%)	6 (9)	3 (10)	3 (8)	n.a.
IIA, *n* (%)	4 (6)	1 (3)	3 (8)	n.a.
Chronic TCMR, *n* (%)	1 (1)	1 (3)	0 (0)	n.a.
Concurrent GN, *n* (%)	14 (20)	9 (29)	5 (13)	0.092
IgA nephropathy, *n* (%)	7 (10)	5 (16)	2 (5)	n.a.
Immune-complex GN (e.g., MPGN), *n* (%)	6 (9)	4 (13)	2 (5)	n.a.
Membranous GN, *n* (%)	1 (1)	0 (0)	1 (3)	n.a.
Thrombotic microangiopathy, *n* (%)	4 (6)	3 (10)	1 (3)	0.203

ABMR, antibody-mediated rejection; cg, transplant glomerulopathy; g, glomerulitis; GN, glomerulonephritis; IQR, interquartile range; MPGN, membranoproliferative glomerulonephritis; n.a., not applicable; ptc, peritubular capillaritis; PTC, peritubular capillaries; TCMR, T cell-mediated rejection.

with respect to eGFR slopes as an accepted trial endpoint in kidney transplantation is ongoing and several recent studies have addressed this issue (8, 9, 21–23). In trials studying chronic kidney disease, eGFR slopes were recently accepted as a surrogate endpoint by the FDA, but their value in late ABMR remains elusive (12).

We evaluated early eGFR slopes with respect to their association with future allograft loss using LME models and analyzed them together with clinically meaningful covariables applying Cox regression and accounting for the competing risk of death. Landmarks excluding all events before their setpoint were placed at the end of the observation periods of interest to avoid overfitting of our model. Here we show, that an eGFR decline of only 1 mL/min/1.73 m^2 per year within 12 to 24 months after biopsy with late ABMR was associated with an elevated risk of 10% (12-month slope) to up to 30% (24-month slope) for future allograft loss. Our findings thus implicate, that certain cutoffs in early post-iBx eGFR decline in patients with late ABMR might be defined as surrogate endpoint for allograft loss in future interventional trials. This might be of interest, since our aim was to specifically identify the value of early eGFR slopes in patients late after transplantation, whereas other studies have focused on eGFR decline within the earlier periods after transplantation as a surrogate endpoint for graft survival (24).

Very recently, Irish et al. (9) have carried out a retrospective multicenter study as part of a modeling exercise, applying a joint model for longitudinal data to the 12-month eGFR slope after a Bx with late active ABMR in order to simulate what impact different scenarios of eGFR slope-stabilization would have on predicted graft survival. The authors elegantly demonstrated that a stabilization of the eGFR slope of 30, 50, or 75%, based on the mean 12-month post-Bx slope, would lead to significantly improved graft survival after 2, 3, 4, and 5 years. Their concept might help to guide sample size calculations for the design of adaptive clinical trials in ABMR, based on putative eGFR slope-improvements potentially mediated by study drug interventions (9).

Our finding that the mean pre-iBx eGFR slope showed a steeper decline compared to the post-iBx eGFR slope, but was not significantly associated with graft survival, was unexpected. The reason for the smoothening of the eGFR slope after biopsy is unclear, but the effect of kidney function stabilization after biopsy was also shown in large studies such as the DeKAF Study, that next to other study questions also investigated the course of troubled kidney transplants with no intervention (25). Explanations for this finding in our study might be adaptation of baseline immunosuppression after the diagnosis of late ABMR, enhanced medical adherence or effects of anti-rejection treatment on ABMR concurring with a substantial rate of TCMR cases (41%) in our cohort. Although the majority of these concurring TCMR cases were borderline lesion (26%), we have tried to account for this by including TCMR in our multivariate model.

Our study also revealed other factors being differently distributed between patients who experienced graft loss compared to patients who did not experience graft loss within our observation period. Most factors associated with graft loss were reflecting a high likelihood of pre-sensitization, such as having received a prior kidney transplant or the use of ATG

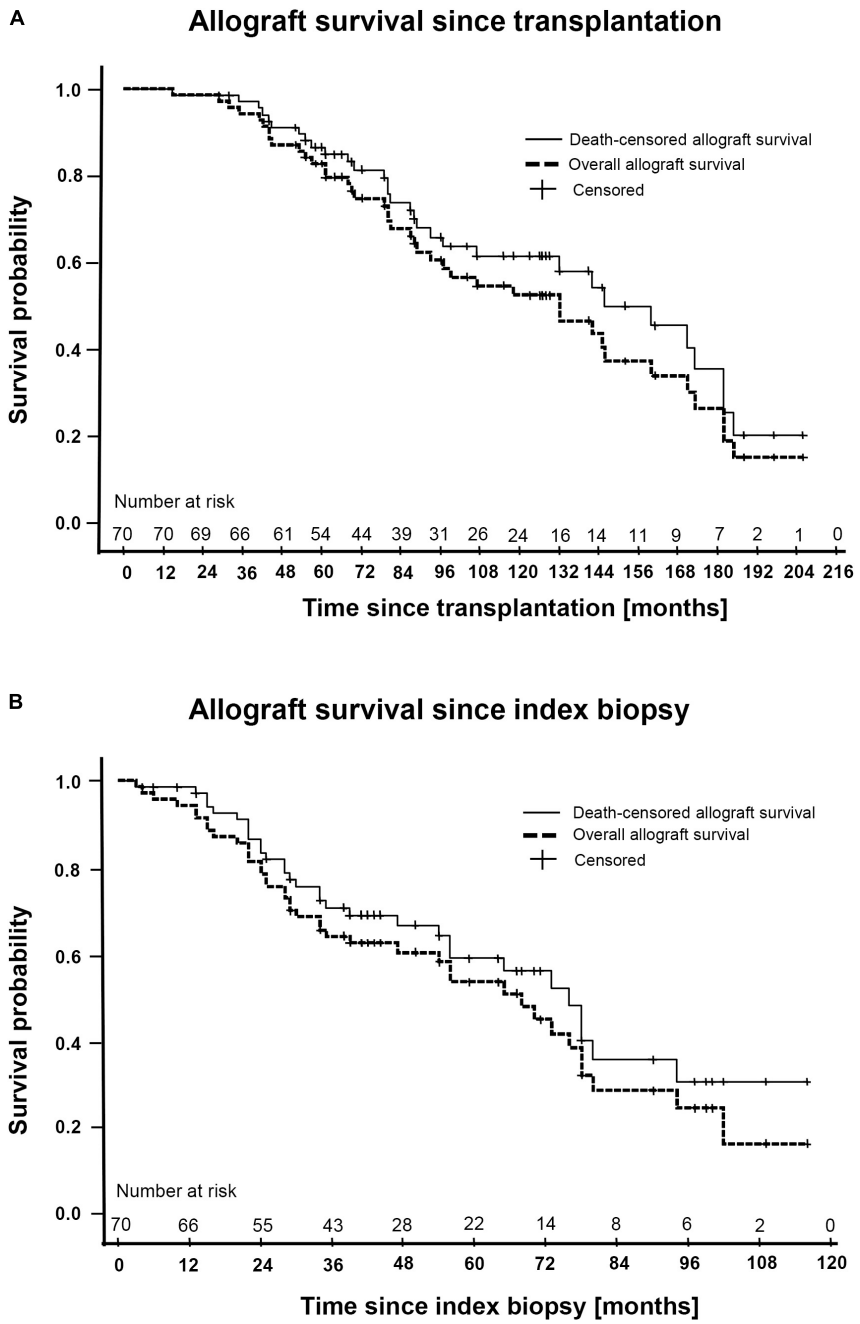

FIGURE 2 | Kaplan-Meier plots of the total cohort (*N* = 70) including overall- and death-censored allograft survival since transplantation **(A)** and since index biopsy **(B)**.

as induction agent. Others have found that graft survival in patients with *de novo* DSA was worse compared to patients with preformed DSA (26). In our cohort this analysis is precluded by the fact that before Luminex testing in 2009 only patients with a CDC-PRA $\geq$ 40% received induction with ATG, while patients with a CDC-PRA $<$ 40% that nowadays might reveal a significant DSA when tested with Luminex did either receive an anti-IL-2 antibody or no induction therapy at all.

Another covariable that was significantly associated with graft loss was the occurrence of GN. This finding is not surprising, since it has already been shown, that the occurrence of a GN alone after transplantation is associated with reduced graft survival (27–29). However, in our model this covariables did not fulfill the proportional hazards assumption, which we corrected for, and resulted in a very high HR with an associated wide 95% confidence interval. Our explanation for this lies within the very

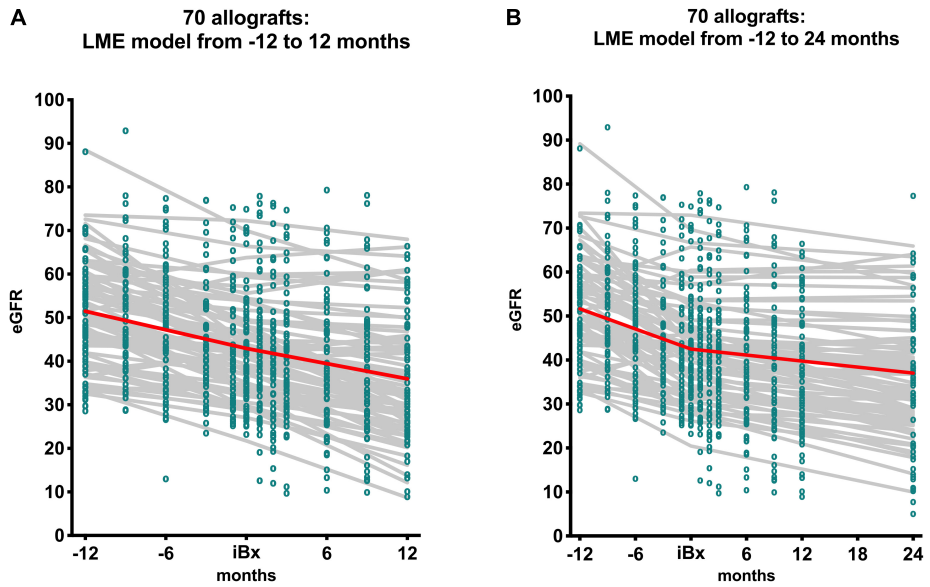

FIGURE 3 | It shows the single eGFR measurements (green dots), the individual eGFR trajectories (gray solid lines) as well as the overall trajectory (red solid line) of the total cohort (*N* = 70). The linear mixed effects model (LME) was carried out over a course of 2 years **(A)** from −12 to 12 months with intercept at time of iBx and over the course of 3 years **(B)** from −12 to 24 months with intercept at the time of iBx. The shown overall trajectory is based on the fixed effects of the LME, whereas the individual trajectories also include the random effects.

high event rate after biopsy in an overall small subset of patients with this biopsy finding, thereby leaving only few cases in the later observation period at risk (30). In our opinion, this covariable is of importance when selecting patients for a clinical trial in ABMR, since on one hand their eGFR slope often shows a rapid decline and the future event of allograft loss is highly likely, but on the other hand it is unclear if any ABMR treatment will also have an impact on the course of a recurrent or *de novo* GN, making this concurrent diagnosis questionable for inclusion into interventional trials in ABMR.

