TRANSPLANTATION OF MARGINAL ORGANS: IMMUNOLOGICAL ASPECTS AND THERAPEUTIC PERSPECTIVES

EDITED BY: Caner Süsal, Christophe Legendre, Thomas Friedrich Mueller and Peter Schemmer

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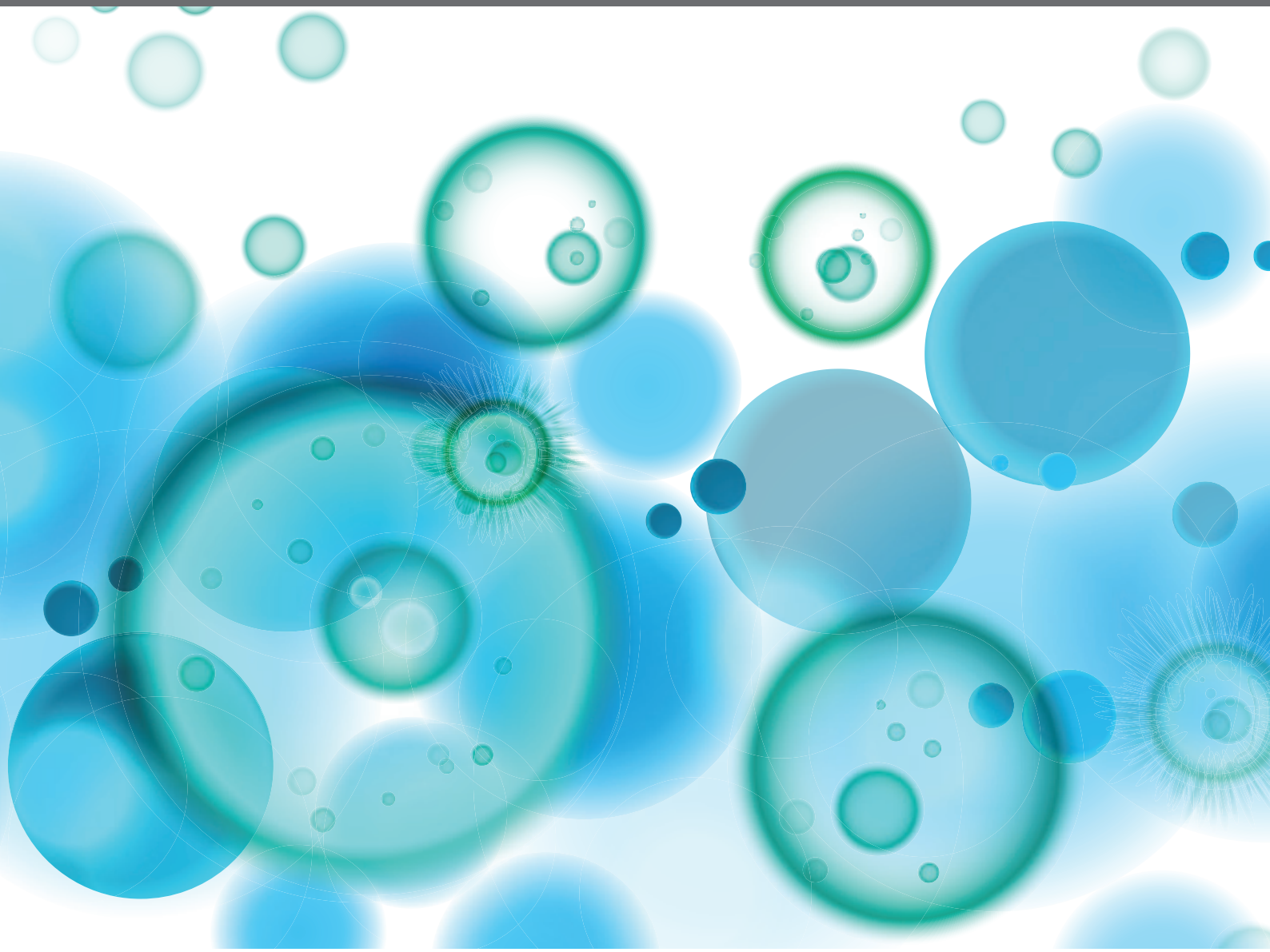

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TRANSPLANTATION OF MARGINAL ORGANS: IMMUNOLOGICAL ASPECTS AND THERAPEUTIC PERSPECTIVES

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EDITORIAL
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Editorial: Transplantation of Marginal Organs—Immunological Aspects and Therapeutic Perspectives

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Keywords: organ transplantation, marginal donors, expanded criteria donors, machine perfusion, organ allocation, graft survival, delayed graft function, antigen silencing

Editorial on the Research Topic

Transplantation of Marginal Organs—Immunological Aspects and Therapeutic Perspectives

In organ transplantation, shortage of available grafts has resulted in a continuously growing use of kidneys, livers, lungs, and hearts from elderly donors, often with comorbidities. The percentage of ≥60-year-old deceased kidney donors reported to the Collaborative Transplant Study (CTS) from Europe was 21% during 2000 to 2001 and increased to as high as 42% during 2016 to 2017 (1). Due to a decreased number of functional tissue and inflammation-mediated alloantigen expression, these marginal organs from expanded criteria donors (ECD) are considered to be more vulnerable to immune attack and show impaired survival rates. Different criteria are used in different organ types to define ECDs. In kidney transplantation, besides a donor age above 59, a history of hypertension, increased creatinine and cerebrovascular cause of death are key factors impacting the quality of the deceased donor organ. Articles in this Research Topic highlight the problems that are encountered in transplantation of marginal organs and propose novel diagnostic and methodological approaches, including improvement of organ allocation strategies, use of alternative therapeutic regimens and utilization of modern machine perfusion (MP) techniques that enable estimation of organ quality, preconditioning of donors, depletion of immune cells and genetic silencing of alloantigens.

Gerbase-DeLima et al. underline with their large single-center analysis of more than 5,000 kidney transplantations the importance of the awareness of risk that is associated with different combinations of donor and recipient age. They report lower death censored graft and patient survival in recipients of kidneys from elderly donors and higher graft but lower patient survival in elderly recipients. Also factors, such as time on dialysis and HLA match, influenced outcome differentially in different recipient and donor age combinations. Noble et al. describe in their comprehensive review immunological aspects of kidney transplantation in elderly recipients with organs from marginal donors and highlight therapeutic options that could help to prevent side effects, such as toxicity and development of cancer. Echterdiek et al. report, despite a negative trend in demographic parameters, an improving outcome of kidneys from ≥70-year-old donors in Europe over the years, most probably due to an improving physiology of elderly donors. This finding could encourage to increase the donor pool also in other parts of the world, such as the United States, where organs from elderly donors are currently discarded at a high rate.

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Eurotransplant (ET) Senior Program (ESP) is an "old-for-old" allocation scheme in which kidneys from ≥65-year-old deceased donors are allocated independent of HLA matching to ≥65-year-old recipients locally in order to prevent further ischemic injury in an already vulnerable organ during the transport to a better matched recipient. Increasingly more kidney transplantations are currently performed *via* ESP and their percentage among all performed deceased donor kidney transplantations rose in ET countries from 9% in 2000 to 18% in 2019 (2). Dreyer and de Fijter advocate in their review the introduction of a restricted form of matching in ESP for only the HLA-DR locus instead of the three HLA loci that are considered in regular kidney allocation. They claim that this procedure can be performed locally without prolonging the cold ischemia time (CIT) and is expected to result in less rejections and mortality. Prolonged CIT is also in liver transplantation a critical factor that affects graft and patient survival. Lozanovski et al. found in their CTS analysis of more than 40,000 liver transplant recipients that the negative influence of CIT on outcome is much stronger in patients with hepatitis C-related cirrhosis than for example in patients with alcoholic cirrhosis or in patients with hepatocellular carcinoma and low "model of end-stage liver disease" scores. They, therefore, suggest that original disease should also be implemented as a criterion in allocation of livers.

Due to the general trend of increasing donor age, delayed graft function (DGF) is observed in a growing proportion of recipients of deceased donor kidney transplants. Morath et al. analyzed the influence of DGF in association with pre-sensitization in kidney transplantations performed between 2008 and 2017. Besides the known non-immunological factors, such as high donor age and prolonged ischemia time, the pre-transplant presence of broad alloantibody reactivity was found to be a significant predictor of DGF, also in this more recent era of transplantation during which sensitive antibody testing was practiced. Development of DGF itself doubled the risk of graft loss which, however, increased further if the patient had HLA or donor-specific HLA antibodies before transplantation. These findings indicate that special measures are necessary during allocation of organs from elderly donors, especially to pre-sensitized patients.

Robust measures of organ quality are required for reliable prediction of successful outcomes in the use of marginal organs. However, organ quality is difficult to assess, in particular, within the narrow window of time to transplantation. In this setting molecular diagnostics could complement the clinical and histopathological evaluation of tissue quality by capturing additional information on immune- and non–immune-mediated injury as well as repair mechanisms. Von Moos et al. reviewed the current state of quality assessment in donor kidneys using clinical scores, histopathology and perfusion characteristics with an emphasis on molecular analyses. They describe shortcomings of available methods and studies. The review highlights advances made in the integration of molecular analyses with clinical data and future studies necessary to perform. Along the same line, Hruba et al. investigated in different donor categories transcriptome profiles of allograft biopsies with borderline changes. When compared with standard criteria or living donor kidneys, ECD kidneys showed higher expression of transcripts related to inflammation and extracellular matrix remodeling. These changes are expected to aggravate alloimmune responses and influence outcomes. Boissier et al. compared the cellular components and the transcriptomic and vasculogenic profiles in the peri-renal adipose tissue of kidneys from ECDs and optimal donors. Peri-renal adipose tissue of ECDs was found to display an age-dependent inflammatory signature that was associated with early graft dysfunction. NK-cell subsets were recruited differentially in the peri-organ environment of kidney grafts from elderly donors. This novel evidence indicate that peri-renal adipose tissue, which can be gained non-invasively and timely, represents a valuable source of donor material to assess inflammatory changes that affect organ quality and function. Corradetti et al. highlight in their case report cholesterol embolism as a rare but severe adverse event that can occur in transplantation of marginal donor kidneys and report its successful treatment with the prostaglandin I2 analog iloprost. Causality still has to be shown here and controlled studies are necessary to assess the true value of the iloprost therapy.

MP is on the way of becoming a core technology in the use of ECD organs that allows not only the estimation of organ quality prior to transplantation but also enables intervention for improved preservation, (re)conditioning and regeneration of organs. Furthermore, it has the potential for a medical revolution toward organ engineering. Resch et al. review in detail the beneficial use of oxygenated hypothermic and normothermic MP and describe findings which indicate that MP prolongs the graft preservation time significantly and allows, during the perfusion procedure, treatments generating chimeric organs and enabling immunological changes, defatting, reduction of inflammation and elimination of hepatitis C. They state that improved graft function after MP can most likely be explained by amelioration of ischemia/reperfusion injury (IRI) and assessment of the graft's viability and function prior to transplantation. Kvietkauskas et al. summarize in their review experimental studies and clinical trials on MP-associated treatment strategies and focus hereby on inhibition of allorecognition pathways. Specifically, they show evidence that MP protects the organ from inadequate activation of innate immunity by decreasing IRI. Unfortunately, established clinical standards in MP-protocols are still missing.

In several experimental models, MHC-silenced cells were shown to be protected against allogeneic immune responses (3, 4). Yuzefovych et al. demonstrate a sub-normothermic ex vivo perfusion system in rats which enables the delivery of lentiviral vectors that encode small hairpin RNAs to permanently silence MHC antigens. If feasible and safe in humans, generation of immunologically silenced organs raise great expectations to solve the major problems of organ transplantation, such as rejection and side effects of immunosuppressive regimens. Donor hearts have significant leukocyte reservoirs which can activate recipient leukocytes and initiate acute rejection upon transplantation.

Critchley et al. demonstrate in an experimental porcine heart transplantation model that, as compared to static cold storage, hypothermic MP with cardioplegic solution reduces immunogenicity of the organ significantly *via* elimination of resident leukocytes. Besides a pro-inflammatory cytokine pattern, a pro-survival- and reduced ischemia-related profile was observed after hypothermic MP that could explain the improvement in graft viability.

In addition to MP-technology, the clinical expertise of transplant surgeons is of great importance to both accept and allocate organs from ECDs to suitable recipients. Kahn et al. compared the outcome of liver transplants from ECDs versus non-ECDs and show evidence that, if a well-standardized allocation strategy based on clinical facts is used, the outcome of ECD livers is not compromised with a 90-day mortality of only 3.6%.

Dezfouli et al. compared, in a large animal model, anti-inflammatory effects of preconditioning of kidney donors after brain death with different therapeutic regimens. Interestingly, oral administration of calcineurin inhibitors as well as inhibitors of mammalian target for rapamycin decreased TNF-alpha expression more effectively than the routinely administered intravenous steroids, indicating that it would be worth to investigate in additional studies the protective effect of oral donor preconditioning on IRI.

The present Research Topic proves that transplantation of ECD organs has become a major issue in organ transplantation and describes solutions to the problem.

AUTHOR CONTRIBUTIONS

CS, TFM, CL, and PS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Kidneys From Elderly Deceased Donors—Is 70 the New 60?

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There is a growing shortage of kidney donors leading to extended transplant waiting times associated with increased mortality. To expand the donor pool, clinicians nowadays regularly accept organs from elderly donors, including those aged ≥70 years. There is only limited and conflicting data whether kidneys from these elderly donors allow for satisfactory allograft outcome rates. To asses this question, the 5-year death censored graft survival of 116,870 adult first deceased donor kidney allograft recipients that were transplanted at European centers between 1997 and 2016 and reported to the "Collaborative Transplant Study" were analyzed using Kaplan–Meier analysis and country stratified Cox regression. The combinations of the two transplant periods 1997–2006 and 2007–2016 with the donor age categories 18–49, 50–59, 60–69, and ≥70 years were considered. From 1997–2006 to 2007–2016, the median donor age increased from 50 to 55 years and the proportion of kidneys from ≥60-year-old donors rose from 24.1 to 38.8%. At the same time, the proportion of kidneys from ≥70-year-old donors more than doubled (6.7 vs. 15.4%). Between 1997–2006 and 2007–2016, the 5-year graft survival improved in all donor age categories. During 2007–2016, the 5-year death censored graft survival of kidneys from ≥70-year-old donors was comparable to that of kidneys from 60 to 69-year-old donors during 1997–2006. This was true both for younger recipients (18–64 years) and older recipients (≥65 years). Among the younger recipients, 45–64-year-old recipients showed the best death censored graft survival rates for kidneys from old donors. In the country-stratified Cox regression analysis, compared to the reference of grafts from 18 to 49-year-old donors, the hazard ratio for grafts from ≥70-year-old donors during 2007–2016 was 1.92, exactly the same as the hazard ratio for grafts from 60 to 69-year-old donors during 1997–2006. Our analysis indicates that within only one further decade (1997–2006 vs. 2007–2016) the 5-year death censored graft survival of kidneys from ≥70-year old donors improved to the level of kidneys from 60 to 69-year-old donors in the previous decade.