Strengths of our study are the rigorous inclusion and exclusion criteria and the bottom-down selection process from > 2,500 biopsies spanning over two decades, that allowed us to build a representative cohort in the field of late ABMR. Furthermore, we set our focus on the verification of DSA at the time of biopsy and by testing historic sera, we were able to generate a highly granular and well-characterized patient cohort. Also, our collective included patients who were administered various different anti-rejection treatment modalities, which in clinical reality is a frequent situation when including patients into trials for late ABMR. Most of these treatments included the proteasome inhibitor bortezomib and the CD20 antibody rituximab for which it has already been shown that treatment of late ABMR is unsuccessful (18, 31).

To address the retrospective nature of this study with all the known causes for bias, we applied a well-designed statistical model that included various carefully selected covariables, accounted for competing risks, and set landmarks in order to account for immortal time bias. The latter also precluded the model to use event data within the period of the early eGFR course after biopsy to avoid overconfident outcome prediction.

Limitations of our study are the retrospective design, which makes it hard to decipher the unclear impact of ABMR management in patients from earlier transplant eras, when treatment algorithms were widely missing. Also, the change of Banff classification over time and the focus on different entities such as C4d could have led to different therapeutic decisions in these patients. Surprisingly, in our 24-month, but not in our 12-month eGFR slope model we found that C4d-positivity was not associated with a higher risk for graft loss, which is in contrast with other studies, but in our study might be explained by the fact that these patients could have undergone a higher post-Bx surveillance by the treating physicians and received more

TABLE 4 | eGFR slopes.

Parameters	Estimate	95% confidence interval		p-value
Total cohort (*n* = 70)		**Lower**	**Upper**	
−12 to 12 months LME model				
Intercept at iBx [ml/min/1.73 m^2]	42.3	40.1	45.8	<0.001
eGFR slope pre-iBx [ml/min/1.73 m^2]	−8.5	−11.4	−5.6	<0.001
eGFR slope post-iBx [ml/min/1.73 m^2]	−6.9	−9.8	−4.2	<0.001
−12 to 24 months LME model				
Intercept at iBx [ml/min/1.73 m^2]	42.5	39.6	45.4	<0.001
eGFR slope pre-iBx [ml/min/1.73 m^2]	−9.2	−12.1	−6.2	<0.001
eGFR slope post-iBx [ml/min/1.73 m^2]	−5.5	−7.3	−3.6	<0.001

iBx, index biopsy; eGFR, estimated glomerular filtration rate; LME, linear mixed effects model.

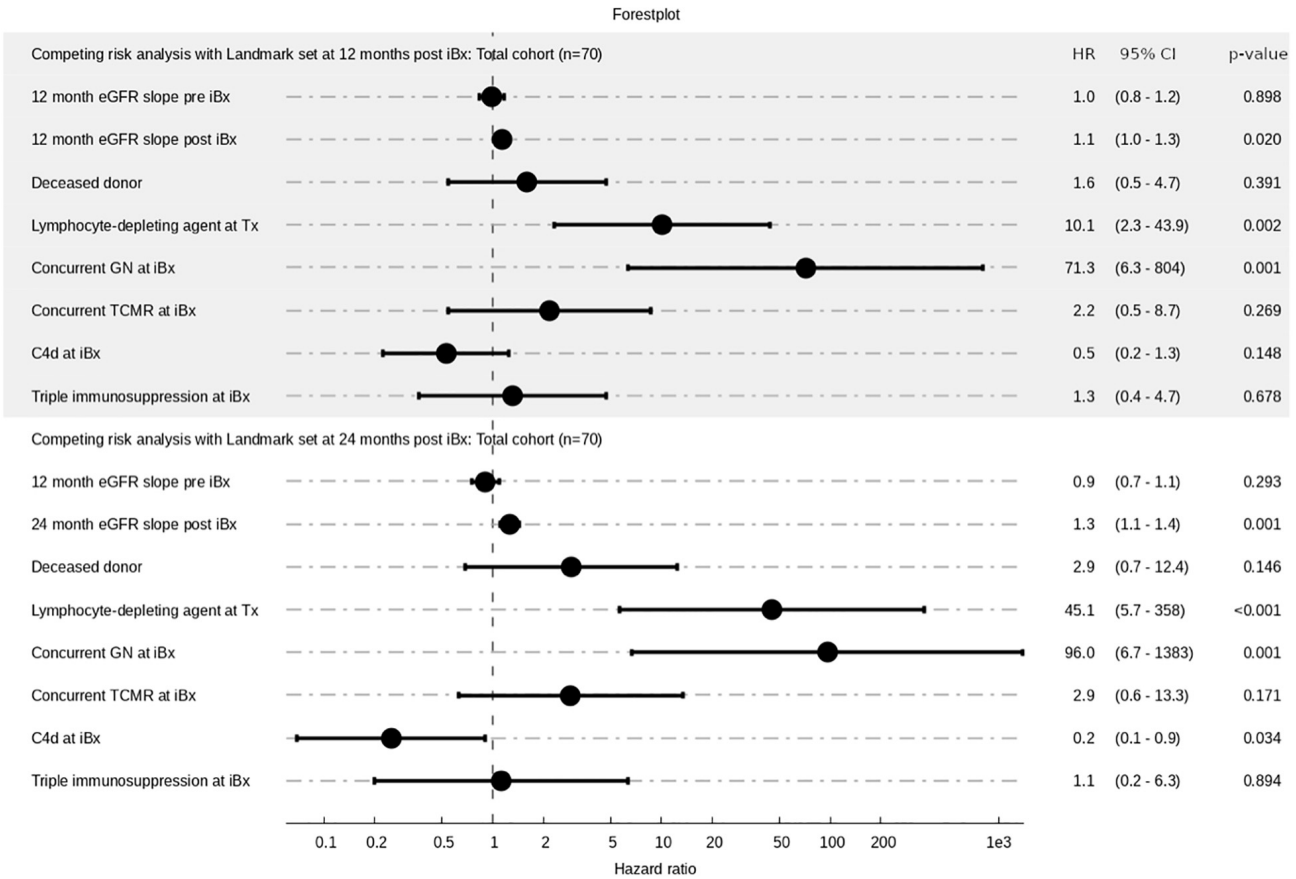

FIGURE 4 | Forest plots for the total cohort (*N* = 70) with landmarks set at 12 or 24 months after iBx to avoid immortal time bias. Hazard ratios (HR's) and their corresponding 95% confidence intervals (95% CI) are shown on the right. HR of 1 shows indifference of the variable regarding risk for allograft loss. GN, glomerulonephritis; TCMR, T cell-mediated rejection.

intense, and–in our cohort–also widely varying treatments (32). Other important limitations are the single-center design and a missing validation cohort. Lastly, we decided to also include patients, where no antibody-verification was possible at the time-of-biopsy, which reflects the fact that some ABMR cases may occur that miss the presence of classic anti-HLA DSA. Even though the majority of these cases in our study were mostly due to incomplete HLA typing of either donor or recipient, the histologic picture of ABMR could potentially also have been mediated by non-HLA DSA or NK cell-mediated missing-self processes (1, 33, 34).

In conclusion, our study is in line with the study by Irish et al. (9), showing that the early eGFR slope after biopsy-confirmed late ABMR can offer a valid surrogate endpoint for future allograft loss. We also detected GN concurrent with ABMR and the use of ATG at the time of transplantation, reflecting pre-sensitization status at our center, to be associated with a high risk of allograft loss. Future prospective data on eGFR slopes from large multicenter studies such as the IMAGINE trial (NCT03744910) may be used to further validate the early eGFR slope after biopsy-proven late ABMR for its implementation as a robust endpoint in clinical trials in transplantation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the Medical University of Vienna. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AB, AK, GAB, and FE designed the study, collected the data, analysed the data, and wrote the primary draft of the manuscript. HR, NK, JK, RS, GF, IF, SW, ŽK, GB, RR-S, KM, ME, MW, SH, and KD provided the materials needed for analysis, collected the

data, and critically revised the manuscript. FE, GB, and GAB provided funding for the project. All authors agreed to the final submission of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.817127/full#supplementary-material

Supplementary Figure 1 | Bar charts with percentages of the HLA class I and class II antigens that the corresponding DSA were directed against. Since some patients had > 1 DSA within either HLA class, percentages do not perfectly match with the given percentages of class I and/or class II DSA at the time of iBx in **Table 2** and **Supplementary Table 2**.

Supplementary Figure 2 | Kaplan-Meier plots of the total cohort (*N* = 70) for patient survival since transplantation **(A)** and since index biopsy **(B)**.