Keywords: kidney transplantation, marginal donor, expanded criteria donor, elderly donor, death censored graft survival, donor age

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INTRODUCTION

Kidney transplantation is the therapy of choice for patients with end stage renal disease (ESRD) and is associated with improved survival rates also in elderly recipients aged ≥70 years (1, 2). Donation from a living donor provides the best outcome rates; however, in many cases there is no living donor available, leaving patients to wait for an organ from a deceased donor whilst staying on maintenance dialysis. Due to a widespread shortage in donor organs, the waiting time for a deceased donor kidney often amounts to several years (3). At the same time, maintenance hemodialysis is associated with a mortality that is up to 10 times greater than the mortality of the general population, reaching up to 20% per year (4). This dilemma has urged clinicians to increase the donor pool by accepting kidneys from suboptimal donors. First in 2002, these donors were categorized as expanded-criteria donors (ECD) (5). ECD were defined as either aged 60 years or older at time of death or as aged 50–59 years with two of the following three criteria (a) history of hypertension, (b) serum creatinine >1.5 mg/dl, or (c) death by cardiovascular accident.

There is ample evidence that kidneys from ECD have worse survival rates than kidneys from standard criteria donors (SCD) (6). Nonetheless, the proportion of ECD has strongly increased in the last decade, especially in Europe, amounting to almost 50% of all deceased donors in recent years (7, 8). Nowadays, clinicians regularly transplant kidneys from deceased donors aged 65 and older: in 2018, 25% of all deceased donor kidneys transplanted within the Eurotransplant (ET) region came from donors aged ≥65 years (7). In this aging donor population with—by nature—an increased amount of (potentially unknown) comorbidities, donor selection has become even more important. There is limited and partly conflicting data whether kidney transplants from donors aged 70 or older result in satisfactory allograft outcome rates. Some transplant centers have reported encouraging results for kidneys from ≥70-year-old donors with graft survival rates comparable to kidneys from younger donors by using pre-implantation biopsies and proceeding with either single or dual-kidney transplantation or discarding the organs, depending on the biopsy results (9, 10). However, graft survival rates of kidneys transplanted within the European Senior Program (ESP) (comprising—by definition—only donors aged ≥65 years) have been shown to be slightly worse than the graft survival rates in the regular Eurotransplant Kidney Allocation System (ETKAS) (11). One large study in the United States (US) also showed significantly worse outcomes for kidneys from donors aged 70 years and older when compared to donors aged 50–69 (12). However, no study so far has assessed how the graft survival rates of kidneys from donors aged ≥70 years that were transplanted in recent years compare to survival rates of kidneys from coeval as well as younger donors obtained in the past. To evaluate this matter, we analyzed outcome data from the international Collaborative Transplant Study (CTS) by combining transplant period and donor age.

MATERIALS AND METHODS

Study Design

First deceased donor kidney transplants in adult recipients and donors (age ≥18 years) reported to CTS were analyzed (www.ctstransplant.org). Multi-organ transplants (e.g., kidney and pancreas) were excluded. Analysis was limited to data from transplant centers in Europe (209 centers from 23 countries). The combination of the two transplant periods 1997–2006 and 2007–2016 with the following four donor age categories 18–49, 50–59, 60–69, and ≥70 years were considered. Moreover, kidney transplants from the two transplant periods were also stratified according to four recipient age categories: 18–44, 45–54, 55–64, and ≥65 years.

Statistical Analysis and Outcome

The primary endpoint was 5-year death censored graft survival. Categorical variables were assessed using Fisher's exact test or chi-squared test. For continuous variables, the median with interquartile range (IQR) as well as the mean with standard deviation (SD) are shown. Mann–Whitney–*U*-test was used for statistical analysis of continuous variables. Survival rates were illustrated using the Kaplan–Meier method. Hazard ratios of the influence of the donor age categories with 95% confidence intervals (CI) were calculated with multivariable Cox regression. Analyses were stratified by country to eliminate confounding by different country-based allocation strategies. Other parameters such as donor/recipient comorbidities, cold ischemia time, duration of dialysis, induction therapy, sensitization status, or race were deliberately not considered for Cox regression analysis as the primary goal was to show the real-life changes in 5-year death censored graft survival between the two transplant periods for the different donor age groups. To exclude the influence of age-matched allocation strategies, separate analyses in the subgroups of 18–64 and ≥65-year-old recipients were also performed. The survival rate of the 18–49-year-old donors in the period 1997–2006 served as reference.

Two tailed *P*-values of <0.05 were considered statistically significant. Statistical analysis was conducted using the software IBM® SPSS® Statistics version 25.0 (SPSS Inc., IBM Corporation, Somers, NY, USA).

RESULTS

In total, 116,870 patients were assessed, 59,158 in the transplant period 1997–2006 and 57,712 patients in the transplant period 2007–2016.

The demographics of study patients from both periods are summarized in **Table 1**. The median donor age increased from 50 years during 1997–2006 to 55 years during 2007–2016 ($P < 0.001$). Within the donor population, the proportion of 60–69-year-old donors increased significantly from 17.3% during 1997–2006 to 23.4% during 2007–2016 ($P < 0.001$). The absolute number of donors aged ≥70-years more than doubled (3,996 during 1997–2006 vs. 8,874 during 2007–2016) and their relative

TABLE 1 | Demographics of study patients.

Characteristic	Unknown (%)	Transplant period		*P*
		1997–2006 *n* = 59,158	2007–2016 *n* = 57,712	
Recipient sex	0.0			<0.001
Female		22,185 (37.5)	20,806 (36.1)	
Male		36,964 (62.5)	36,894 (63.9)	
Recipient age (years)	–			<0.001
Median [IQR]		51 [40–60]	56 [46–64]	
Mean ± SD		49.6 ± 12.8	53.9 ± 12.8	
18–64		51,387 (86.9)	44,128 (76.5)	<0.001
≥65		7,771 (13.1)	13,584 (24.5)	
Donor age (years)	–			<0.001
Median [IQR]		50 [38–59]	55 [45–65]	
Mean ± SD		48.2 ± 14.9	54.0 ± 15.0	
18–49		29,477 (49.8)	20,050 (34.7)	<0.001
50–59		15,441 (26.1)	15,255 (26.4)	
60–69		10,244 (17.3)	13,533 (23.4)	
≥70		3,996 (6.7)	8,874 (15.4)	
Cause of donor death	5.8			<0.001
Trauma		15,445 (27.9)	9,549 (17.4)	
Cerebrovascular		34,179 (61.8)	35,009 (63.8)	
Other		5,658 (10.2)	10,276 (18.7)	
Donation after cardiac death	3.2	2,218 (3.9)	7,395 (13.2)	<0.001
Donor history of hypertension	3.4	7,194 (12.5)	8,598 (15.4)	<0.001
Cold ischemia time (hours)	7.9			<0.001
Median [IQR]		17 [13–21]	14 [11–18]	
Mean ± SD		17.7 ± 7.0	14.8 ± 5.6	
HLA-A+B+DR mismatches	10.9			<0.001
Mean ± SD		2.9 ± 1.4	3.3 ± 1.4	
0–1		8,199 (15.1)	5,139 (10.3)	<0.001
2–4		39,330 (72.3)	34,605 (69.5)	
5–6		6,816 (12.5)	10,051 (20.2)	

IQR, interquartile range; SD, standard deviation; numbers in brackets represent percentages if not otherwise indicated.

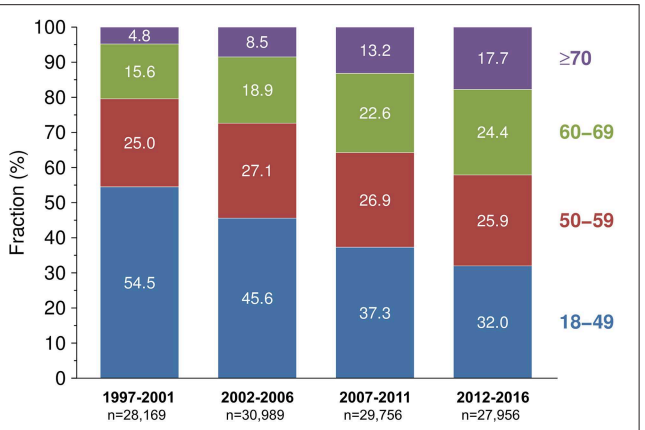

FIGURE 1 | Development of donor age in European adult recipients of first deceased donor kidney transplants across different time periods.

proportion rose from 6.7 to 15.4% ($P < 0.001$). The median recipient age also increased over time (51 vs. 56 years; $P < 0.001$). **Figure 1** visualizes the development of donor age in 5-year intervals over the course of the 20 years assessed: From 1997–2001 to 2012–2016, the proportion of ≥70- as well as 60–69-year-old donors increased from 4.8 and 15.6% to 17.7 and 24.4%, respectively. This was paralleled by a decline of 18–49-year-old donors from 54.5 to 32.0% ($P < 0.001$).

Furthermore, the total number and especially the number of 5–6 HLA-mismatched transplants increased significantly between 1997–2006 and 2007–2016 (2.9 vs. 3.3 and 12.5 vs. 20.2%, respectively; $P < 0.001$ for both comparisons). The donors had significantly more often a history of hypertension (12.5 vs. 15.4%; $P < 0.001$), the cause of donor death was significantly less often trauma (27.9 vs. 17.4%; $P < 0.001$), and donation after cardiac death became more frequent (3.9 vs. 13.2%; $P < 0.001$). Cold ischemia time was the only parameter which improved, i.e., it decreased in median from 17 to 14 h ($P < 0.001$).

Although a negative trend was evident in the majority of the demographic parameters, the 5-year death censored graft survival improved significantly across all donor age groups from 1997–2006 to 2007–2016, including younger recipients aged 18–64-years as well as older recipients aged ≥65-years (**Figure 2**). In detail: in 18–64-year-old recipients, the 5-year death censored graft survival of kidneys from ≥70-year-old donors during 2007–2016 was superior compared to kidneys from 60 to 69-year-old donors during 1997–2006 (82.9% [95% CI 81.2–84.4%] vs. 79.7% [95% CI 78.7–80.6%], log rank $P < 0.001$, **Figures 2A,B**). The 5-year death censored graft survival of kidneys from 60 to 69-year-old donors in 2007–2016 improved to the level of kidneys from 50 to 59-year-old donors in 1997–2006 (84.4% [95% CI 83.5–85.2%] vs. 84.1% [95% CI 83.4–84.7%], $P = 0.27$).

In ≥65-year-old recipients, the 5-year death censored graft survival of kidneys from ≥70-year-old donors during 2007–2016 was similar to kidneys from 60 to 69-year-old donors transplanted during 1997–2006 (78.6% [95% CI 77.3–79.8%] vs. 79.1% [95% CI 77.3–80.7%], $P = 0.60$, **Figures 2C,D**). Likewise, the 5-year death censored graft survival of kidneys from 60 to 69-year-old donors transplanted during 2007–2016 was comparable to kidneys from 50 to 59-year old donors during 1997–2006 (84.7% [95% CI 83.4%−85.9%] vs. 85.7% [95% CI 83.4%−87.6%], P=0.45). The same results were obtained when comparing all cause graft survival among the different donor age groups across the two transplant periods—both in young recipients (18–64 years) and old recipients (≥65 years; **Figure 3**).

The influence of recipient age on 5-year death censored graft survival was also assessed for the two different transplant periods (**Figure 4**). Except for 18–44-year-old recipients, kidneys from young donors (aged 18–59 years) showed similar survival rates in all recipient age groups of the two considered transplant periods (global log rank $P = 0.87$ and $P = 0.82$, respectively). Kidneys

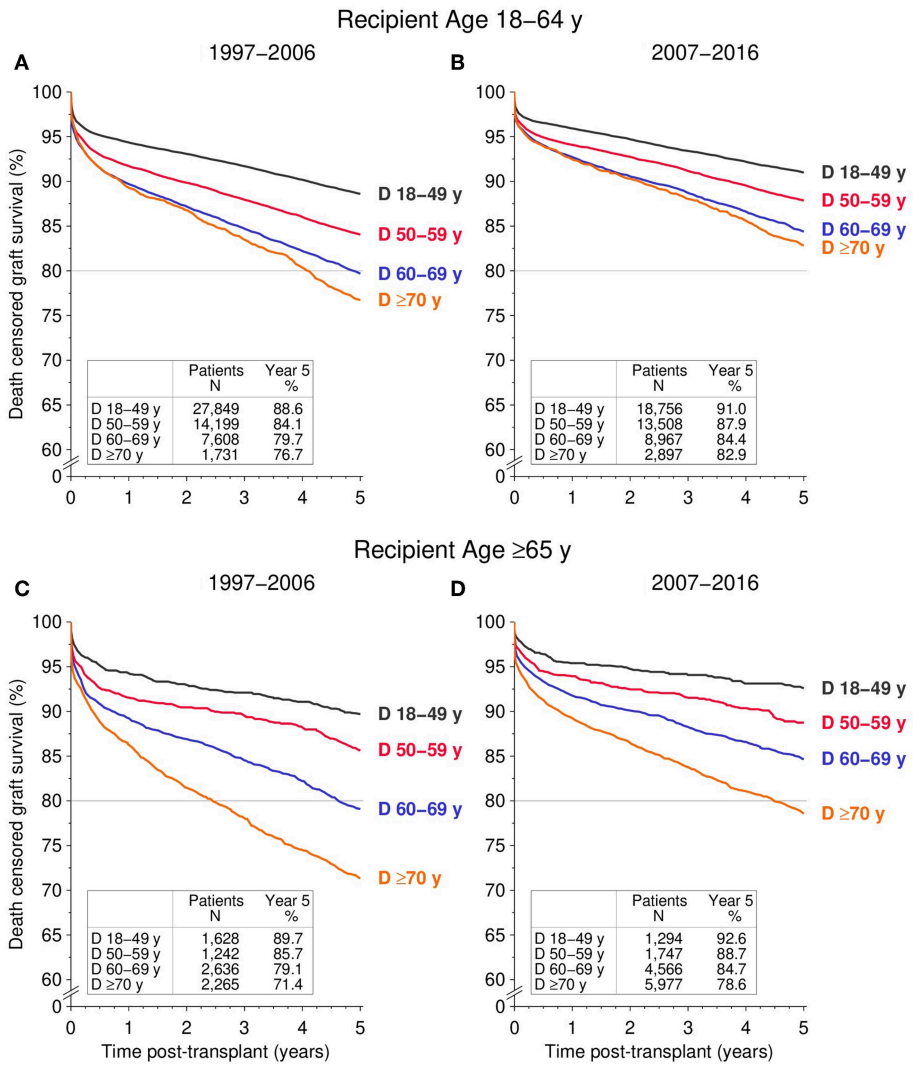

FIGURE 2 | Influence of donor age (D) on death censored graft survival during the first 5 post-transplant years, stratified by recipient age and transplant period. **(A,B)** Display 5-year death censored graft survival for recipients aged 18–64 transplanted during the **(A)** 1997–2006 and **(B)** 2007–2016 period; **(C,D)** display 5-year death censored graft survival for ≥65-year-old recipients for **(C)** 1997–2006 and **(D)** 2007–2016 (all global log rank $P < 0.001$).

from older donors (aged ≥60 years) had significantly worse 5-year death censored graft survival rates in 18–44-year-old as well as in ≥65-year-old recipients compared to 45–64-year-old recipients, regardless of transplant period (all log rank $P < 0.001$).