Supplementary Figure 3 | It shows the single eGFR measurements (green dots), the individual eGFR trajectories (gray solid lines) as well as the overall trajectory (red solid line) of the antibody-verified cohort (*N* = 55). The linear mixed effects model (LME) was carried out over a course of 2 years **(A)** from −12 to 12 months with intercept at time of iBx and over the course of 3 years **(B)** from -12 to 24 months with intercept at the time of iBx. The shown overall trajectory is based on the fixed effects of the LME, whereas the individual trajectories also include the random effects.

Supplementary Figure 4 | Forest plots for the antibody-verified cohort (*N* = 55) with landmarks set at 12 or 24 months after iBx to avoid immortal time bias. Hazard ratios (HR's) and their corresponding 95% confidence intervals (95% CI) are shown on the right. GN, glomerulonephritis; TCMR, T cell-mediated rejection.

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Histologic and Molecular Patterns in Responders and Non-responders With Chronic-Active Antibody-Mediated Rejection in Kidney Transplants

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Introduction: There is no proven therapy for chronic-active antibody-mediated rejection (caABMR), the major cause of late kidney allograft failure. Histological and molecular patterns associated with possible therapy responsiveness are not known.

Methods: Based on rigorous selection criteria this single center, retrospective study identified 16 out of 1027 consecutive kidney transplant biopsies taken between 2008 and 2016 with pure, unquestionable caABMR, without other pathologic features. The change in estimated GFR pre- and post-biopsy/treatment were utilized to differentiate subjects into responders and non-responders. Gene sets reflecting active immune processes of caABMR were defined *a priori*, including endothelial, inflammatory, cellular, interferon gamma (IFNg) and calcineurin inhibitor (CNI) related-genes based on the literature. Transcript measurements were performed in RNA extracted from stored, formalin-fixed, paraffin-embedded (FFPE) samples using NanoString™ technology. Histology and gene expression patterns of responders and non-responders were compared.

Results: A reductionist approach applying very tight criteria to identify caABMR and treatment response excluded the vast majority of clinical ABMR cases. Only 16 out of 139 cases with a written diagnosis of chronic rejection fulfilled the caABMR criteria. Histological associations with therapy response included a lower peritubular capillaritis score (p = 0.028) along with less glomerulitis. In contrast, no single gene discriminated responders from non-responders. Activated genes associated with NK cells and endothelial cells suggested lack of treatment response.

Conclusion: In caABMR active microvascular injury, in particular peritubular capillaritis, differentiates treatment responders from non-responders. Transcriptome changes in NK cell and endothelial cell associated genes may further help to identify treatment response. Future prospective studies will be needed which include more subjects, who receive standardized treatment protocols to identify biomarkers for treatment response.

Clinical Trial Registration: [ClinicalTrials.gov], identifier [NCT03430414].

Keywords: kidney transplantation, chronic-active ABMR, Banff classification, transcriptome, eGFR slope, therapy response

INTRODUCTION

Antibody-mediated rejection (ABMR) is the major cause of late kidney allograft failure (1). Early transplant survival rates have significantly improved over the last decades, in particular due to advances in human leukocyte antigen (HLA) matching and immunosuppression leading to a significant decrease in T-cell mediated rejections (TCMR) and acute ABMRs. However, for chronic rejection processes mediated by anti-HLA antibodies effective treatments are missing. The diagnosis of chronic-active ABMR (caABMR) is most likely associated with a progressive decrease in allograft function leading to near certain transplant failure (2).

The histology-based diagnosis of transplant glomerulopathy with microvascular inflammation together with donor-specific antibodies (DSA) is frequently seen as "kiss of death" for the kidney transplant. The lack of proven, effective therapies causing either a nihilistic approach, i.e., not changing therapy to avoid side-effects of over-immunosuppression, passively monitoring the progressive decline in function or a trial of various rejection therapies with anecdotal cases in mind of functional and morphological improvements (3).

Hence, the diagnosis of caABMR leaves the clinician (and the patient) with a profound uncertainty both in regard whether to treat at all, i.e., which cases are likely to respond to therapy and with which therapy.

The identification of prognostic features of potential treatment responsiveness is needed to justify and guide treatment. Key hurdles to identify these biomarkers are the often ambiguous, heterogeneous cases and diagnoses of caABMR impacted by a multitude of parallel disease processes (4, 5). This is further complicated by the lack of solid criteria for treatment response *vs.* non-response (6), the heterogeneity of treatment approaches (3, 7–10) and the dynamic of immune-mediated injury and response not being captured by histopathology alone. Molecular profiling might detect changes not seen by morphology or clinical markers (11–16).

On this background, we decided to identify features of cases of caABMR that responded and did not respond to therapy based on a rigorous, highly "puristic" approach. The selection of cases was seen as critical, i.e., only pure, unquestionable cases of caABMR with sufficiently documented pre- and post-biopsy courses and treatment responses were chosen. This highly selective, "cherry picking" approach, however, excluded the vast majority of clinical ABMR cases, in particular those with likely ongoing other pathology processes such as glomerulonephritis, TCMR, viral infections, cases suspicious but not definite for ABMR according to Banff criteria, early rejections, incomplete clinical or laboratory data, repeat biopsies or cases without change in immunosuppressive treatment.

Stored tissue samples of these highly selected cases of treated, pure caABMR with pre-defined response criteria were processed and analyzed according to their transcript expression profiles. We hypothesized that transcript changes of an *a priori* defined set of genes, reflecting the active immune processes of ABMR, might better identify ABMR cases that improve on treatment to those that do not respond. The gene selection was literature-based and focused on genes related to endothelial function, natural killer (NK) cells, and inflammatory processes (16–18).

The objective of our study is to identify in a retrospective analysis features that differentiate caABMR treatment responders from non-responders defined by a significant treatment-associated change in the estimated glomerular filtration rate (eGFR) slope.

MATERIALS AND METHODS

Sample Cohort

The study was approved by the cantonal ethics committee (KEC, BASEC number 2017-02130) of Zurich. This retrospective, observational, longitudinal cohort study reviewed all biopsies performed in kidney transplant patients at the University Hospital of Zurich between 01.01.2008 and 31.12.2016 with histologically confirmed caABMR (based on the Banff 2017 classification) (19).

Follow-up data included serum creatinine, proteinuria (assessed by protein/creatinine ratio in spot urine expressed in g/mmol), donor-specific HLA antibody development, medication use, level of immunosuppression, date of transplantation, date of biopsy, treatment received post-biopsy. In addition, data on age, gender, primary kidney disease, and deceased or living kidney transplant were also collected. Exclusion criteria were: age at transplantation <18 years, combined organ transplantation (incl. kidney-pancreas, kidney-liver), incomplete laboratory and/or clinical data, recurrence of the initial disease, insufficient biopsy material for transcript analysis, and documented refusal of data analysis for research purpose.

Immunosuppressive Therapy Regimens

The baseline, maintenance immunosuppression of our patients consisted of a calcineurin inhibitor (CNI; cyclosporine or tacrolimus), an anti-proliferative agent (mycophenolic acid or azathioprine), and in some cases prednisone. Induction therapy was done with either basiliximab or anti-thymocyte globulin.

Treatment of ABMR was not standardized and based on an increase in immunosuppression. The lack of standardization is reflected in the variety and combinations of treatments applied: dose increase ± drug conversion (from cyclosporine to tacrolimus, azathioprine to mycophenolic acid) ± addition of steroid bolus, immunoadsorption, plasmapheresis, intravenous immunoglobulins (5, 10, 20–23), rituximab, and/or bortezomib (24).

All rejection therapies given within 2 months post-biopsy were recorded and classified into nine different regimens. A single patient may have been treated with more than one therapy regimen.

Classification and Selection of the Primary Set of "139 Antibody-Mediated Rejection Biopsies"

All transplant kidney biopsies, performed between 01.01.2008 and 31.12.2016, were pre-screened (n = 1027) and in a step-wise selection process the final set of biopsies was identified (see **Figure 1**).

In a first step biopsies of patients who did not give a general research consent (n = 21) and biopsies of patients with combined organ transplantation (n = 11, of those 8 kidney-pancreas and 3 kidney-liver transplantations) were excluded (n = 32 in total).

In a second step the remaining 995 biopsies were classified in 6 groups according to the written pathology diagnosis. The selection based on "descriptive words" rather than scores was chosen because in only a fraction of biopsies, taken over this extended period of time, Banff scores were available. In addition the Banff classification has changed over time.

Group 1 included all biopsies with chronic ABMR only (n = 61), group 2 all biopsies with active ABMR only (n = 34), and group 3 all biopsies with chronic changes only (such as non-specific interstitial fibrosis and tubular atrophy) without signs of ABMR (n = 427). Patients with biopsy diagnoses stating both chronic and active ABMR changes were included in group 12 (n = 10), those with both active ABMR and other chronic changes were included in group 23 (n = 13), those with both chronic ABMR changes and other chronic changes in group 13 (n = 10). Some biopsy diagnoses stated active ABMR and chronic ABMR and other chronic changes at the same time, these biopsies were included in group 123 (n = 11). All biopsies which had another diagnosis and could not be included in one of the groups above were excluded (n = 429).

Following these selection criteria a total of 139 "ABMR biopsies" were identified based on their descriptive diagnosis in words, i.e., described as either active and/or chronic ABMR (groups 1, 2, 12, 13, 123, 23). All 109 patients of these 139 "ABMR biopsies" were contacted and asked for consent.

Identification of Therapy Responders and Non-responders

The individual serum creatinine and eGFR (25) slopes before and after these 139 biopsies were plotted to identify clinical therapy responders and non-responders. Through linear regression analysis based on the Mitch curve the trajectory of the curves 6 months before and 6 months after the biopsy time point were computed. Therapy response was defined as a slower rate of loss or a gain in eGFR from the pre- to post-biopsy/treatment periods. Treatment non-response was defined as no change or a more rapid loss of eGFR from the pre- to post-biopsy/treatment periods.