In the Cox regression analysis of death censored graft loss stratified by country, the 5-year graft loss of kidneys from 18 to 49-year-old donors transplanted in the 1997–2006 period was taken as reference (**Table 2**). When all recipients were analyzed together, the hazard ratio for graft loss of kidneys from ≥70-year-old donors during 2007–2016 was 1.92 (95% CI 1.80–2.05), the same as the hazard ratio for kidneys from 60 to 69-year-old donors during 1997–2006 (95% CI 1.81–2.03; $P = 0.96$). In addition, the hazard ratio for kidneys from 60 to 69-year-old donors during 2007–2016 was the same as the hazard ratio for kidneys from 50 to 59-year-old donors during 1997–2006 (1.45, 95% CI 1.37–1.54 and 1.38–1.53, respectively; $P = 0.96$).

In 18–64-year-old recipients, the hazard ratio for graft loss of kidneys from ≥70-year-old donors during 2007–2016 was lower (1.68, 95% CI 1.51–1.87) compared to that of kidneys from ≥70-year- as well as 60–69-year-old donors during 1997–2006 (2.38; 95% CI 2.13–2.65; $P < 0.001$ and 1.93; 95% CI 1.81–2.06; $P = 0.017$, respectively) and slightly (but significantly) worse than the hazard ratio for kidneys from 50 to 59-year-old donors during 1997–2006 (1.45; 95% CI 1.37–1.54; $P = 0.008$). The hazard ratio for kidneys from 60 to 69-year-old donors during 2007–2016 was comparable to that for kidneys from 50 to 59-year-old donors during 1997–2006 (1.46, 95% CI 1.37–1.57 and 1.45; 95% CI 1.37–1.54, respectively; $P = 0.83$).

In ≥65-year-old recipients, the hazard ratio for graft loss of kidneys from ≥70-year-old donors during 2007–2016 was lower compared to ≥70-year-old donors during 1997–2006 (2.15, 95% CI 1.80–2.57 and 2.70, 95% CI 2.24–3.25, respectively; $P < 0.001$)

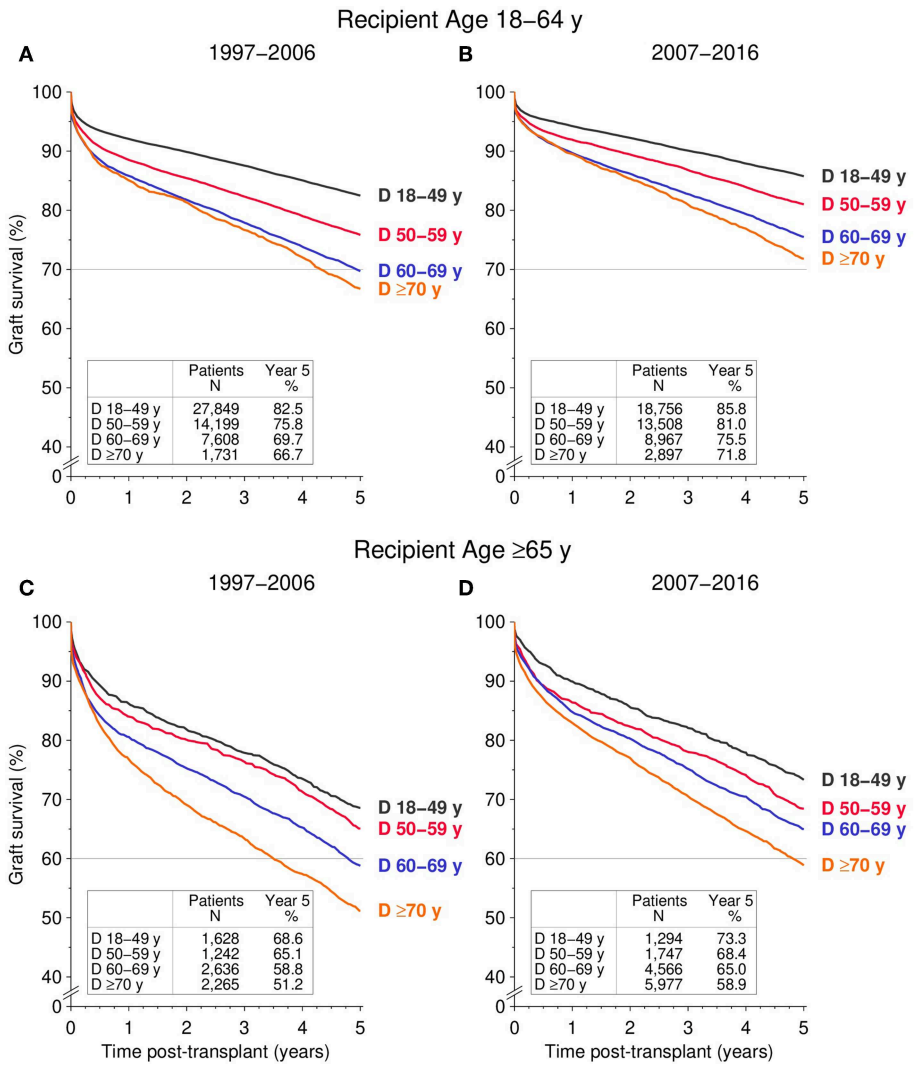

FIGURE 3 | Influence of donor age (D) on all cause graft survival during the first 5 post-transplant years, stratified by recipient age and transplant period. **(A,B)** Display 5-year graft survival for recipients aged 18–64 transplanted during the **(A)** 1997–2006 and **(B)** 2007–2016 period; **(C,D)** display 5-year graft survival for ≥65-year-old recipients for **(C)** 1997–2006 and **(D)** 2007–2016 (all global log rank $P < 0.001$).

and only slightly (but not significantly) worse compared to 60–69 year-old-donors during 1997–2006 (1.96, 95% CI 1.63–2.36; $P = 0.097$). The hazard ratio of kidneys from 60 to 69-year-old donors during 2007–2016 and that of kidneys from 50 to 59-year-old donors during 1997–2006 were nearly equal (1.53, 95% CI 1.27–1.83 and 1.52; 95% CI 1.21–1.90, respectively; $P = 0.95$).

DISCUSSION

We could demonstrate that within only one decade the 5-year death censored graft survival rates of kidneys from donors aged ≥70 years improved to a level that was comparable to the graft survival of kidneys from donors aged 60–69 years in the previous decade. Moreover, as may have been expected—a significant 5-year increase in median donor age was observed during the same time period. Remarkably, the proportion of donors aged ≥70 years more than doubled from 6.7 to 15.4%. Regardless of the changes in donor age distribution, graft survival improved significantly in all donor age groups over the assessed time period.

Increasing donor age is widely recognized as one of the most important risk factors for poor kidney allograft survival (13–15). As a consequence, the discard rate of kidneys from elderly donors is strongly elevated, especially in donors aged ≥65 years (10, 16–18). Nonetheless, we were able to show that the graft survival of kidneys from donors aged ≥70 years improved, in the short time interval of one decade, to the level previously seen for kidneys from donors aged 60–69 years. At the same time, the graft survival of kidneys from 60 to 69-year-old donors improved to the level of 50–59-year old donors from the previous decade. In times of universal organ shortage, these are remarkable findings especially considering the high organ discard rate in old donors mentioned above. Of course, it has to be pointed out that kidneys

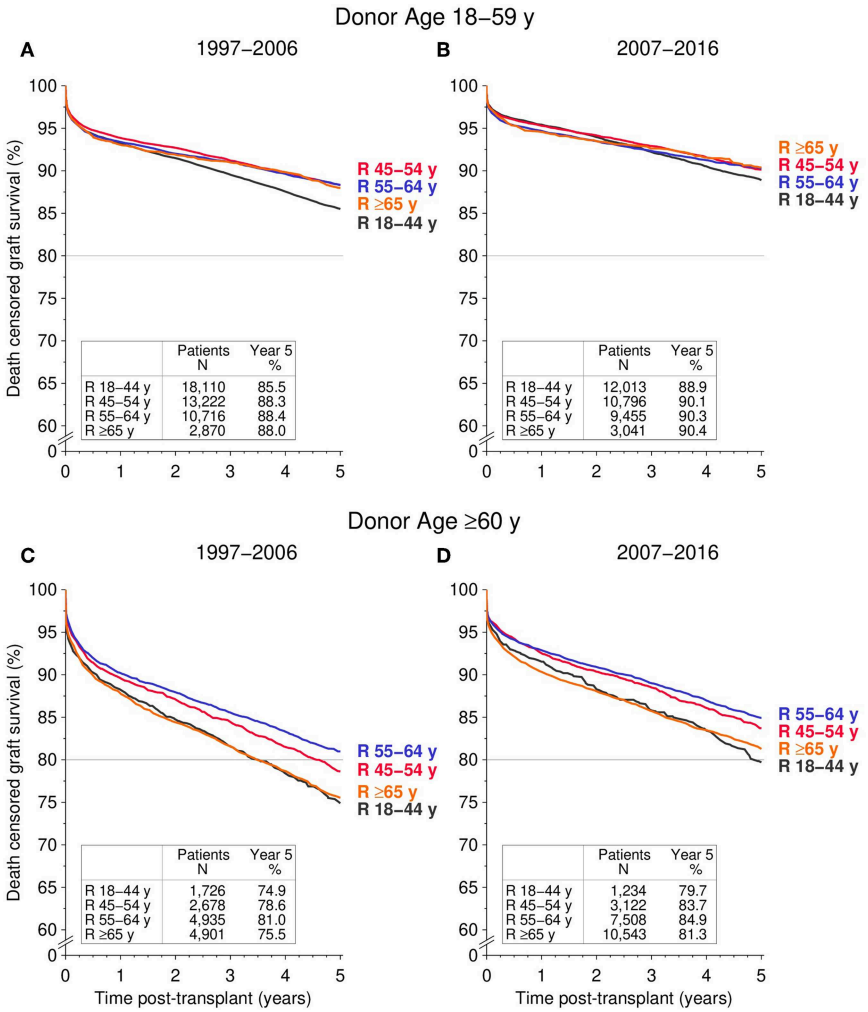

FIGURE 4 | Influence of recipient age (R) on death censored graft survival during the first 5 post-transplant years, stratified by donor age and transplant period. **(A,B)** Display 5-year death censored graft survival for donors aged 18–59 transplanted during the **(A)** 1997–2006 and **(B)** 2007–2016 period; **(C,D)** display 5-year death censored graft survival for ≥60-year-old donors for **(C)** 1997–2006 and **(D)** 2007–2016 [global log rank **(B)** $P = 0.12$; **(A,C,D)** $P < 0.001$].

from younger donors still perform distinctly better than kidneys from older donors and that increasing donor age remains a negative predictor of graft survival. However, the absolute and relative improvements in 5-year death censored graft survival and all cause graft survival of kidneys from older donors over the course of just one decade are astonishing. This is an important, reassuring finding for clinicians when deciding on whether to accept an organ offer from an elderly donor or not.

Several previous publications had shown fairly poor survival rates for kidney grafts from old donors transplanted into young recipients (11, 12, 19). However, our data show, that the hazard ratio of kidneys from ≥70-year-old donors transplanted into 18–64-year-old recipients decreased—within only one decade—from 2.38 to 1.68, which is better than the hazard ratio reported for 60–69-year-old kidney donors during 1997–2006 (1.93) and only slightly worse than the hazard ratio reported for 50–59-year-old kidney donors (1.45). It needs to be pointed out though that not all young recipients fare alike with kidneys of older donors. We demonstrate that it is the group of 45–64-year-old recipients that show the best 5-year death censored graft survival rates whereas 18–44-year-old recipients have a significantly reduced graft survival. Therefore, if kidneys from older donors (age ≥60 years) are transplanted into younger recipients (<65 years), they should be chosen primarily for the group of 45–64-year-old recipients. Moreover, out data indicate that a strict old-for-old allocation concept puts 45–64-year-old recipients at a disadvantage as they also profit from a ≥60-year-old-donor.

We can only speculate about the main factors that are responsible for the improved graft survival rates: post-transplant surveillance has improved, ranging from more frequent, in some centers to even per-protocol kidney biopsies with more standardized histological evaluation; close surveillance of individualized immunosuppressive drug levels, regular screening for development of donor-specific antibodies, effective antiviral prophylaxis and better diagnosis and treatment of concomitant, cardiovascular and renal risk factors. Furthermore, there have

TABLE 2 | Results of the Cox regression analysis for influence of donor age on death censored graft loss during the first 5 post-transplant years.

Transplant period and donor age		*N*	HR	95% CI	*P*-value
All recipients					
1997–2006	18–49 years	29,477	1 (ref)	–	–
	50–59 years	15,441	1.45	1.38–1.53	<0.001
	60–69 years	10,244	1.92	1.81–2.03	<0.001
	≥70 years	3,996	2.53	2.35–2.73	<0.001
2007–2016	18–49 years	20,050	0.80	0.75–0.85	<0.001
	50–59 years	15,255	1.10	1.03–1.17	0.003
	60–69 years	13,533	1.45	1.37–1.54	<0.001
	≥70 years	8,874	1.92	1.80–2.05	<0.001
Recipients 18–64 years					
1997–2006	18–49 years	27,849	1 (ref)	–	–
	50–59 years	14,199	1.45	1.37–1.54	<0.001
	60–69 years	7,608	1.93	1.81–2.06	<0.001
	≥70 years	1,731	2.38	2.13–2.65	<0.001
2007–2016	18–49 years	18,756	0.80	0.75–0.85	<0.001
	50–59 years	13,508	1.10	1.03–1.17	0.006
	60–69 years	8,967	1.46	1.37–1.57	<0.001
	≥70 years	2,897	1.68	1.51–1.87	<0.001
Recipients ≥65 years					
1997–2006	18–49 years	1,628	1 (ref)	–	–
	50–59 years	1,242	1.52	1.21–1.90	<0.001
	60–69 years	2,636	1.96	1.63–2.36	<0.001
	≥70 years	2,265	2.70	2.24–3.25	<0.001
2007–2016	18–49 years	1,294	0.77	0.59–1.02	0.064
	50–59 years	1,747	1.23	0.99–1.55	0.066
	60–69 years	4,566	1.53	1.27–1.83	<0.001
	≥70 years	5,977	2.15	1.80–2.57	<0.001

Hazard ratios (HR) with 95% confidence interval (CI) of donor age are shown.

been advances in the pre- and peri-transplant period including more sensitive alloantibody detection, revised allocation procedures and improved kidney storage and preservation. The impact of different immunosuppressive agents on graft survival is controversial with some studies suggesting superior outcomes with tacrolimus and mycophenolate but other large studies showing no difference (20–24). What seems more important is to tailor the choice of immunosuppressive agents to the immunological risk profile of each patient as well as to consider individual patient risk factors such as co-morbidities and the clinical course after transplant, especially in elder recipients (25). All factors mentioned above might—to varying degrees—have contributed to the improved survival rates in transplants from elderly donors (26–28).

The general increase in 5-year death censored graft survival is even more noteworthy considering the increased immunological risk that clinicians were willing to take in the more recent transplant period. The mean number of HLA-mismatches increased significantly, from 2.9 during 1997–2006 to 3.3 during 2007–2016. Furthermore, the number of kidney transplants with 5 or 6 HLA-mismatches increased from 12.5 to 20.2%. It has been well-documented that the number of HLA-mismatches is strongly associated with worse long-term graft survival (29, 30). Apparently, the aforementioned improvements both in the peri- and post-transplant management seem to have outweighed the enhanced immunological risk.

There have been previous studies on kidney transplantations from elderly donors aged ≥70 years. Several Italian and British studies have shown that performing pre-implantation biopsies of donor kidneys aged ≥70 years and then proceeding with either dual or single transplantation or discarding the organs depending on the histological evaluation resulted in kidney graft survival rates that were equal to survival rates of organs from younger donors (9, 10, 31). In contrast, studies on the European Senior Programme have reported slightly worse survival rates for kidneys from donors aged ≥65 years when compared to the regular ETKAS programme (11). There is also a large study from the United States that presented inferior survival data for kidneys from donors aged ≥70 years (12). Of note, in all these studies the survival of kidneys from older donors was compared to that of younger donors from the same time period. Our data are novel as we compared the (death censored) graft survival of different kidney donor age groups to the same age cohorts transplanted 10 years earlier. This allowed us to appreciate the significant improvements, especially for elderly donor kidneys, that have been achieved over the last 20 years. Our data also stress that this improvement was necessary for we have also seen a remarkable change in kidney donor characteristics in Europe. The median donor age increased to 55 years and 17.7% of donors were ≥70 years old during 2012–2016. At the same time, the proportion of 18–49-year-old donors decreased from 55% in 1997–2001 to 32% in 2012–2016. Nowadays, ECD seem to have almost become the new average donor, at least in Europe. Interestingly, these trends are not observed in the US where kidneys from ≥65-year-old donors still comprise <5% of the donor pool with no upward trend during the last decade (18). In contrast, our data from European centers demonstrate that even kidney allografts from donors aged ≥70 years can be accepted with good outcome rates for selected recipients.

Our study has several limitations. First, we cannot fully exclude a potential center selection bias as the data in our dataset were not collected at random but rather stemmed from the participants of the CTS, a voluntary network of transplant centers worldwide. However, the data of this study originated from more than 200 centers in 23 European countries, comprising a total of 116,870 patients. About two thirds of the data set came from countries, where all (or nearly all) of the countries' transplant centers report their data to CTS. Moreover, the CTS has excellent follow-up completeness rates of 97% 1 year and 95% 5 years post-transplant (32). Therefore, we consider the impact of a potential center selection bias to be marginal. Second, our study focussed on donor age. We deliberately did not consider other parameters that are known to be associated with graft survival such as donor/recipient comorbidities (arterial hypertension/diabetes mellitus), duration of dialysis, cold ischemia time, or number of HLA-mismatches in the Cox regression analysis. This was done as we wanted to illustrate the real-life improvements in graft survival for kidneys from old donors that have been achieved

within just one decade irrespective of potential changes in other variables. However, looking at the parameters that were available within the CTS, except for cold ischemia time, all factors with a negative impact on graft survival were more frequent in the more recent transplant period (number of HLA-mismatches, donor history of arterial hypertension, donation after cardiac death, cerebrovascular accident as cause of death, high recipient age). Cold ischemia time was the only measured parameter that was in favor of the second transplant period as it was found to have diminished from 1997–2006 to 2007–2016. We are aware that this brief overview is by no means equivalent to a full regression analysis correcting for potential confounders; however, from the data available we have no evidence that the pattern of our results could be due to a strong bias. Third, we focused our analysis primarily on death censored graft survival and did not report on patient survival. We chose to do so because the life expectancy of kidney transplant patients increased over the course of the 20-year time period assessed in the paper (33). However, this fact itself already improves patient survival thus impairing a correct analysis of this parameter. Death censored graft survival purely reflects graft function independent of patient survival data which is why we chose to focus on it. Importantly, the all cause graft survival data reported by us confirm the findings from the analysis of death censored graft survival. Forth, we do not report follow-up data beyond 5-years post-transplant, because the long-term data available for the second transplant period (2007–2016) is still rather incomplete after year 5. Hence, we do not know if the findings demonstrated in this study will persist long-term. However, the hazard ratio of (death censored) graft survival usually remains approximately constant after the first-year post-transplant, suggesting that the long-term effects will be similar to our findings 5-years after transplantation.

In conclusion, we demonstrate that within only one decade, namely from 1997–2006 to 2007–2016, the 5-year death censored graft survival of kidneys from ≥70-year-old donors improved to a level of kidneys from 60 to 69-year-old donors in the previous decade. The same improvement was observed also for kidneys from 60 to 69-year-old donors compared to kidneys from 50 to 59-year-old donors transplanted one decade earlier. Considering the unmet lack of donor organs, these results may help to further expand the kidney donor pool especially for recipients aged ≥45 years.

DATA AVAILABILITY STATEMENT

The raw data are available upon request to the Collaborative Transplant Study in accordance with the consents of the patients, the participating transplant centers and registries.

ETHICS STATEMENT

The Collaborative Transplant Study involving human participants was reviewed and approved by the ethics committee of the Medical Faculty of Heidelberg University (No. 083/2005) and performed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles in the currently valid version. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FE, VS, BD, and CS drafted and wrote the manuscript. FE, VS, JL, DK, UH, and CS conceived the idea of the manuscript. BD and CS provided the CTS database and performed statistical analysis. All authors reviewed and edited the manuscript and provided final approval for publication.

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REVIEW
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Transplantation of Marginal Organs: Immunological Aspects and Therapeutic Perspectives in Kidney Transplantation

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Recent data from the World Population Prospects projects that, by 2050, nearly all regions in the world will have a quarter or more of the population aged 60 and above. Chronic kidney disease (CKD) has a high global prevalence (∼13%) worldwide, and the prevalence of chronic kidney disease and end-stage kidney disease increase with age. Kidney transplantation remains the best therapeutic option for end-stage kidney disease, offering a survival benefit in comparison with dialysis maintenance for most patients. This review focuses on immunological aspects of kidney transplantation in older patients and marginal donors, i.e., 60 years or older deceased kidney donors or 50–59 years old deceased kidney donors with comorbidities. Clinical outcomes of kidney recipients in terms of renal and patient survival are more than acceptable even for patients over 70. In this population, the first cause of graft loss is death with a functional graft. However, the inherent issues of these transplantations are the acceptance or refusal of frail kidney from an old donor and the increased immunogenicity of these organs in balance with potential frail and immunosenescent recipients. Finally, the immunosuppressive regimen itself is a challenge for the future of the transplant, to prevent adverse effects such as nephrotoxicity and higher risk of infections or cancer in a population already at risk. Belatacept may have a good place in the immunosuppressive strategy to improve efficacy and the safety posttransplantation.

Keywords: kidney transplantation, extended criteria donors, aging, immunosenescence, graft survival

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INTRODUCTION

Chronic kidney disease (CKD) has a high global prevalence worldwide. The prevalence of CKD and end-stage kidney disease (ESKD) increase with age: 27.6% between 60 and 70 years old and 34.3% above 70 years old when taking into account the five stages of CKD (1).

Kidney transplantation is the best therapeutic option for ESKD. Results of kidney transplantation in terms of morbidity and mortality, life quality, and cost effectiveness are better as compared to hemodialysis or peritoneal dialysis (2). However, kidney transplantation, as well as all other solid organ transplantations, is confronted with an organ shortage. To increase the pool of organ donors, the American United Network for Organ Shortage decided to accept organs from Extended Criteria Donors (ECD). The term marginal kidney was replaced by ECD kidney

for the first time in 1997 by Kauffman (3). In 2002, a clear definition was given: ECD are defined by deceased donors aged 60 years or older and 50–59 years old deceased donors with at least two of the three following criteria: cerebrovascular cause of death, terminal serum creatinine higher than 1.5 mg/dl (132.6 μmol/L), or history of hypertension (4, 5). Other definitions and aspects of "marginal kidneys" have been studied by different authors such as kidney fibrosis based on histopathology, dual kidney transplantation, donation after cardiac death (DCD), and discarded kidneys (6–9). In 2019, in Europe, ∼30% of potential donors are ECD. In North America, ∼24% of potential donors are ECD, and nearly 40% of these kidneys are discarded each year (10). ECD kidneys do not follow the classical allocation system of standard kidneys and allow to shorten the time on waiting list at the expense of a better graft (11–13).

MECHANISMS OF ORGAN AGING

Aging has been described as the decline of physiological integrity due to an accumulation of damages, deterioration of proteins, and organelle functions (14). We use the term of senescence to relate biological and functional changes in cells due to aging. Senescence, which is a state of permanent cellular cycle arrest, may occur following a decline over time of cell proliferation capacity as shown by Hayflick (15). Different stimuli may trigger this cellular phenotype such as cells undergoing major DNA damages, telomere dysfunction, and oxidative stress (16). To prevent the risk of malignant transformation, cells may undergo apoptosis, or become senescent. The senescent state is mediated by two cellular pathways: p53/p21 and p16INK4a/pRB pathways (17). This phenotype is also a proinflammatory phenotype, with a high level of inflammatory cytokines and chemokines secretion [e.g., interleukin (IL)-6, IL-8, IL-1]. This induces chronic inflammation in the organs (18).

Senescence in renal cells may be described at different levels using a top–down approach. At a genetic level, Kim et al. described a set of age-related genes (985) in kidneys in 74 healthy patients from 27 to 92 years old (19). Most of these genes showed increased activity and were shared both in the kidney medulla and cortex. Those age-related genes were also shared in other human tissues. These genes involved in kidney aging are for instance the *mortalin-2*, which encodes the heat shock protein 70. Other genes prevent kidney aging, such as the one encoding the *insulin-like growth factor receptor*. However, it is unclear if senescence and age-related genes activations in the organs are genetically or epigenetically inherited. A recent study assessed aging signature in 563 human kidney transcriptomes using next generation RNA sequencing correlated with genomic data and epigenomic data in kidney and non-renal tissues. Finally, the authors identified a total of 19 kidney age-related genes. Five of them were kidney specific (*EDH3*, *ERP27*, *MAP4*, *PPPAR3C*, and *SNX24*). However, these results are preliminary, and to our knowledge, no other team have reproduced this association. Ten of them were associated with biological and clinical signs of aging. Testis-specific Y-like 5 (*TSPYL5*) was the gene with the most significant association with aging (20). *TSPYL5* is one of the nucleosome proteins and plays a role in transcriptional regulation, cell cycle, and probably in cellular senescence (21, 22).

At a molecular level, many mechanisms of kidney aging have been described and well-reported in the review published by López-Otín et al. (14). One of them implies autophagy dysregulation. Autophagy is a physiological process in which cytoplasmic proteins and organelles are non-selectively degraded. Autophagy is critical for terminally differentiated podocytes that are rarely renewed. Autophagy dysregulation results in the accumulation of intracytoplasmic proteins. This eventually results in podocyte degeneration, responsible for age-related glomerulosclerosis and proteinuria (23). Another mechanism of kidney aging is the mitochondrial dysfunction theory causing overproduction of reactive oxygen species, oxidative stress, and age-related damages (24).

At a structural level, aging is related to renal anatomic alterations. Main changes observed in aging kidney are sclerosis (focal and global glomerulosclerosis, tubular atrophy and interstitial fibrosis, arteriosclerosis), nephron hypertrophy, and decline in the number of functional nephrons (25, 26). These modifications lead to renal mass decrease of ∼10% per decade and decrease in plasma flow and tubular damages (27). The majority of renal cells are permanently renewed, but podocytes have a limited capacity of regeneration due to their terminally differentiation (28, 29). Podocyte senescence largely contributes to renal aging. The cortex shrinks and the medulla increase in size, with an increased number of renal cysts (30).

At a clinical level, aging leads to glomerular filtration rate (GFR) decline. It has been estimated that, after the fourth decade, a decline of GFR occurs that ranges between 0.63 and 0.75 ml/min/year with kidney aging (26, 31). However, nephrosclerosis and cortical atrophy failed to explain the entirety of the GFR decrease with age (25).