Selection of Cases of "32 Antibody-Mediated Rejection Treatment Biopsies"

In the third selection step all 139 "ABMR biopsies" with a written diagnosis of "ABMR" were analyzed to identify those fulfilling the criteria for evaluation of treatment response or non-response. Altogether 107 biopsies were excluded, some had more than one of the exclusion criteria:

- No rejection therapy, i.e., no documented increase or addition of immunosuppressive treatment at time of biopsy (n = 13 biopsies).
- Suspected ABMR, i.e., biopsies that did not qualify as full picture of ABMR according to Banff criteria in the written pathology diagnosis (n = 14).
- Concomitant tumor, i.e., biopsy in a patient with a tumor that impacted the treatment decision (n = 1).
- Early biopsies, i.e., biopsies taken within the first 3 post-transplant months (n = 18).
- On dialysis, i.e., biopsy taken while the patient was already on dialysis (n = 1).
- Improvement before biopsy, i.e., cases with improvement in kidney function before the biopsy was taken and without any rejection therapy (n = 35).
- Lack of creatinine/eGFR measurements, i.e., biopsy cases that did not have at least 3 creatinines/eGFRs measured in each period, the 6 months before and 6 months after the biopsy, respectively (n = 40).
- Repeat biopsies, i.e., only one biopsy per patient was selected to avoid overlaps and putting too much weight on a single patient case (n = 8).

After this step a total set of 32 "ABMR treatment biopsies" met all the inclusion criteria.

Scoring and Selection of the Final Set of "16 Chronic-Active Antibody-Mediated Rejection Biopsy Cases"

In the last selection step the set of 32 "ABMR treatment biopsies" were reread by our nephropathologist to be scored according to the definitions of the Banff 2017 classification (19). Biopsies displaying diagnostic features of either a mixed rejection phenotype (TCMR and ABMR), an active ABMR process without

FIGURE 1 | Algorithm of the biopsy selection process. cABMR, chronic antibody mediated rejection; aABMR, active antibody mediated rejection; IF/TA, interstitial fibrosis and tubular atrophy; ICT, information and communication technology; caABMR, chronic-active antibody mediated rejection fulfilling Banff 2017 criteria; ptc, peritubular capillaritis; g, glomerulitis; cg, transplant glomerulopathy; C4d, complement split product.

the chronic component, clear signs of a *de novo* or recurrent glomerulonephritis or cases in which patients did not specifically consent to transcriptome studies (6 patients out of 32) were excluded. After this rigorous selection step a remaining set of 18 biopsies showed the three diagnostic criteria of caABMR, i.e., ABMR chronicity, antibody interaction, and DSA ± C4d staining. As in two biopsy cases not enough FFPE tissue was left over for high quality RNA-processing the final set consisted of "16 caABMR cases" for transcriptome measurements (see also **Figure 1**).

Gene Selection for Transcriptome Measurements in Chronic-Active Antibody-Mediated Rejection Cases

Genes of interest were selected *a priori* based on the known molecular immunopathology of the antibody-mediated rejection process. The diagnostic hallmark of ABMR is the injury to the microcirculation (26). The endothelium is presumed to be the primary target of antibodies leading to cell injury associated with pathways and signals of inflammation (18, 27–29), migration of myeloid cells and NK cells (17, 18, 27, 30–32) and interferon gamma (IFNg) related injury responses (27, 33). In addition, genes associated with calcineurin inhibitor (CNI) toxicity, that might impact chronicity changes, were also chosen. A total of 44 target genes, representing key pathways and structures associated with ABMR, and 4 house-keeping genes, were selected. Grouping and in detail description of the individual genes is summarized below and in **Supplementary Table 1**:

Group 1–Endothelium-associated genes: CDH5, CDH13, COL13A1, DARC, ECSCR, GNG11, ICAM2, MALL, PECAM1, PGM5, RAMP3, RAPGEF5, ROBO4, TM4SF18, VWF, THBD, SELE, PLAT, and TEK.

Group 2–Inflammation-associated genes: OSM, OSMR, SAA1, IL6, IL6R, HMGB1, CLEC4E, and IL1B.

Group 3–Cellular response-associated genes: myeloid cells KLF4, PPM1F, NK cells CCL4c, CD160, YME1L1, FGFBP2, GNLY, CX3CR1, KLRD1, KLRF1, SH2D1B, and TRDV3.

Group 4–Interferon-gamma inducible genes: CXCL10, CXCL11, and PLA1A.

Group 5–Calcineurin inhibitor toxicity: TNFSF12 and TNFRSF12A.

Group 6–House-keeping genes: ACTB, LDHA, HPRT1, and GAPDH.

Transcriptome Measurements of the Formalin-Fixed, Paraffin-Embedded Samples of Chronic-Active Antibody-Mediated Rejection Cases

To obtain high quality transcriptome measurements in stored FFPE tissues we chose the NanoString^{TM} technology that allows robust mRNA analysis without further amplification (34). For NanoString^{TM} analysis around 100 ng total RNA is needed, i.e., 3–4 cuts of 20 um each from the FFPE block. After deparaffinization RNA was extracted and its quality checked. Based on the target mRNA sequence two specially designed oligonucleotides A and B were synthesized for each one of the 48 target mRNAs. Oligonucleotide A specifically binds the target mRNA sequence and a unique fluorescent barcode reporter specific for the target mRNA sequence. Oligonucleotide B specifically binds the target mRNA sequence and a universal capture tag able to fix the target mRNA on a plate. Forty-eight target-mRNA-specific oligonucleotides $A_{1,2,0.48}$ and oligonucleotides $B_{1,2,0.48}$ were synthesized by Integrated DNA Technologies^{TM}. Each 7 μL of the oligonucleotides A (present at 0.6 nM) and B (present at 3 nM) were mixed with 70 μL containing the unique fluorescent "barcode" reporter tag and the universal capture tag (provided by NanoString^{TM}), generating a so called Master Mix^{TM}. For hybridization 8 μL of the Master Mix^{TM} were added to 7 μL of FFPE RNA sample with subsequent incubation at 67°C for minimum 16 h. In a purification process all the mRNA/oligonucleotides not bound to a plate (through the universal capture tag) were washed away. After an alignment of all the hybridized unique fluorescent barcodes bound to the target mRNA in an electromagnetic field, analysis followed: the number of mRNA copies bound to their unique fluorescent barcode was counted.

Statistics

Numerical variables were described with the mean ± standard deviation (SD). Characteristics of responders and non-responders were compared with *t*-tests for numerical variables and Chi-square-tests for categorical variables. For all calculations, *p*-values less than 0.05 were considered significant. The creatinine/eGFR curves were approximated with linear regression analysis. Data was analyzed using JASP version 0.10.2, Excel version 15.38, and XLSTAT version 20.2. Heatmap analysis of gene expression was performed with the nSolver NanoString^{TM} Analysis Software Version 4.0.70. Principal component analyses and hierarchical clustering was computed with XLSTAT version 20.2.

RESULTS

Identification of Biopsies With a "Pure" Histological Diagnosis of Chronic-Active Antibody-Mediated Rejection and a Treatment Response That Could Be Classified

As shown in **Figure 1** a total of 139 (13.5%) out of the 1027 kidney transplant biopsies collected had a written pathology report including antibody mediated rejection. From these "ABMR biopsies" those with incomplete creatinine measurements, improvement of kidney function already before biopsy, a diagnosis of only suspected ABMR, biopsies taken in the first 3 post-transplant months, and biopsies with no rejection treatment were excluded. This reduced the set to 32 "ABMR treatment biopsies." These biopsies were reread and classified according to the Banff 2017 criteria and those with mixed TCMR/ABMR or active ABMR phenotype, low quantity or quality of FFPE sample material and lack of consent to use stored tissue were excluded and left a final set of 16 pure "caABMR" biopsies for transcriptome analysis (**Figure 1**).

Patient Characteristics

The baseline characteristics of the 16 patients and their biopsies with the diagnosis of "caABMR" are given in **Table 1**. Demographics between the groups of 6 non-responders and 10 responders were similar. The recipients had a mean age of 44 years at transplantation and 52 years at time of biopsy; 5 (31%) were female and most kidneys were from deceased donors (63%). Biopsies were taken at a mean of 7.9 years

TABLE 1 | Baseline characteristics.

	Overall	Non-responder	Responder	*P*-value
Number	16	6	10	
Demographics				
Gender (female)	5 (31)	2 (33)	3 (30)	0.889
Age at transplantation [yrs]	44 ± 14	46 ± 18	43 ± 11	0.673
Age at biopsy [yrs]	52 ± 12	53 ± 16	52 ± 10	0.944
Age of transplant kidney at time of biopsy [yrs]	55 ± 15	57 ± 18	54 ± 14	0.693
Post-operative time of biopsy [yrs]	7.9 ± 5.5	6.3 ± 4.2	8.8 ± 6.2	0.408
Donor type (deceased donor)	10 (63)	4 (67)	6 (60)	0.790
Graft survival [yrs]		3.15 ± 2.9	4.0 ± 4.1	0.64
DSA status (positive)	11 (69)	4 (67)	7 (70)	0.889
Written pathology diagnosis				
cABMR	13 (81)	5 (83)	8 (80)	
cABMR + other chronic changes	3 (19)	1 (17)	2 (20)	

Donor specific antigen (DSA) status was considered positive in individuals with preformed and/or de novo DSA with median fluorescence intensity (MFI) > 1000. Values are given as mean ± SD, or as absolute counts (percentage). Characteristics of responders and non-responders were compared with t-tests for numerical variables and Chi-square-tests for categorical variables.

TABLE 2 | Summary of the antibody-mediated rejection therapies in the non-responder and responder groups.