IMMUNOLOGICAL ASPECT OF AGING IN KIDNEY TRANSPLANT RECIPIENTS

Aging, in the immunological field, is associated with the concept of immunosenescence, which was based on the clinical reports of a higher incidence of infection and cancer and a lower efficacy of vaccination in older people (32). In the field of kidney transplantation, older age of recipients is associated with a lower risk of acute rejection as compared to younger recipients (33). The leading cause of death in old recipient is infection, and death is the leading cause of graft loss (34). Moreover, Mendonça et al. reported a rate of 37.6% of acute rejection in younger recipients (<60 years old) as compared to 22.7% in older (≥60 years old; $p = 0.01$), after a median time of 22 months of follow-up (35). In larger cohorts, it has been shown that the absolute risk of acute rejection decreases for each decade of recipient age (36).

On top of aging, kidney transplant recipients suffer from CKDs and ESKD before transplantation. ESKD itself is associated with a higher risk of infections and virus-related cancers as compared to the general population of the same age. In the general population, the absolute rate of cancer mortality increases with age. However, on the contrary, in kidney transplant

patients, the excess risk of cancer-related death decreases with age as compared to the general population. Over 65 years, the absolute risk of cancer-related death is 1.7-fold increased in kidney-transplanted recipients as compared to same age non-transplanted population (37). The mechanism of accelerated immunosenescence in ESKD patients is not clearly understood, but some mechanisms have been assumed: chronic inflammation, oxidative stress, cytomegalovirus (CMV) infection, and epigenetics modifications (38, 39).

The T-cell receptor (TCR) repertoire allows the adaptive immune system to recognize a large number of foreign antigens. The TCR β repertoire is known to decrease almost linearly with age, decreasing from 6.4×10^5 TRBV CDR3 clone types per 10^6 T cells at the age of 16 years to 3.1×10^5 at the age of 62 years. Although the absolute and relative numbers of total $CD3^+$ cells do not differ with age, the percentages of naive $CD8^+$ and $CD4^+$ cells decrease with age (40). Huang et al. assessed the factors that may accelerate the TCR β repertoire contraction. They showed that age, CMV infection, and ESKD were significantly and independently associated with a shrinking of the TCR β repertoire (41). The impact of age on the TCR β repertoire concerned only the $CD8^+$ memory T-cell subset but not the naive T-cell subset.

Other immune cell compartments appear to be affected by aging (42). Impaired B-cells proliferation and antibodies production have been reported. The hypothesis put forward may be IL-2 lower production or T-cell/B-cell interaction dysfunction through CD28 downregulation (43, 44). On contrary, immunosenescence is associated with an increase in cytotoxic natural killer cells capacity with aging. Indeed, some authors reported a decrease in $CD56^{bright}$ subset and an increase in $CD56^{dim}$ subset of natural killer cells, which may play a role in graft antibody-mediated rejection (45).

ESKD also seems to impact the absolute and relative number of different immune cell subsets. Betjes et al. showed that ESKD was associated with a premature immune system aging, i.e., a lower $CD31^+$ naive T-cell number as compared to age-matched healthy individuals and a higher percentage of terminally differentiated activated memory $CD8^+$ T cells (TEMRA cells) (46). ESKD patients may experience an overinduced apoptosis of naive T cells and an insufficient increase in thymic output and compensating proliferation as compared to same aged healthy individuals (46). Chiu et al. demonstrated how ESKD may accelerate immunosenescence. Indeed, they showed that not only $CD8^+$ TEMRA cell frequency was higher in ESKD patients as compared to healthy individuals but also, in multivariate analysis, the level of this senescent phenotype positively correlated with dialysis duration and uremic toxin *p-cresyl* sulfate (47).

Modality of ESKD treatment also impacts immunosenescence as hemodialysis was shown to be associated with a higher level of inflammation as compared to peritoneal dialysis. The chronic inflammation and lymphocyte-sustained activation generated in these patients may accelerate immunosenescence by recruiting new T cells, promote stem cell exhaustion, and explain the lower incidence of observed acute rejection in hemodialysis patients, as compared to peritoneal dialysis patients, before transplantation (39).

The impact of CMV infection on the adaptive immune system homeostasis and immunosenescence is reported in many studies. First, CMV latency is associated with a specific anti-CMV $CD8^+$ T-cell repertoire expansion. In healthy donors, using CMV peptides–HLA tetrameric complexes, it has been shown that this subpopulation may reach 10% of $CD8^+$ T-cell compartment (48, 49). Posttransplantation, this percentage may reach 18% (50). This unbalanced expansion due to CMV is considered to be detrimental to the immune system of individuals. Similarly, to ESKD, CMV infection, and/or latency are associated with a decrease in naive $CD8^+$ T cells and an accumulation of TEMRA cells. Yang et al. showed that a higher anti-CMV IgG level is associated with a lower percentage of total $CD4^+$ and $CD8^+$ T cells but a higher percentage of CCR7-CD45RA T cells (TEMRA cells) in hemodialysis patients (51). These results were comparable to those found in kidney transplant recipients under immunosuppressive regimen. CMV drives a $CD8^+$ T-cell expansion especially $CD8^+$CD28 null and TEMRA CD8+ T cells (52).

Finally, in older transplant recipients, Schaenman et al. showed a decreased number of naive $CD4^+$ and naive $CD8^+$ T cells and an increased number of TEMRA cells and senescent $KLRG1^+$ T cells as compared to younger recipients (53).

IMMUNOLOGICAL ASPECT OF AGING IN KIDNEY TRANSPLANT DONORS

Donor age appears to be an important prognostic factor of long-term outcome after kidney transplantation (54). Nevertheless, the donor age criteria may be misleading when assessed alone (55). In contrast with older recipients, older donors are likely to be more immunogenic. In experimental data, T cells of rats receiving an old graft express a higher level of IFN-γ as compared to those receiving a younger graft. This difference was associated with an accelerated chronic allograft dysfunction (56). de Fijter et al. assessed in a large cohort of kidney transplant recipients the risk factors of acute rejection (57). In a multivariate analysis, donor age ≥50 years old, recipient age <50 years old, and HLA-DR mismatches were significantly associated with a higher risk of acute rejection (risk ratio = 1.53, 1.34, and 2.28 respectively). Interestingly, the risk of acute rejection in older donors was independent of recipient age suggesting other mechanisms than immunosenescence involved.

Aged kidneys have an increased susceptibility to ischemia–reperfusion injury (IRI). The presence of senescent cells in older kidney may result in a reduced tissue regeneration and chronic low level of inflammation. Different mechanisms may explain the reduced tolerance to IRI: impairment of mitochondrial functions which results in a decrease in antioxidant defenses, reduced expression of heat shock protein-70 involved in transmitochondrial transport, and telomere shortening contributing to the increase in the process of senescence (58). Conversely, IRI like hypertension was shown to increase the level of senescence in donor kidney (59).

In the end, the increased level of inflammation and edema induced by IRI in aged kidneys is the root of a stronger immune

response. Indeed, antigen-presenting capacities of dendritic cells seem to increase with age (60). Nevertheless, regarding dendritic cell functions in aging, little is known currently and data in the literature are controversial (61, 62). Moreover, it was shown that, after acute tubular necrosis, there is an increased expression of HLA molecules in tubular cells and accumulation of inflammatory cells (63). Clinically, delayed graft function induced by IRI is associated with a 38% increased risk of acute rejection (64). The impact of IRI on ECD kidneys is significant, and those kidney benefit from machine of perfusion with a lower rate of delayed graft function and higher kidney survival rate as compared to cold storage (65, 66).

CLINICAL RESULTS IN RECIPIENTS OF MARGINAL KIDNEYS

Since the proportions of older patients on the waiting list and ECD have largely increased, many studies assessed the benefit of kidney transplantation in these populations. First, transplantation with kidney from ECD has been associated with a higher survival rate as compared to maintenance of the waiting list in >60 years old recipients (67). In this European study, the 5-year survival rate was 83.6% for recipients of ECD kidney as compared to 67.4% for patients who remained on the waiting list. Recipient's age was the major predictive risk factor of mortality in the early- and late-period posttransplantation with time on dialysis before transplantation and diabetes mellitus (68, 69).

Only few studies assessed the long-term results of recipients receiving a kidney graft from ECD as compared to standard criteria donors (SCDs) (70). In 2015, Aubert et al. assessed the long-term results of graft survival between ECD and SCD in 2,763 recipients in a French cohort and in a validation cohort. ECD was associated with a lower graft survival [hazard ratio (HR) = 1.87 (1.50–2.32), $p < 0.001$] as compared to SCD at 7 years posttransplantation. In the multivariate Cox analysis, ECD, cold ischemia, and presence of donor-specific alloantibodies (DSA) at transplantation were significantly associated with kidney allograft loss. The model was adjusted on donor type (deceased vs. living), presence of diabetes in donor, graft rank, and number of HLA-A/B/DR mismatches (71). Recipients of ECD with circulating DSAs at the time of transplantation had the worse kidney graft outcome with a 4.4-fold increased risk of graft loss as compared to those without DSA.

In 2016, Querard et al. conducted a meta-analysis to assess the results of ECD transplantation. From 29 studies, they estimated the non-adjusted pooled risk ratio of patient survival at 5 years at 1.62 (1.18–2.22) and of death-censored graft loss at 1.69 (1.18–2.34) in favor of SCD as compared to ECD (72). The results largely came from North America studies. Moreover, only a very small number of studies were adjusted with usual confounders. In Europe, the non-adjusted pooled risk ratios were lower than in North America.

Van Ittersum et al. published the results of 3,062 kidney recipients after 7.8 years of follow-up in a European population (73). Six hundred nineteen recipients received an ECD kidney, and 2,443 received a SCD kidney. Recipients from deceased ECD donors had a higher risk of death-censored graft failure [HR = 1.92 (1.63–2.26)] and death [HR = 1.45 (1.26–1.67)] as compared to other recipients (deceased donors with SCD criteria and living donors). At 10 years, ECD criteria was associated with an absolute risk of 16.9% for graft lost and 10.1% for death, as compared to SCD. In a subgroup analysis of recipients of the same study, DCD with ECD criteria had the lower graft and patient survival prognosis. Tomita et al. specifically studied ECD after DCD and did not find an increased overall risk of graft loss as compared to SCD. However, the risk of death-censored graft loss was higher in older ECD and donors with an history of hypertension or cerebrovascular events (74).

However, some published data report excellent results with ECD transplantation as compared to SCD. In the study of Palkoci et al. 50 ECD were compared to 107 ECD kidney recipients. At 1 year, the rate of acute rejection was not statistically different, and at 5 years, the death-censored survival rate was not different (92%, $P = 0.884$) in both groups (75). Another study conducted by Kim et al., which included 42 ECD and 364 SCD, showed higher serum creatinine level at 12 months in ECD, but the survival rate was similar as compared to SCD (76).

THERAPEUTIC STRATEGIES IN ECD KIDNEY TRANSPLANTATION

Different immunosuppressive strategies in ECD recipients may be discussed (**Table 1**). The goal in ECD is to reduce not only the incidence of infections and cancers but also acute rejection in this at-risk population. In induction therapy, rabbit antithymocyte globulin (rATG) has shown lower risk of acute rejection as compared to IL-2 receptor antagonists without an increased risk of death in older recipients and high-risk kidney such as ECD (86). Steroids maintenance or withdrawal has to be weighed between the higher risk of acute rejection and the risk of side effects in older patients. It was shown that an early steroid withdrawal at the time of first discharge posttransplantation was associated with a better adjusted overall graft survival [HR = 1.32 (1.1–1.56), $P = 0.002$) and patient survival [HR = 1.46 (1.16–1.83), $P = 0.001$] but not death-censored graft survival. In a subgroup analysis, these results were confirmed only in the T-cell-depleting induction treatment (thymoglobulin) group but not in the IL-2 receptor blocker (Basiliximab) group (87).

In the field of kidney transplantation, clinicians seek intensively for new immunosuppressive regimens to avoid calcineurin inhibitors (CNIs) nephrotoxicity. In 2011, the US Food and Drug Administration approved the use of belatacept. This drug is a fusion protein that bind CD80/86 onto antigen-presenting cells and thereby blocks effector T cells by preventing interactions with CD28 (88). In the BENEFIT-EXT trial, 543 ECD recipients received either cyclosporine- or belatacept-based regimen (80). At 7 years posttransplantation, mean estimated GFR was 53.9 ± 1.9, 54.2 ± 1.9, and 35.3 ± 2.0 ml/min per 1.73 m^2 for belatacept more intensive, belatacept less intensive, and cyclosporine groups, respectively ($P < 0.001$). This showed the benefit of avoiding CNI nephrotoxicity in those kidneys. Death-censored graft loss and patient survival was similar in

TABLE 1 | Study characteristics of trials evaluating immunosuppressive regimen in expanded criteria donors.

	Reference	Study design	Results
Induction	Gill et al. (77)	Retrospective. rATG or IL2RA or alemtuzumab 14,820 patients	rATG > IL2RA/alemtuzumab in terms of rejection rate and graft survival in high-risk patients
Steroid withdrawal	Aull et al. (78)	Retrospective. 634 patients. 46% ECD	At 5 years: 90.2% patient survival 87.6% DCGS 12.8% acute rejection
	Segolini et al. (79)	88 ECD IL2R + MMF + tacrolimus and steroid reducing or withdrawal	At 3 years: 13.6% rejection rate At 4 years: 96% patient survival 79% graft survival
Belatacept	Durrbach et al. (80) (BENEFIT-EXT)	Prospective. 543 patients. Belatacept vs. CsA IL2RA + MMF + steroids	At 7 years: 73 vs. 78% patient survival 88 vs. 81% DCGS 21 vs. 17% acute rejection
Delayed CNI	Stratta et al. (81)	Prospective. 101 ECD. ATG or Alemtuzumab + MMF + steroids	At 4 years: 12% acute rejection 93% patient survival 83% graft survival
	Arbogast et al. (82)	Prospective. 89 ECD. rATG + MMF + steroids.	At 5 years: 24% acute rejection 88% patient survival 70% graft survival
mTOR inhibitors	Furian et al. (83)	Comparative non-randomized. 31 ECD. rATG + Sirolimus + MMF + steroids	At 1 year: 19% acute rejection 100% patient survival 97% graft survival
	Cruzado et al. (84)	Comparative non-randomized. 42 ECD. rATG + Sirolimus + MMF + Steroids	At 3 years: 8% acute rejection 76% patient survival 90% DCGS
	Ferreira et al. (85)	Prospective randomized. 171 ECD. rATG + tacrolimus + everolimus + steroids vs. MMF	At 1 year: 95 vs. 84% avute rejection 89 vs. 99% DCGS 90 vs. 99% patient survival

rATG, rabbit antithymoglobulin; IL2RA, IL2 receptor antagonist; ECD, expanded criteria donors; MMF, mycophenolate mofetil; CsA, cyclosporin A; DCGS, death-censored graft survival.

all groups except for a higher incidence of posttransplant lymphoproliferative disorders in EBV-negative recipients treated by belatacept. Posttransplantation switch from CNI to belatacept within the first 6 months also seems efficient to improve renal graft function from ECD (89). Mammalian target of rapamycin (mTOR) inhibitors may also be a valuable option to avoid CNI nephrotoxicity, but large randomized and controlled studies are missing (90). Most of non-randomized studies showed acceptable results of graft survival and rejection rate with sirolimus or everolimus in CNI minimization strategies (86). Yet, the benefit of mTOR inhibitors as compared to mycophenolate in ECD patients is controversial (91). Indeed, despite a lower incidence of CMV infection/disease, Ferreira et al. study was prematurely terminated due to a higher incidence of acute rejection, graft loss, and death in the mTOR inhibitor group, i.e., tacrolimus + everolimus as compared to tacrolimus + MPA (85).