Gender	Non-responder	Responder
Increase in dose >20%	3	0
Switch Cyclosporine to Tacrolimus	4	3
Switch Azathioprine to Mycophenolate	0	3
Switch Everolimus to Mycophenolate	1	0
Steroid pulse	5	8
Immunoadsorption	1	0
Plasmapheresis	0	2
IVIG	2	6
Rituximab	0	3
Bortezomib	1	0

IVIG, intravenous immunoglobulin.

post-transplantation, slightly but not significantly later in the responder group compared to the non-responder group (8.8 vs. 6.3 years post-transplantation, resp.). In 11 patients (69%) donor specific anti-HLA antibodies (DSA), preformed and/or *de novo*, with a median fluorescence intensity of >1000 were detected. There was no statistical difference in the total number of DSA or the cumulative total MFI between responders and non-responders, however, there was a trend toward higher DSAs and MFIs in the non-responders (data not shown). The treatments of the ABMR showed a significant heterogeneity without a clear pattern (**Table 2**).

Kidney Transplant Function Before and After Biopsy

The individual slopes of kidney function, based on linear regression of at least 3 eGFR values before and 3 after biopsy, are shown in **Figure 2**. Pre-biopsy the decrease in kidney function over 6 months was significantly greater in the responders (**Figure 2A**) compared to the non-responders (**Figure 2B**), showing a mean slope of a −0.070 vs. −0.005 ml/min × d, resp. (p = 0.010). Post-biopsy over 6 months the decrease in function accelerated in the non-responders and improved in the responders, showing a mean slope of −0.103 vs. 0.035 ml/min × d, resp. (p = 0.023). The mean difference in the slopes before to after biopsy was −0.095 vs. 0.105 ml/min × d for non-responders and responders, resp. (0.002). The detailed numbers are given in **Supplementary Table 2**. As shown in **Table 1** the difference in graft survival did not reach statistical significance, however, there was a trend toward longer graft survival in responders compared to non-responders.

Proteinuria Before and After Biopsy

The mean level of proteinuria pre-biopsy was 0.15 g/mmol (extrapolates to 1.5 g per day) for non-responders and 0.16 g/mmol (extrapolates to 1.6 g per day) for responders, post-biopsy 0.24 g/mmol for non-responders, and 0.2 g/mmol for responders. The levels were not significantly different between pre- vs. post-biopsy or non-responders vs. responders as shown in **Supplementary Table 3**.

Banff Features of Biopsies in Responders vs. Non-responders

Table 3 shows the average Banff scores derived from the biopsies. All 16 biopsies had signs of microvascular inflammation with peritubular capillaritis (ptc 1.31 ± 1.25) and glomerulitis (g 1.79 ± 0.89). In addition they showed hyaline arteriolar changes (ah 2.31 ± 1.01 and aah 1.38 ± 1.03) and marked signs of transplant glomerulopathy (cg 2.46 ± 0.78). The scores for tubulitis (t 0.25 ± 0.45), interstitial inflammation (i 0.33 ± 0.52), intimal arteritis (v 0.07 ± 0.27), and arterial fibrous intimal thickening (cv 0.36 ± 0.75) were low. Peritubular capillaritis was significantly higher in the non-responder compared to the responder group (ptc 2.18 ± 1.17 vs. 0.80 ± 1.03, p = 0.028).

The heat map (**Figure 3**) visualizes the trends in the histopathology patterns. The non-responders show more a phenotype of active microvascular inflammation (ptc and g), whereas chronic microvascular changes characterized by glomerular basement membrane double contours were predominantly seen in the responder group.

Overall, results of histomorphology and scoring according to the Banff 2017 criteria are shown in **Figure 4A**. The principal component analysis indicates that 8 Banff features explain around 61% of variability of the data. Regarding the variables ptc has the strongest weight, is positively correlated with g and to a lesser degree with t and negatively correlated with cg and not correlated with ct and ah. The x-axis differentiates acute changes (toward the right) and chronic changes (toward the left). The cluster of Banff features, shown in the dendrogram (**Figure 4B**), reflects the three major histological groups, active microvascular inflammation (ptc and g), chronic microvascular lesions (cg and ah) and tubulo-interstitial changes (i, t, and ct) with C4d deposition as the strongest outlier. The dendrogram in **Figure 4C** clusters the biopsy samples based on the profiles of 8 Banff features in the 16

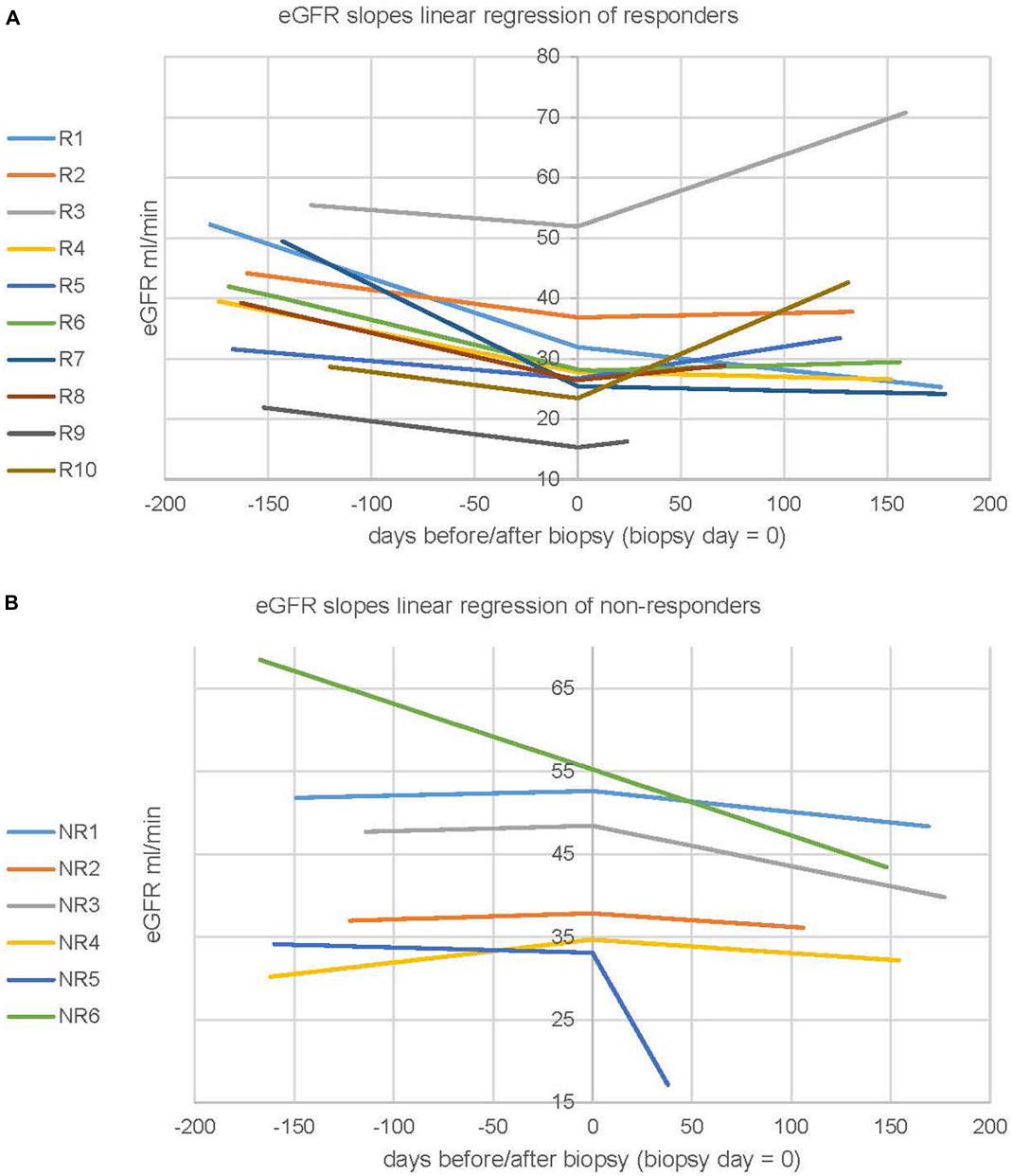


FIGURE 2 | Kidney function before and after biopsy in treatment responders versus non-responders. **(A)** eGFR slopes in 10 therapy responders, **(B)** in 6 non-responders. Every line represents one patient. The linearized slopes were calculated by linear regression of raw data before and after biopsy and plotting of the two curves $y_{pre-biopsy} = m_{pre\text{-}biopsy} * x + q_{pre\text{-}biopsy}$ and $y = m_{post\text{-}biopsy} * x + q_{post\text{-}biopsy}$ where $q_{pre\text{-}biopsy} = q_{post\text{-}biopsy}$ = measured eGFR at biopsy day.

biopsies. Basically, two major groups are clustered, which show a trend toward separating responders from non-responders.

Gene Transcript Profiles in Biopsies of Responders and Non-responders

Transcriptome Quality and Analysis

To have sufficient sample quantity and quality in the transcriptome reading the NanoString™ technology requires in FFPE samples a A260/280 ratio of >1.8 (protein contamination), a A260/230 ratio of >1.8 (organic contaminants), a minimal concentration of 20–60 ng/ul of RNA and at least 50% of the sample being greater than 300 nucleotides in length. Only 2 out of 18 biopsies could not be analyzed because of a too low RNA concentration, average concentrations, ratios and fragment lengths are given in **Supplementary Table 4**. Overall there was good RNA quality achieved and there were no significant differences between the responder and non-responder groups.

The average transcript levels and fold change of genes between responders and non-responders is given in

TABLE 3 | Banff scores characteristics.

Banff score	Overall	Non-responder	Responder	*P*-value
t score	0.25 ± 0.45	0.33 ± 0.52	0.20 ± 0.42	0.582
i score	0.19 ± 0.43	0.33 ± 0.52	0.10 ± 0.32	0.277[a]
ti score	0.69 ± 0.70	0.50 ± 0.55	0.80 ± 0.79	0.428
ptc score	1.31 ± 1.25	**2.18** ± 1.17	**0.80** ± 1.03	**0.028**
v score	0.07 ± 0.27	0.17 ± 0.41	0.00 ± 0.00	[b]
cv score	0.36 ± 0.75	0.17 ± 0.41	0.50 ± 0.93	0.429[a]
g score	1.79 ± 0.89	2.20 ± 1.10	1.56 ± 0.73	0.207
cg score	2.46 ± 0.78	2.00 ± 0.71	2.75 ± 0.71	0.090
mm score	0.44 ± 0.90	0.17 ± 0.41	0.60 ± 1.08	0.365
ci score	0.94 ± 0.57	1.00 ± 0.63	0.90 ± 0.57	0.748
ct score	0.94 ± 0.57	1.00 ± 0.63	0.90 ± 0.57	0.748
ah score	2.31 ± 1.01	2.50 ± 1.25	2.20 ± 0.92	0.585
aah score	1.38 ± 1.03	1.67 ± 1.03	1.20 ± 1.03	0.396
c4d score	1.25 ± 1.44	1.00 ± 1.55	1.40 ± 1.43	0.607
i-IFTA score	1.78 ± 1.20	2.00 ± 0.00	1.71 ± 1.38	[c]

Banff scores of responders and non-responders were compared with t-tests.
Values are given as mean ± SD.
Significant values are given in bold.
t, tubulitis score; i, interstitial inflammation score; ti, total cortical inflammation score; ptc, peritubular capillaritis score; v, vasculitis score; cv, arterial fibrous intimal thickening score; g, glomerulitis score; cg, transplant glomerulopathy (glomerular basement membrane double contours) score; mm, mesangial matrix expansion score; ci, interstitial fibrosis score; ct, tubular atrophy; ah, arteriolar hyalinosis score; aah, hyaline arteriolar thickening score; C4d, complement split product; IFTA, interstitial fibrosis–tubular atrophy.
[a]*Levene's test is significant ($p < 0.05$) suggesting a violation of the equal variance assumption.*
[b]*Variance of the Banff score v is equal to zero after grouping on responder and non-responder.*
[c]*Number of observations < 2 for i-IFTA after grouping on responder and non-responder.*

Supplementary Table 5. The transcript levels of the 44 target genes showed a similar expression pattern in therapy responders vs. non-responders. After adjustment for multiple testing no gene was significantly differently expressed between the two therapy groups, although NK cell related genes were showing a tendency to be relatively more expressed in non-responders. The genes of each pathophysiological category showing the biggest fold change in gene expression were: VWF in the endothelial gene group (being expressed 1.6-fold in responders), CLEC4E in the inflammatory gene group (being expressed 1.7-fold in responders), CD160 in the NK cell related gene group (being expressed 2.2-fold in non-responders), and CXCL10 in the IFNg related group (being expressed 1.5-fold in non-responders). The maximal normalized fold change in gene expression between non-responders and responders was a 2.2-fold change, seen with CD160.

The unsupervised hierarchical clustering, regarding similarities and dissimilarities between the different genes, identified clusters that largely reflect the inflammatory and immunological pathways (**Figure 5A**). The dendrogram clusters genes toward pathophysiological classes with endothelial related genes being grouped together and partly separated from NK cells/inflammation related genes, which cluster together. The IFNg-inducible genes also group together. CNI toxicity related genes also order very closely. Interestingly, the two myeloid cells related genes are grouped separately from each other. OSM is known to induce IL6, the two genes appear together in the dendrogram; almost the same applies for their receptors OSMR and IL6R. The NK cell related CCL4c gene is known to be induced by IFNg and groups together with IFNg-inducible genes. The clustering supports that the selected gene groups associate with the different immunological pathways. This is not biologically surprising, however, serves to support the validity of the analysis of the RNA derived from the stored FFPE samples.

Figure 5B shows the clustering of the biopsies based on the expression patterns of the 44 *a priori* selected genes. As shown, the clusters do not separate responders from non-responders. The 16 samples are distributed randomly among the clusters.

Gene expression was visualized through heatmap analysis (**Figure 6**). The similarity metric and linkage method used in the dendrogram reflects the correlation between the biopsies and genes. A trend toward higher endothelial gene expression was observed in non-responders. As expected, hierarchical clustering demonstrated that genes from the same functional category (such as endothelial, NK-cell related, etc.) behaved similarly.

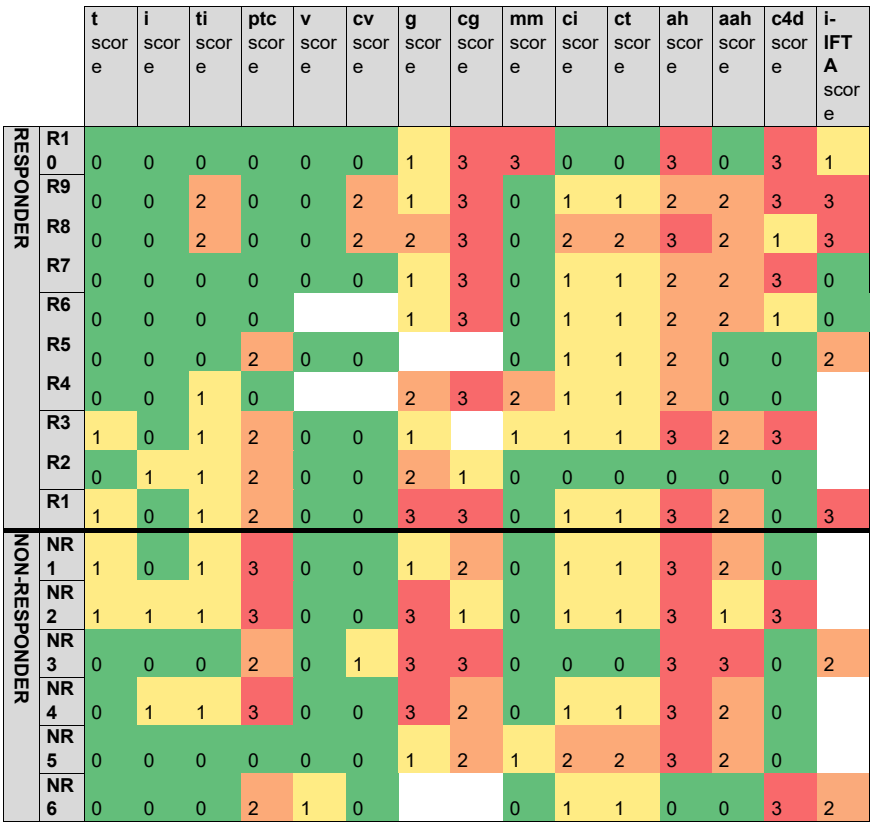
DISCUSSION

Late allograft failure due to chronic rejection caused by donor-specific antibodies remains the key problem for allograft survival, not only for kidney but also other solid organ transplants (26). Currently, there is still no effective treatment for caABMR and the phenotype of a graft that might respond to a potential therapy is unknown (5, 35). Hence, clinically the most important question for patients with a histological diagnosis of chronic rejection is who will benefit from treatment which is not risk-free.

This retrospective study was based on the hypothesis that analysis of pure cases of caABMR, not disturbed by overlapping pathologies, is needed to identify features associated with treatment response vs. no-response. In addition, a rigorous definition of treatment response was chosen based on the trajectory of eGFR pre- and post-biopsy/treatment. Further, considering immune-mediated processes as drivers of caABMR, transcriptome measurements were added to the histopathology readings and an unbiased approach was chosen by defining gene sets *a priori*.

Due to this reductionist, very selective methodological approach, 16 "pure" caABMR cases were identified, of whom 10 responded and 6 did not respond to various therapies. Overall, active microcirculation injury, in particular histopathological severity of peritubular capillaritis, and a trend toward increased glomerulitis and transcriptome changes of activated NK and endothelial cell related genes were associated with non-response to treatment.

The rigorous selection process used here is both a strength and a weakness in that the diagnosis of ABMR was as robust as it was possible to be but the subject number was very small impacting the likelihood to detect subtle or significant changes and stratification by treatment group. The robust clinical diagnosis was based on multiple creatinine measurements before



		t score	i score	ti score	ptc score	v score	cv score	g score	cg score	mm score	ci score	ct score	ah score	aah score	c4d score	i-IFTA score
RESPONDER	R10	0	0	0	0	0	0	1	3	3	0	0	3	0	3	1
	R9	0	0	2	0	0	2	1	3	0	1	1	2	2	3	3
	R8	0	0	2	0	0	2	2	3	0	2	2	3	2	1	3
	R7	0	0	0	0	0	0	1	3	0	1	1	2	2	3	0
	R6	0	0	0	0			1	3	0	1	1	2	2	1	0
	R5	0	0	0	2	0	0			0	1	1	2	0	0	2
	R4	0	0	1	0			2	3	2	1	1	2	0	0	
	R3	1	0	1	2	0	0	1		1	1	1	3	2	3	
	R2	0	1	1	2	0	0	2	1	0	0	0	0	0	0	
	R1	1	0	1	2	0	0	3	3	0	1	1	3	2	0	3
NON-RESPONDER	NR1	1	0	1	3	0	0	1	2	0	1	1	3	2	0	
	NR2	1	1	1	3	0	0	3	1	0	1	1	3	1	3	
	NR3	0	0	0	2	0	1	3	3	0	0	0	3	3	0	2
	NR4	0	1	1	3	0	0	3	2	0	1	1	3	2	0	
	NR5	0	0	0	0	0	0	1	2	1	2	2	3	2	0	
	NR6	0	0	0	2	1	0			0	1	1	0	0	3	2

FIGURE 3 | Overview of Banff scores of responders versus non-responders. t, tubulitis; i, interstitial inflammation; ti, total cortical inflammation; ptc, peritubular capillaritis; v, vasculitis; cv, arterial fibrous intimal thickening; g, glomerulitis; cg, transplant glomerulopathy; mm, mesangial matrix expansion; ci, interstitial fibrosis; ct, tubular atrophy; ah, arteriolar hyalinosis; aah, hyaline arteriolar thickening; C4d, complement split product; IFTA, interstitial fibrosis–tubular atrophy.

and after biopsy permitting assessment of eGFR trajectories, reclassification of the diagnosis by the pathologist according to the Banff 2017 criteria, and exclusion of cases with suspected or mixed rejection or overlapping other pathologies. In addition, the retrospective nature of the study in a single center, the lack of an untreated control group, and the heterogeneity in treatments limits any generalizability of our findings. These limitations are shared with many other ABMR treatment studies (5, 15, 36). Another inherent limitation in all ABMR studies is the lack of knowledge whether existing treatments actually work or whether clinical trajectories simply reflect the natural history of the disease in an individual patient. As AMBR remains an important clinical conundrum, we aimed to obtain as "pure" a cohort of patients as possible to reduce potential confounding. The reality is that diagnosis is challenging and there are no established treatments. Treatments are therefore individualized and in such a retrospective study as ours this is an inherent limitation that cannot be controlled for given the small numbers. We, however, felt that the likelihood of bias would be greater if we included less rigorously defined cases than if we included more cases and attempted to stratify by presence of DSA or treatment received. In this case, given the clinical importance of ABMR, we still suggest that our findings are relevant and illustrate the complexity of such a study and will permit improved planning of future studies.