Despite all these clinical results of ECD vs. SCD, kidney transplantation with ECD remains a valuable option. Indeed, in North America, Ojo et al. showed that ECD transplantation improve patient survival over maintenance dialysis treatment with an increase of 5 years in life expectancy (92). These results were consistent in the European population (67).

RISK STRATIFICATION

In 2009, Rao et al. published the kidney donor risk index based on the Scientific Registry of Transplant recipients in the North American population (93). The kidney donor risk index appears to be an interesting tool to stratify the risk and estimate outcomes posttransplantation based on 14 donor and transplant factors associated with death and graft failure. This score is currently used in the United States to allocate kidney graft for single kidney transplantation or dual kidney transplantation (94). KDPI score was assessed also in European cohorts of high-risk donor–recipient pairs and was efficient to improve the graft outcome prediction (95).

In Europe, the Eurotransplant senior program (ESP) was created to improve transplant allocation and shorten the time on waiting list. It was designed to allocate kidney from ≥65

years old donors to ≥65 years old recipients regardless of HLA matching but with a focus on reducing the cold ischemia time (96). Frei et al. published the 5-year results of the ESP and showed that death-censored graft survival of ESP patients was similar when compared to old donor giving to other any recipients (67% survival) but was lower as compared to any aged donor giving to old recipients (81%). These results were obtained at the price of higher incidence of acute rejection (97). Results from the Dutch Organ Transplant Registry, which is part of Eurotransplant and ESP, showed a 5-year death censored graft survival of 83.8% in DBD and 75.3% in DCD (98). In this old recipient population, delayed graft function was a strong risk factor of death (+40% risk) and of rejection (+57%) and DSA development (99).

CONCLUSIONS AND PERSPECTIVES

Kidney transplantation of "marginal donors" to old recipients implies different specificities: immunosenescence of recipients and higher risk of complications (i.e., infections and cancers), higher immunogenic response of older kidneys and increased susceptibility to IRI, and worse outcome than SCD kidneys. Nevertheless, older patients still benefit from transplantation rather than remaining in the waiting list. New immunosuppressive regimens and strategies such as costimulation blockade, early steroids withdrawal, and CNI minimization strategies may be useful to improve patient and renal outcomes in ECD recipients. The goal in the future will be to minimize CNI-associated toxicity such as nephrotoxicity, cardiovascular morbidity and mortality, and malignancy in the particular population of ECD recipients. To achieve this goal, we need to improve the risk stratification before clinicians allocate a kidney from ECD to an old recipient. New randomized studies need to be done in ECD transplantation.

AUTHOR CONTRIBUTIONS

JN performed most of the literature search and wrote the manuscript. TJ, PM, and CS contributed to the literature search and carefully read the manuscript. LR finalized the manuscript.

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frontiers in Immunology

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Genetic Engineering of the Kidney to Permanently Silence MHC Transcripts During *ex vivo* Organ Perfusion

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Organ gene therapy represents a promising tool to correct diseases or improve graft survival after transplantation. Polymorphic variation of the major histocompatibility complex (MHC) antigens remains a major obstacle to long-term graft survival after transplantation. Previously, we demonstrated that MHC-silenced cells are protected against allogeneic immune responses. We also showed the feasibility to silence MHC in the lung. Here, we aimed at the genetic engineering of the kidney toward permanent silencing of MHC antigens in a rat model. We constructed a sub-normothermic *ex vivo* perfusion system to deliver lentiviral vectors encoding shRNAs targeting β2-microglobulin and the class II transactivator to the kidney. In addition, the vector contained the sequence for a secreted nanoluciferase. After kidney transplantation (ktx), we detected bioluminescence in the plasma and urine of recipients of an engineered kidney during the 6 weeks of post-transplant monitoring, indicating a stable transgene expression. Remarkably, transcript levels of β2-microglobulin and the class II transactivator were decreased by 70% in kidneys expressing specific shRNAs. Kidney genetic modification did not cause additional cell death compared to control kidneys after machine perfusion. Nevertheless, cytokine secretion signatures were altered during perfusion with lentiviral vectors as revealed by an increase in the secretion of IL-10, MIP-1α, MIP-2, IP-10, and EGF and a decrease in the levels of IL-12, IL-17, MCP-1, and IFN-γ. Biodistribution assays indicate that the localization of the vector was restricted to the graft. This study shows the potential to generate immunologically invisible kidneys showing great promise to support graft survival after transplantation and may contribute to reduce the burden of immunosuppression.

Keywords: transplantation - kidney, organ engineering, HLA, gene therapy, lentiviral vector, organ perfusion

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INTRODUCTION

Kidney transplantation remains the best treatment for end stage renal diseases. However, the limited availability of organ donors contribute to increased waiting times and high morbidity and mortality on the waiting list. In Germany there are currently 7,526 patients waiting on a kidney transplantation with waiting times exceeding 10 years (DSO 2018) (1). Despite the advances in histocompatibility and transplant immunology, the success of kidney transplantation relies on the use of powerful immunosuppressive agents that predispose the transplanted patients to infections and malignancies (2, 3). Furthermore, still a considerable number of patients develop graft failure (4–6). The endothelium supports the renal vasculature and modulates crucial mechanisms such as inflammation or thrombosis contributing to the appropriate organ function. The impairment of the endothelium or the mesenchymal transition of the endothelial cells supporting fibrosis is a major cause for acute or chronic allograft rejection. After transplantation, the endothelium represents the frontier between the graft and recipient, thereby being the most important immune checkpoint (7–9). The discrepancies at the human leukocyte antigen (HLA) loci between donors and recipients remain the major cause for antibody medicated rejection (10). HLA expression on the renal endothelium serves as a strong antigenic stimulus and is simultaneously a main driver and a target of allogeneic immune responses (11). Allograft loss after kidney transplantation is the result of a tight and synergistic interplay between innate and adaptive immune responses. This involves complex molecular mechanisms based on T and B-cell activation, autophagy, apoptosis, and inflammatory responses (12). Interaction of HLA with T-cell receptor activates T-cell immune responses that may directly target the allograft or induce the *de novo* formation of donor specific antibodies (DSA). Remarkably, approximately 63% of late kidney allograft dysfunction is a consequence of antibody mediated rejection (ABMR) (13). Donor specific antibodies (DSA) targeting HLA class I antigens support inflammation and induce proliferation. Also, DSA specific for HLA class II are often correlated to chronic allograft rejection and may play an important role in necrosis of endothelial cells (14, 15). Recently, *ex vivo* normothermic organ perfusion has emerged as a promising biotechnological platform to preserve and assess organ quality. An increasing number of studies also suggests the potential of normothermic perfusion to improve quality, resuscitate, and eventually repair the organ (16). Organ gene therapy offers the possibility to modulate intragraft gene signatures involved in renal pathologies or graft survival. Nevertheless, *in vivo* non-viral or viral gene therapeutic approaches have shown so far very low efficiencies and lack of organ specificity. The use of lentiviral vectors allows a permanent genetic modification of cells and tissues, but beside difficulties in their large-scale production and purification so far representing an obstacle to the genetic modification of large solid vascularized organs, *in vivo* approaches lack specificity and are prone to off-target effects (17). Therefore, viral vector-mediated transduction during *ex vivo* organ perfusion may offer a promising approach to generate stable genetically engineered organs (18). Previously, we have demonstrated that silencing HLA expression reduce the strength of allogeneic immune responses *in vitro* and *in vivo* (19–22). Hence, in this study we aimed to induce a stable genetic modification of the kidney during *ex vivo* organ perfusion. In particular, in the interest of a precise regulation, we used RNA interference to downregulate MHC class I and II transcript in a rat kidney transplant model as a strategy to reduce the immunogenicity of the allograft. This could offer many new opportunities in transplant settings and contribute to gender and diversity equality.

MATERIALS AND METHODS

Lentiviral Vector Constructs and Vector Production

The pRRL.PPT.eFS.pre lentiviral vector plasmid encoding for the sequence of a secreted form of luciferase from Oplophorus gracilirostris (NanoLuc, NL) as a reporter gene was used for cloning of an RNAi cassette (18). The cassette consisted of U6 and H1 promoter sequences regulating expression of shRNAs targeting rat beta2-microglobulin (β2m; shβ2m: 5′-GGAAAGAAGATACCAAATA-3′) and rat class II transactivator (CIITA; shCIITA: 5′-GGATATGGAAATGGATGAAGA-3′), respectively. Thus, the ultimate construct was designed to silence rat MHC I and rat MHC II genes expression. A vector containing a sequence for a non-sense shRNA (shNS) was used as a control. Lentiviral vector particles were produced in HEK293T cells cultured in HYPERFlask Cell Culture Vessels (Corning, New York, USA). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 1% glutamine and 2% penicillin-streptomycin until a confluence of 80–90% and then transfected. For the transfection, the shRNA-encoding vector, as well as psPAX2 and pMD2.G plasmids were mixed with polyethylenimine (Polysciences, Warrington, PA, USA). Afterwards, this mix was applied onto the HEK293T cells and incubated for 64 h. Then, the cell culture supernatants were collected and centrifuged at 20,000 g, 16°C for 3 h. Pellets of viral vector particles were resuspended in Williams' Media E (WME) (Thermo Fisher Scientific, Waltham, MA, USA), divided in 1 ml aliquots and stored at −80°C. Viral vector titration was performed by p24 enzyme-linked immunosorbent assay (Cell Biolabs, San Diego, CA, USA).

Transduction of Rat Kidneys With Lentiviral Vectors During *ex vivo* Perfusion

Rat kidneys were perfused in a system designed to allow for the constant roller-pump driven warm oxygenated perfusion (refer to **Supplementary Material** for the perfusion system details). Kidneys were perfused with WME media supplemented with 5% BSA, 0.007 M creatinine and 30 mM HEPES, as well as 500 μg/ml Cefazoline, as previously described (23). After 10 min of cold storage upon retrieval, the organs were connected to the perfusion system via a fragment of aorta and renal artery and allowed to gradually rewarm to 26–29°C, while being perfused with gradually increasing pump speed for another 15 min. At this stage the flow rate typically reached 3–5 ml/min and the

pressure increased to 30–45 mmHg. Then, 0.8 mg protamine sulfate (Sigma–Aldrich, St. Louis, USA) and 1.5×10^{11} shβ2m- and shCIITA-encoding or shNS-encoding vector particles were injected into the system. Afterwards, the kidneys were perfused at further increasing speed and temperature for about 20–25 min until the parameters were stabilized at 80–95 mmHg and 31–32°C, respectively. The entire perfusion time with the viral vector was 2 h. Afterwards, the organs were perfused for 15 min with WME-based perfusion solution containing EDTA-treated blood, followed by another 10 min of washing with WME-based perfusion solution only. During the last two post-vector perfusion stages, the kidneys were cooled down to 12–13°C and placed on ice for transportation to the operating room for transplantation.

Experimental Animals

All animal experiments were conducted according to the German Animal Welfare law and approved by the local authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany). The animals were bred in house and provided by Institute of Laboratory Animals of Hannover Medical School, Hannover, Germany.

Left kidneys were retrieved from Lew.1W(WP)/HanZtm rats and transplanted to Lew/NHanZtm in an orthotropic allotransplantation setting (**Supplementary Material** contains surgery details). Donors and recipients were 8–9 weeks old males. A group of donor kidneys ($n = 5$) was transduced with the shNS lentiviral vector as a control and another group was transduced with the shβ2m and shCIITA-encoding vector ($n = 7$) during the *ex vivo* perfusion prior to transplantation. Blood samples were collected from the recipient rats before transplantation and weekly thereafter by puncturing the retrobulbar venous plexus with EDTA-coated microtubes (Sarstedt, Nuembrecht, Germany). Plasma was stored at −80°C until needed. In addition, urine collection for 6 h during daytime in metabolic cages was done prior and after ktx in weekly intervals. The urine was stored at −80°C until analysis. A follow up duration was 6 weeks. Afterwards, the recipients were sacrificed in deep general anesthesia and several organs were retrieved. Brain, lung, heart, liver, left native recipient kidney, transplanted kidney, spleen, intestine and bone marrow tissue samples were collected, frozen in liquid nitrogen or preserved in RNA later (Sigma, St. Louis, MO, USA) and stored at −80°C after sacrification. The middle part of kidneys was fixed in 3.5–3.7% pH-neutral buffered formaldehyde (Otto Fischar, Saarbruecken, Germany) and embedded in paraffin for further histological analysis.

Detection of Secreted NanoLuc Luciferase Reporter Gene Expression

Levels of secreted NanoLuc Luciferase reporter gene in plasma and urine samples were measured with help of Nano-Glo Luciferase Assay System (Promega, Madison, USA) according to the manufacturer's protocol. Briefly, 5 µl of plasma or urine samples was diluted 1:10 with phosphate-buffered saline and an equal volume of Nano-Glo Luciferase Assay Reagent was added. The bioluminescence signal, generated as a result of NanoLuc Luciferase interaction with its substrate furimazine, was measured with a luminometer (Berthold Technologies, Zug, Switzerland) after 3 min of incubation.