The analysis of the different cases of caABMR was based on clinical markers, such as change in kidney function and degree of proteinuria, histopathology readings and gene expression profiles. Therapy response was defined by the change in eGFR slope from before compared to after therapy. In our study, kidneys with a greater degree of eGFR deterioration before biopsy were more likely to be responders, likely due to a more acute and therefore more treatable process and/or more aggressive therapy. In seeming contradiction may be the observation of more chronic changes in the glomeruli of responders. We do not have a clear explanation for this finding, however, it is known from native kidneys that impairment of GFR correlates better with tubulo-interstitial compared with glomerular injury, which may therefore not contradict our findings here. In fact, although there was a high heterogeneity in the treatment types, comparable with the literature (10, 37), antibody removal and/or rituximab were predominantly applied in the responder group (24). However, it is difficult to differentiate cause and effect. Whether presumed responsiveness lead the physician to add these treatment options or whether these therapies induced the response is in a retrospective, non-randomized analysis not possible to answer. It is unlikely that rituximab and/or plasmapheresis or a combination of rituximab with intravenous immunoglobulins are highly effective treatments of caABMR,

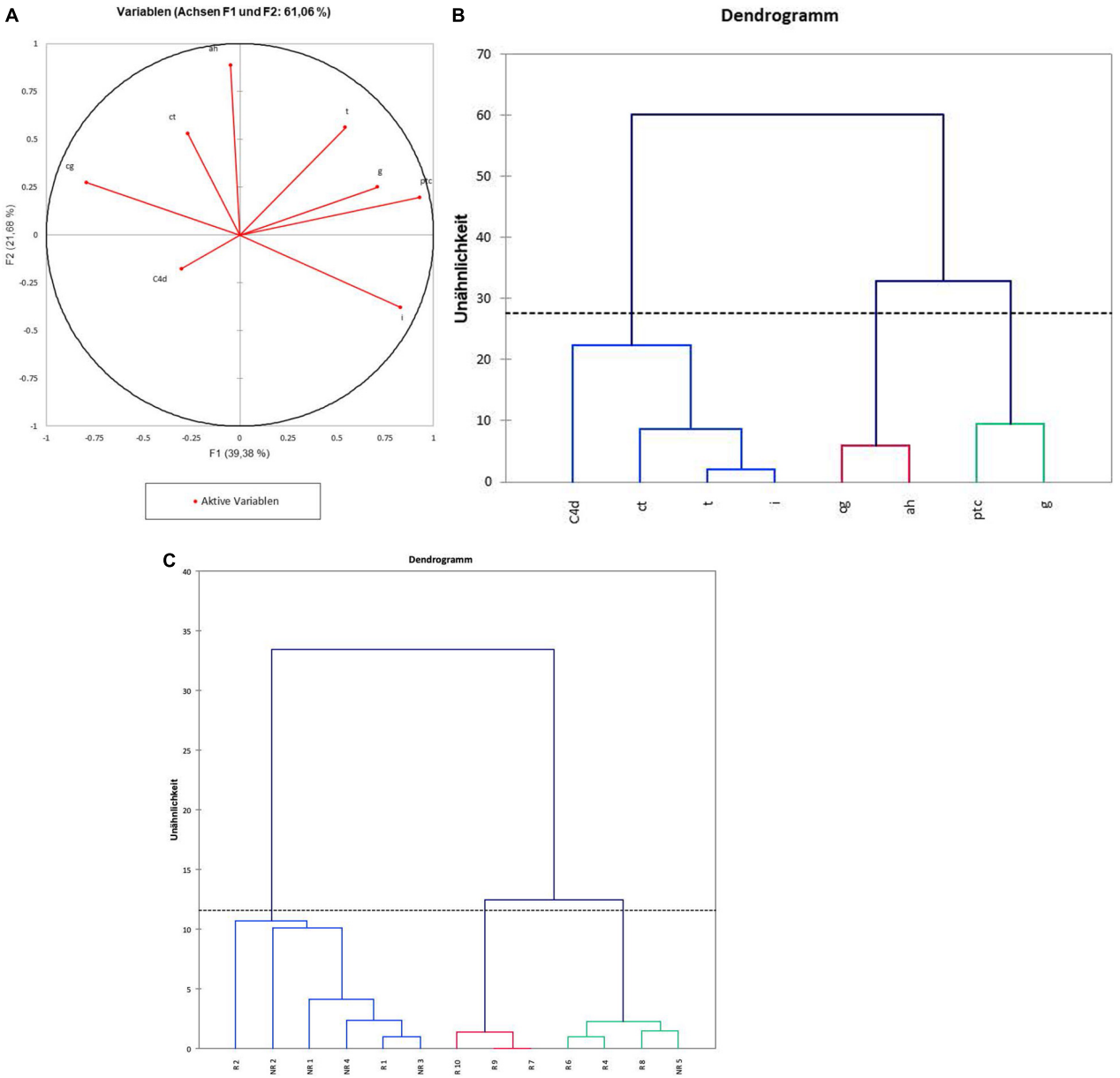


FIGURE 4 | Clustering of histopathology in the caABMR biopsies. **(A)** Principal component analysis indicating distribution and correlation between Banff scores. **(B)** Unsupervised hierarchical clustering of the Banff characteristics based on the samples and **(C)** of the biopsies based on 8 Banff features. Due to high Euclidian distances 3 samples did not include in the analysis.

based on the current literature [(38, 39), Triton Study by Moreso 2018]. Multiple studies indicate a lack of significant and predictable effects of these treatments (40) or any current treatment at all (41).

Proteinuria indicates kidney damage and is associated with mortality in kidney transplant patients (42–44). In our study, proteinuria showed a more pronounced increase after biopsy in non-responders compared to responders. This indicates that the injury process is more active and continuous in non-responders, in agreement with the poorer graft survival seen with proteinuria in patients with caABMR (36, 44, 45). However, the proteinuria values were quite variable across the different patients and the two treatment groups. Our findings indicate that pre-biopsy proteinuria levels are not likely indicating treatment responsiveness. As clinically expected a higher DSA burden and lower graft survival was seen in non-responders compared to responders, however, these differences did not reach statistical difference.

Interestingly, peritubular capillaritis, a histological marker of active microcirculation injury, was significantly increased in the non-responder group. The biological validity of this finding was supported by a parallel increase in glomerulitis scores, again a