Analysis of the Cytokine Secretion Profile in the Course of Kidney Perfusion

Cytokine levels of rat IL-1α, MIP-1α, IL-6, EGF, IL-10, IL-12p70, IFN-γ, IL-17, IL-18, MCP-1, IP-10, MIP-2, TNF-α, and RANTES were measured in the perfusate samples using magnetic multiplex bead technology and serum matrices (Merck Millipore, Schwalbach, Germany). The perfusate samples collected at 5, 30 min, 1 and 2 h time points after starting the perfusion were centrifuged at 1,500 rpm for 5 min at room temperature and stored at −80°C until the cytokines analysis was performed. The samples were incubated with the beads according to the manufacturer's instructions. The beads were acquired using a Luminex 100/200 device (Luminex Corp., Austin, TX, USA) and the cytokine concentrations were calculated using the Xponent software version 3.1 (Luminex Corp.).

Real-Time Polymerase Chain Reaction

Renal tissue samples stored in RNA later were used for total RNA isolation with RNeasy Mini Kit (Qiagen, Hilden, Germany) followed by reverse transcription using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to characterize β2m (Rn00560865_m1; Thermo Fisher Scientific, Waltham, MA, USA) and CIITA (Rn01424725_ m1; Thermo Fisher Scientific) transcript levels in amplification reaction with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). GAPDH was chosen as endogenous control for normalization (Rn01775763_g1; Thermo Fisher Scientific). All samples were measured in triplicate with StepOnePlus Real-Time PCR System and data processed with StepOnePlus Software v2.3 (Applied Biosystems).

Lactate Dehydrogenase Activity in Kidney Perfusion Solution

Perfusate samples collected at 5, 30, 60, 90, and 120 min time points in the course of kidney perfusion were centrifuged as described before and stored at −80°C until lactate dehydrogenase (LDH) activity was measured with Cytotoxicity Detection Kit (LDH) (Roche, Basel, Switzerland) according to the manufacturer's instructions. Optical density units of the colorimetric reaction of iodonitrotetrazolium conversion into a red colored formazan were used for comparing LDH release at different time points during kidney perfusions.

Histology

Formaldehyde-fixed renal tissue samples were embedded in paraffin for cutting. Five micrometer sections were prepared and stained with haematoxylin and eosin (H&E). Pathological evaluation of all relevant renal structures including glomeruli, tubuli, blood vessels, and interstitial tissue was performed. A special attention was given to characteristic features of acute tubular injury (ATI) such as tubular swelling, edema and distension, brush border loss, tubular epithelial lucency,

flattening, pyknosis, nuclei loss, luminal debris, and tubular necrosis (epithelial cell death).

Lentiviral Vector Biodistribution Assay

Six weeks after transplantation, the animals were sacrificed and tissue samples of brain, lung, heart, liver, native kidney, transplanted kidney, spleen, intestine, and bone marrow were collected and frozen in liquid nitrogen as described before. These samples were used for genomic DNA (gDNA) isolation with NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Isolated gDNA was used in a polymerase chain reaction (PCR) to amplify a 296 bp fragment of the genome-integrated lentiviral vector sequence. BIO-X-ACT Short Mix (Bioline, London, UK) and the primers 5′-AATTCGGTTAAGGCCAGGGG-3′; 5′-GCTGTGCGGTGGTCTTACTT-3′ were used for amplification. The PCR product was then separated by electrophoresis next to Quick-Load Purple 100 bp DNA Ladder (New England Biolabs, Ipswich, USA) on a 2% agarose gel with GelStar Nucleic Acid Gel Stain (Lonza, Basel, Switzerland). Images were captured with ChemiDoc MP Imaging System (BioRad, Hercules, CA, USA) and the band intensities were calculated by densitometric analysis using the BioRad Image Lab 6.0.1 software.

Statistical Analyses

Data are presented as mean ± standard deviations (SD). For comparison of two groups the Student's *t*-test was used. Comparison of multiple groups with two independent variables was performed by two-way-ANOVA. $p < 0.05$ were considered significant. Statistical analyses were performed using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Lentiviral Vector Mediated Transgene Delivery Into Rat Kidneys During *ex vivo* Sub-normothermic Perfusion and Subsequent Transplantation

Ex vivo kidney perfusion (EVKP) creates a unique opportunity to genetically engineer the organ. Here, we combine EVKP with lentiviral transduction strategies to genetically modify a rat kidney. For this purpose, we constructed a perfusion system (**Figure 1A**) to accommodate a rat kidney and allow *ex vivo* perfusion with warm oxygenated WME-based perfusion solution (**Figure 1B**) and monitoring of major perfusion parameters. In this miniature EVKP system, flow rates of 9–12 ml/min and pressure of 80–95 mmHg under sub-normothermic conditions (32°C) were achieved. Saturation of O_2 (sO_2) in the perfusion solution of 65–70% was achieved using a silicone tubing oxygenator submerged in a Büchner flask supplied with carbogen (**Supplementary Figure 1**). In addition, the perfusion circuit enabled the injection, transport and delivery of lentiviral particles into the rat kidney. Transduced kidneys were subsequently transplanted into allogenic recipients. Experimental design of the study is depicted in **Figure 1C** and summarized as follows: (1) kidney retrieval from the donor; (2) genetic modification of the kidney with the lentiviral vector during 2 h of EVKP; (3) kidney transplantation (ktx) into the recipient; (4) six weeks monitoring of the transgene expression in the recipient after ktx.

Detection of the Transgene Expression Post-transplantation

NanoLuc Luciferase Reporter Gene Expression

The lentiviral vector constructs encoding for shNS or shβ2m and shCIITA sequences used in this study also contained the sequence

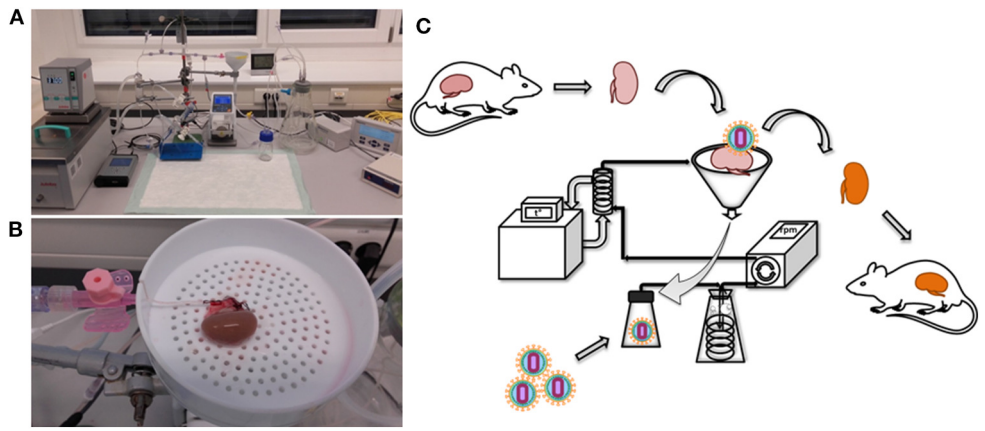

FIGURE 1 | Rat kidney perfusion system for *ex vivo* perfusion and organ genetic engineering. The photographs show the EVKP system **(A)** and the rat kidney connected to the perfusion system via cannulation of the renal artery **(B)**. Schematic representation of the experimental design of the study, including kidney explantation, genetic modification with lentiviral vectors and transplantation **(C)**. Retrieved kidney was placed in the organ container and the renal artery was cannulated and connected to the system. Perfusate flowed from the renal vein into a reservoir for recirculation. Vector particles were injected into the reservoir. A peristaltic pump induced the circulation of the perfusate from the reservoir toward the kidney passing by an oxygenator and glass heat-exchanger. The oxygenated and warmed perfusate entered the kidney via renal arterial cannula.

for secreted NanoLuc Luciferase (NL) as a reporter gene. Kidney transduction was measured by evaluation of bioluminescence activity in the body fluids of animals transplanted with genetically engineered kidneys. In comparison to levels of bioluminescence detected in the pre-transplant plasma samples, all animals transplanted with a graft perfused with lentiviral vectors encoding for NL showed an increase in relative luminescence units (RLU) already 1 week post-transplantation 2.67×10^4 to 1.24×10^6 RLU above the pre-transplantation baseline levels. Bioluminescence in plasma samples was detectable during the entire monitoring period and showed 9.64×10^3 to 3.76×10^5 RLU at week 6 (**Figure 2A**). In addition, weekly urine samples were collected from 8 transplanted rats (3 shNS and 5 shβ2m and shCIITA) (**Figure 2B**). In urine, bioluminescence increased 1 week post-transplantation, similar to plasma, but reached their peak at week 3 showing 2.06×10^6 RLU above the pre-transplantation baseline. Although, the bioluminescence activity was detectable in urine samples during the entire monitoring time the RLU decreased toward 6 weeks and varied between 1.35×10^3 and 1.56×10^6 (**Figure 2B**) at the end of the observation time. Means of NL bioluminescence detected in urine and plasma of animals transplanted with shβ2m and shCIITA or shNS renal grafts are shown in **Figures 2C,D**.

Gene Expression Regulation by shβ2m and shCIITA-Encoding Lentiviral Vector

Increase in NL bioluminescence levels in plasma and urine are indicators of a successful transduction of the renal tissue during EVKP. Hence, MHC class I and II-related transcript levels were evaluated after 6 weeks post-transplantation with shNS- or shβ2m and shCIITA-expressing kidneys. A downregulation of up to 71% in β2-microglobulin levels was detectable in the kidneys engineered for the expression of shβ2m in comparison to shNS-expressing grafts (**Figure 3A**). Similarly, rat CIITA transcript levels were decreased by 70% in the kidneys perfused

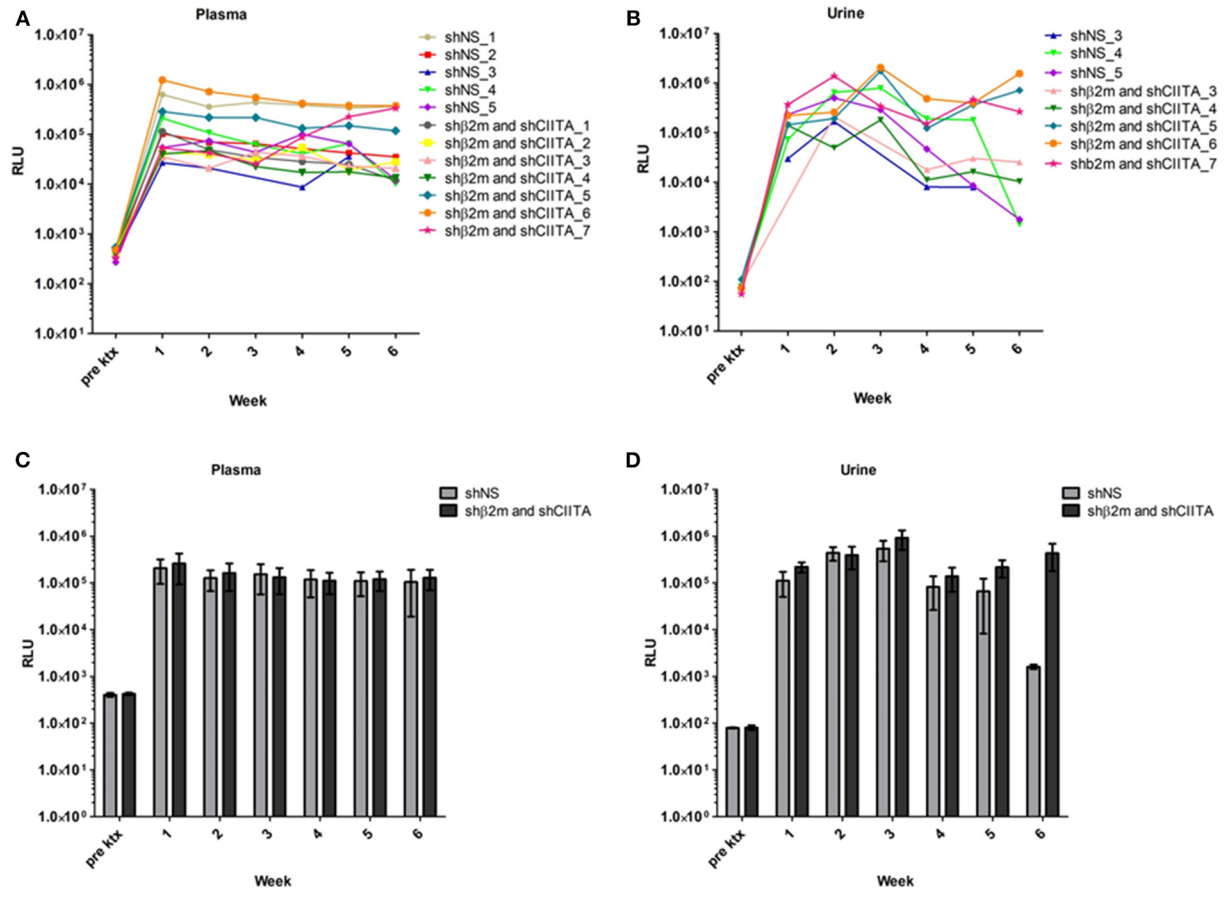

FIGURE 2 | Genetic modification of the kidney during *ex vivo* perfusion. Bioluminescence detected in plasma of the rats transplanted with shβ2m and shCIITA or shNS genetically engineered kidneys. The graphs depict relative luminescence units (RLU) of the secreted NanoLuc Luciferase (NL) reporter gene activity before transplantation and in the course of 6 weeks post-transplantation monitoring **(A)**. Urine bioluminescence levels of the animals transplanted with shβ2m and shCIITA or shNS genetically engineered kidneys. Pre-transplantation NL reporter gene activity levels and NL activity values during 6 weeks after the surgery are shown **(B)**. Mean of RLU detected in plasma of the rats transplanted with shβ2m and shCIITA ($n = 7$) or shNS-expressing ($n = 5$) kidneys (Mean ± SD) **(C)**. Mean of RLU measured in urine of animals transplanted with shβ2m and shCIITA ($n = 5$) or shNS-expressing ($n = 3$) kidneys (Mean ± SD) **(D)**. Pre ktx—pre-transplantation. Statistical analysis was performed by two-way ANOVA. No statistical significance was observed between shNS and the MHC-silence groups.