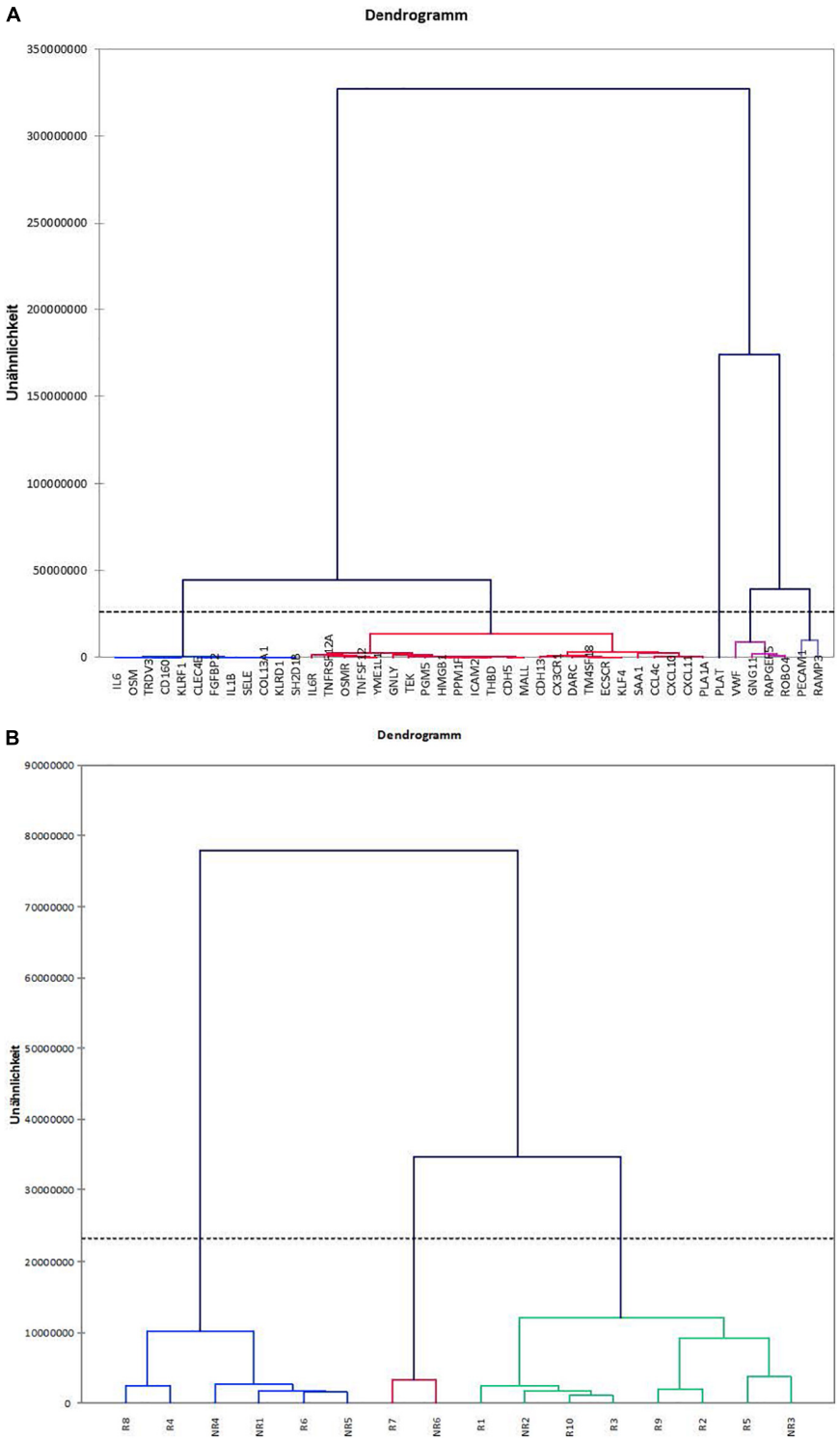


FIGURE 5 | Clustering of transcriptomes in the caABMR biopsies. **(A)** Unsupervised hierarchical clustering of the 44 gene transcripts. The dendrogram shows the clustering of the 44 genes according to similarities and dissimilarities. **(B)** Dendrogram of distribution of responders and non-responders based on gene expression.

feature of active microvascular injury (19). This indicates that a high severity of the active endothelial injury process makes a treatment response unlikely. The degree of microvascular injury is a known risk factor for future graft loss (5, 19). In contrast, to our surprise, transplant glomerulopathy was increased in responders. This might indicate that decreased activity of the

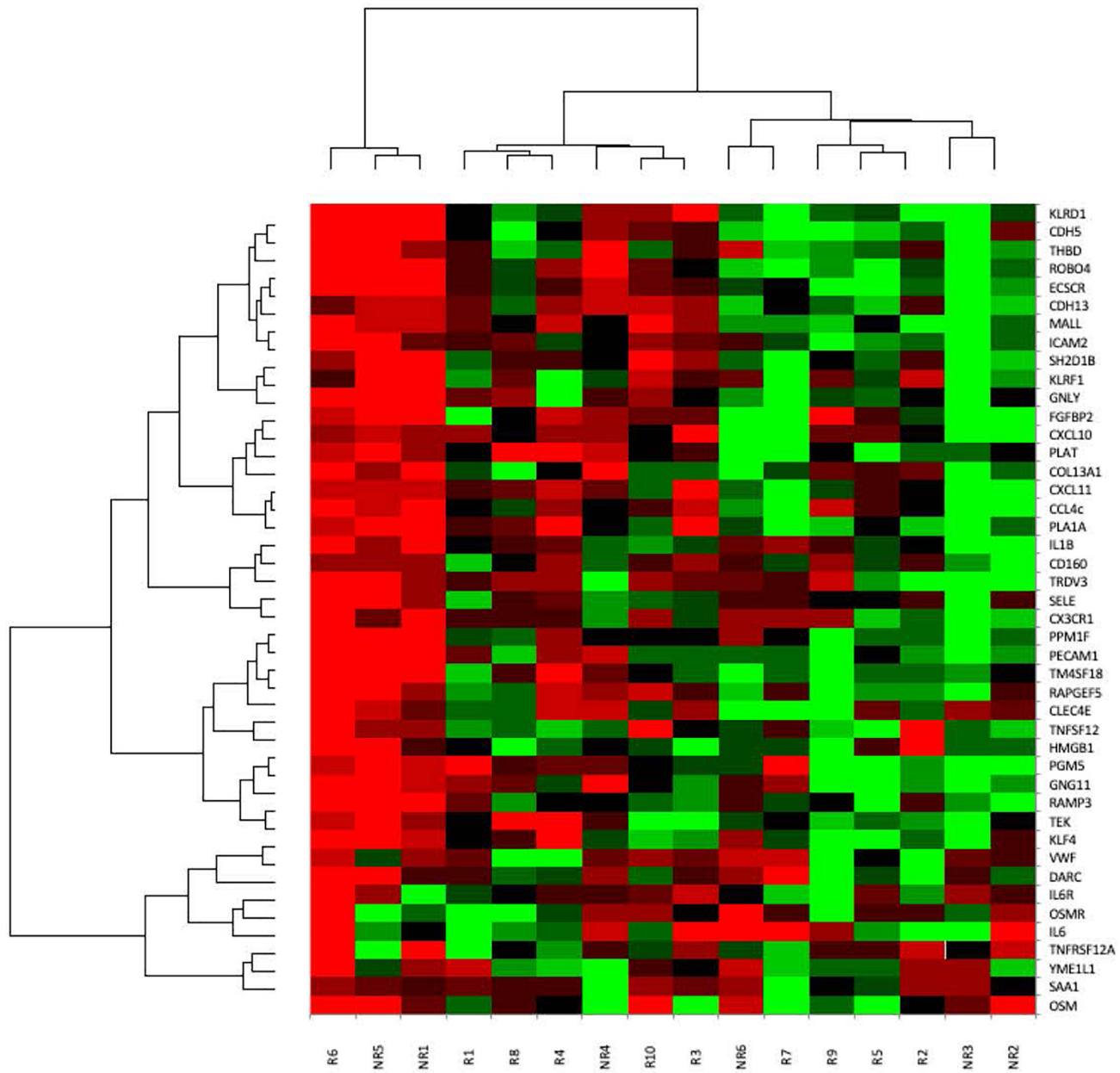


FIGURE 6 | Heatmap analysis of gene expression. Columns represent the 16 different samples, rows the 44 analyzed genes. High gene expression is shown in green, low gene expression in red. Similarity metric was calculated with uncentered Pearson correlation for patients/biopsies and genes; average linkage was the used linkage method.

humoral immune attack and a more chronic phenotype in the vasculature reflects an exhaustion of the rejection process and a repair process in the allografts. However, transplant glomerulopathy has been associated with an increased risk of graft failure (19, 46, 47). Another explanation might be that we defined treatment response by a positive change in the GFR slope during the first 6 months post-biopsy. Hence, the improvement might reflect a transient positive anti-inflammatory effect of the rejection therapy (36). The long-term effect of transplant glomerulopathy might be negative.

Gene expression profiles did not significantly differ between responders and non-responders. However, there was a tendency toward increased endothelial and NK-cell related gene expression in non-responders compared to responders. This would fit with the histological findings of increased peritubular capillaritis. The higher peritubular capillaritis and endothelial gene expression in non-responders may capture the ongoing marked tissue injury not reversible by current ABMR therapies (17, 36, 48).

For the gene expression analysis we have selected, based on literature and pathophysiology, an *a priori* defined set of genes

(16–18, 27) and some additional genes of interest. The analysis of gene expression is well-established, highly reproducible, cost-effective and technically advanced (27). However, limitations of that technology have to be considered. A difference in gene transcription does not necessarily imply a difference in protein level, the mechanisms involved in the various pathways still are not completely understood (49) and vascular or glomerular structures are underrepresented in the whole genome transcriptome profile (since 80% of the cortical tissue in the kidney is tubulo-interstitial tissue). Furthermore, the number of nephrons, the mismatch of donor recipient size, the degree of glomerulosclerosis, and the past medical history (such as diabetes and hypertension) are not likely to be fully reflected in the transcriptome and require the integration of clinical history and histopathology (14). Last but not least inter-laboratory variability and lack of powered studies in gene expression analysis also represent a problem (50).

Hence, the lack of finding significant differences of transcriptome changes in our study might be due to the intrinsic limitations of this technology. In addition, the *a priori* selection of genes might not capture all important pathway changes associated with the rejection process. Another possibility is that the measurements with NanoString^{TM} technology in FFPE samples, some of them of considerable age, might lack the sensitivity and specificity to detect minor changes between possible responder and non-responder profiles. Nevertheless, it was reassuring to see that the biologically defined functional gene groups clustered together in our analysis. This supports the quality of our measurements despite the considerable age of the stored biopsy samples. A major problem is the limited number of cases.

Moreover, transcriptome measurement of a single biopsy represents a snapshot of a process going on for a longer period of time and may not be sufficient. Earlier and repeated biopsies may allow to discover changes over time that reflect tissue injury and regenerative capacity and required to identify clinical responders versus non-responders.

In conclusion, a discrimination of responder vs. non-responder cases in caABMR has not yet been achieved (5, 8, 14, 15, 26, 27, 36, 43). Our results reflect the general lack of successful treatments of caABMR, partly due to the difficulty to identify possible responders from non-responders. We could show that it is possible to measure transcriptome changes in stored FFPE samples and that functional groups of genes cluster together. The rigorous selection process of clear caABMR cases indicated that the majority of biopsies are reflecting overlapping clinical diagnoses and different processes. In addition, it underlined the difficulty to classify biopsy samples based on histopathology. The identification of the severity of microcirculation injury, reflected by histological peritubular capillaritis and endothelial transcriptome activation, indicates the potential to use this for treatment guidance. However, the number of identified pure cases of caABMR in our study is too small for robust recommendations. Nevertheless, approach and results indicate key elements necessary in a future study, in particular it should be prospective, with biopsies characterized by histopathology and molecules and with rigorous functional end-points detecting response to standardized treatment protocols.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192552.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kantonale Ethikkommission Zürich (KEK). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OS and TM participated in design of the work, data collection, data analysis, and writing of the manuscript. OS performed the transcript measurements. AG and DS participated in writing. AG and MR participated in data collection. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.820085/full#supplementary-material

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