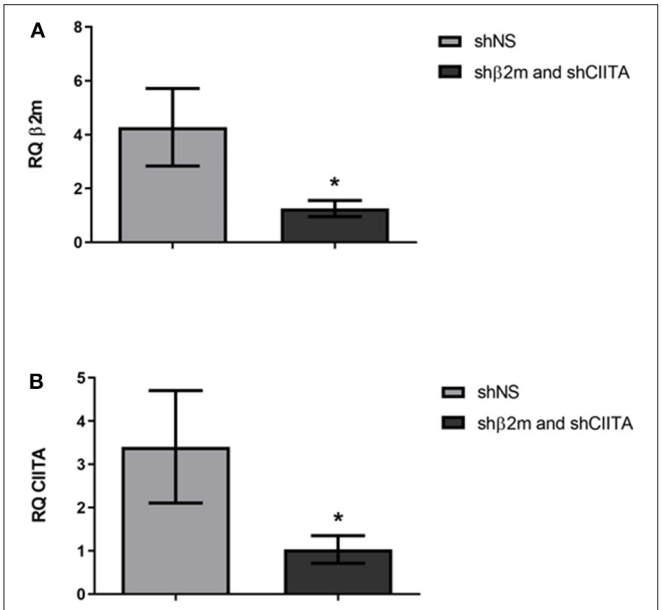

FIGURE 3 | Silencing of β2m and CIITA transcript levels. Relative quantification (RQ) of the β2m gene expression in the kidneys genetically modified with the lentiviral vectors encoding shβ2m and shCIITA ($n = 7$) or shNS ($n = 5$) detected by qRT-PCR (Mean ± SD) **(A)**. RQ values of the CIITA gene transcripts detected by qRT-PCR in shβ2m and shCIITA ($n = 7$) or shNS-transduced ($n = 5$) kidneys (Mean ± SD) **(B)**. Levels of β2m and CIITA gene expression were normalized to GAPDH as housekeeping gene. *$p < 0.05$ (*t*-test).

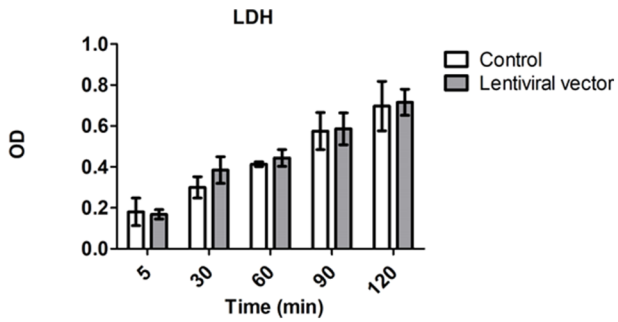

FIGURE 4 | Genetic engineering of the kidney does not cause cell damage. Levels of lactate dehydrogenase (LDH) activity in perfusion solution of the control kidneys ($n = 3$) vs. kidneys subjected to lentiviral vector transduction ($n = 4$) were measured at the beginning (5 min) and every 30 min during EVKP (Mean ± SD). No significant difference in LDH activity was observed between control and lentiviral vector groups as calculated by two-way ANOVA.

with shCIITA-encoding vector particles in comparison to shNS-treated control kidneys (**Figure 3B**). These data indicate that the lentiviral vector harboring shβ2m and shCIITA and applied during EVKP has the potential to simultaneously downregulate the expression of MHC class I and II-related transcript levels.

Assessment of the Kidney Tissue Quality and Integrity in the Course of Sub-Normothermic *ex vivo* Perfusion With Lentiviral Vectors

Lactate Dehydrogenase Activity and Histological Analysis

Levels of lactate dehydrogenase (LDH) have been used as a marker for tissue integrity (24). In order to estimate the level of potential tissue damage induced by the presence of lentiviral vector particles in the perfusion solution during EVKP, we selected LDH as a tissue damage marker and measured its activity in kidney perfusates. Levels of LDH activity increased with time during EVKP. But importantly, no significant differences in the perfusate LDH levels were observed between kidneys perfused with lentiviral particles and control kidneys perfused only with medium at 5, 30, 60, 90, and 120 min time point (**Figure 4**). Histopathological findings of the renal tissue samples exposed to the lentiviral vector encoding for shβ2m and shCIITA during EVKP and control kidney samples perfused only with medium were comparable. Both showed potentially reversible mild to moderate acute tubular injury with overall intact renal morphology. No vector-specific damage in the kidneys perfused with the lentiviral vector was detected (**Figure 5**). These data suggest that application of lentiviral vectors for *ex vivo* kidney genetic engineering under conditions of sub-normothermic perfusion does not cause additional tissue damage in comparison to kidneys perfused without vector.

Cytokines Secretion Profile

Cytokines are important immunomodulatory agents during immune responses after transplantation (25). Therefore, we have characterized potential alterations in the kidney cytokine secretion profile during EVKP in presence or absence of lentiviral vector particles. In comparison to kidneys perfused without vectors, no IL-17 or IFN-γ and lower concentrations of IL-12 and MCP-1 were detectable in kidneys perfused with vector particles during the entire perfusion time. Levels of IL-6 were lower during early perfusion time with lentiviral vectors, but increased at later time point (2 h) to similar concentrations as detected in the kidneys perfused without vectors. In contrast, the secreted levels of IL-10, MIP-1α, MIP-2, IP-10, TNF-α, and EGF were significantly increased in perfusates of kidneys exposed to the lentiviral particles, but only at later time point (2 h). No differences were observed in the secretion patterns of RANTES in kidneys perfused with or without lentiviral vectors. IL-1α and IL-18 were not detected in any of the samples at any time point (**Figure 6**). These data suggest that perfusion with lentiviral vectors induce an alteration in the pattern of secretion of cytokines.

Biodistribution Assay

Ex vivo organ perfusion permits the precise genetic engineering of the target organ, strongly reducing the risk for undesired off-target effects or adverse reactions due to not modifying the cells or other organs. Hence, after transplantation of genetic engineered kidneys, we have assessed different organs for the presence of the lentiviral vector. The vector could not be found in any other organ and was exclusively restricted to the modified renal graft (**Figure 7**).

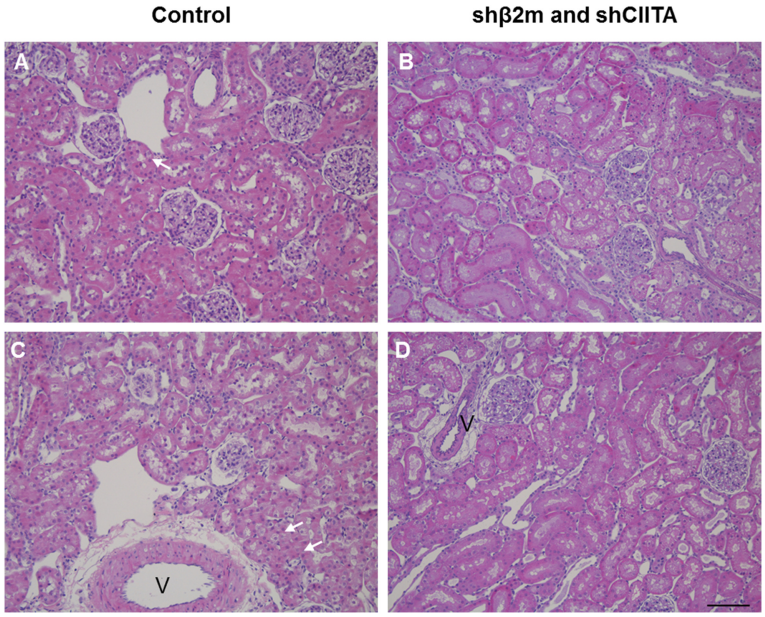

FIGURE 5 | Representative images of perfused kidneys, H&E stain. Perfused control kidneys **(A,C)** or kidneys perfused with lentiviral vector **(B,D)** showed mild to moderate acute tubular injury characterized by tubular vacuolization (arrows, v, vessel). Overall renal morphology was intact in both groups. Images represent individual kidneys from 4 different rats (bar: 100 μm).

DISCUSSION

In this study, we have demonstrated that the kidney can be genetically engineered in a permanent manner toward reduction of its immunogenicity.

Organ Gene Therapy

Recently, gene therapeutic approaches have demonstrated to be successful in the treatment of several diseases such as inherited retinal dystrophies, cancer, hemoglobinopathies and neuromuscular diseases using non-viral or viral-based vector technologies (26–30). Furthermore, several pre-clinical studies show significant progresses in the development of gene therapeutic strategies at organs such as the lung and the liver (31, 32). Selection of the method to deliver of the therapeutic vectors remains an essential and crucial hallmark; on the one hand to achieve organ specificity and high transduction efficiencies and on the other hand to ensure maintenance of organ quality during the genetic engineering process. Despite many efforts, *in vivo* delivery of gene therapeutic vectors has been proven to be inefficient and unspecific (17).

Ex vivo Organ Perfusion in Organ Engineering

Ex vivo normothermic perfusion has been extensively studied during the past decade and it has allowed to monitor function and circulation in marginal organs, such as in case of donation after circulatory death or from extended criteria donors, in kidney, lung, heart and liver transplantation studies and also to deliver certain therapeutic strategies (33–35). During *ex vivo* normothermic perfusion the donated organ undergoes machine perfusion with warm and oxygenated blood or preservation solution prior to transplantation. The feasibility of *ex vivo* normothermic perfusion for long periods such as 24 h was previously demonstrated (36). The prolonged warm perfusion time with maintenance of functionality creates a window of opportunity for various therapeutic interventions. Among those, genetic organ engineering is one of the most promising opportunities to correct monogenetic diseases or support allograft survival after transplantation.

Immuno-Engineering of the Kidney

Recently, we have shown the possibility to engineer the lung endothelium by lentiviral vectors during *ex vivo* normothermic perfusion in a porcine model (18). In a porcine heart study it has been reported that the intravascular delivery of adenoviral (Ad) vectors encoding the luciferase gene led to widespread transgene expression in the allograft (37). The intrabronchial route was also exploited in porcine lungs for genetic modification with human IL-10 encoding Ad vector during *ex vivo* normothermic perfusion (38). Furthermore, it has been reported that glomeruli had been extensively transduced with an Ad vector encoding for β-galactosidase after normothermic EVKP in a porcine model (39). These studies indicate that EVKP creates favorable conditions for genetic organ engineering. In contrast to Ad or Ad-associated vectors, lentiviral vectors enable a permanent transgene expression which might be essential to support long-term graft survival. Human Leukocyte Antigen (HLA) mismatches between donor and recipients remain a major obstacle in allogeneic transplantation. However, ∼38% of

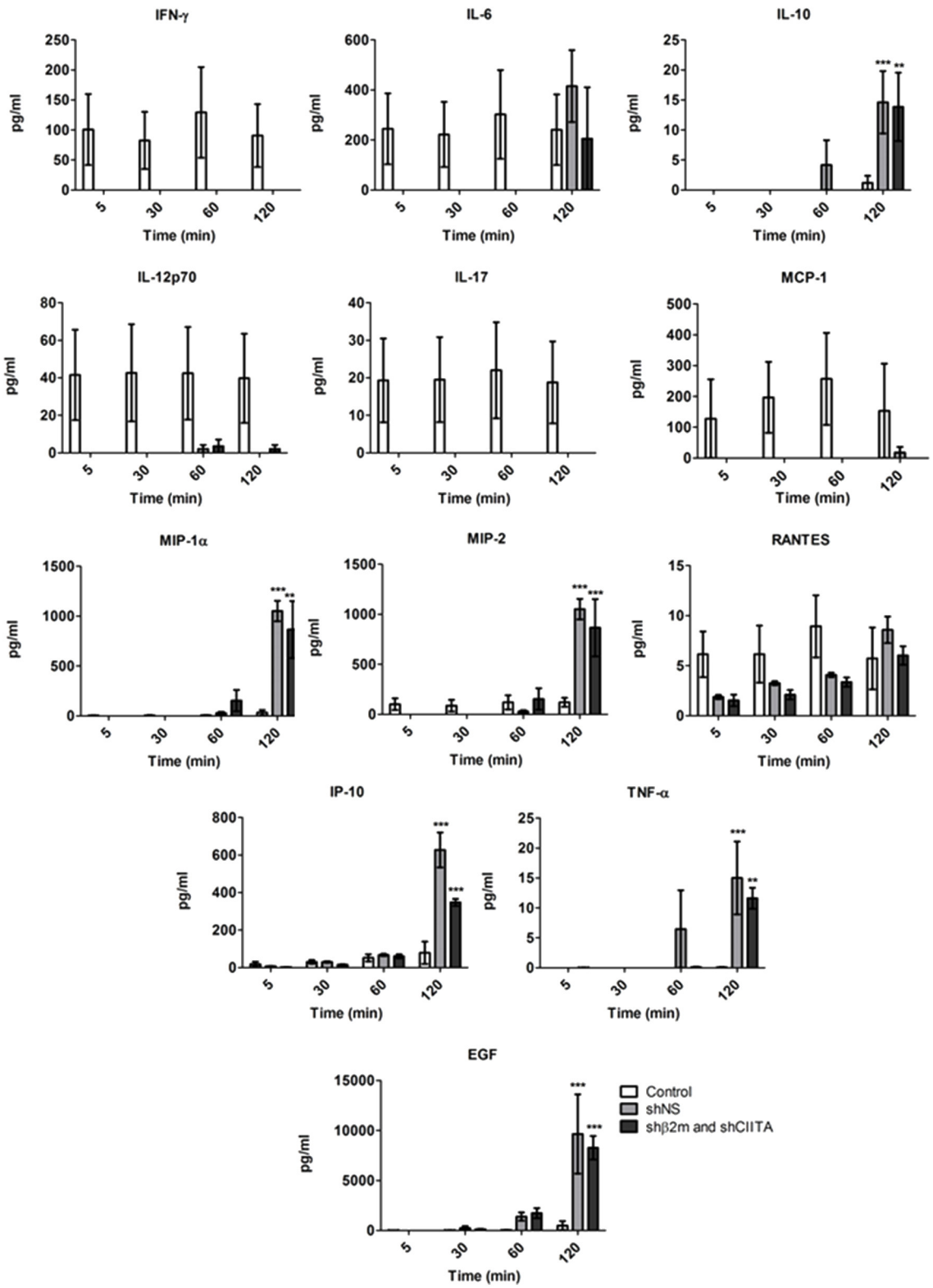

FIGURE 6 | Cytokine secretion signatures during *ex vivo* perfusion and kidney genetic engineering. Cytokine secretion profiles detected in the perfusion solution of the control kidneys ($n = 4$) and kidneys exposed to the shβ2m and shCIITA ($n = 3$) or shNS-encoding ($n = 3$) lentiviral vectors during EVKP (Mean ± SD). ***$p < 0.001$, **$p < 0.01$ (shβ2m and shCIITA or shNS vs. control, two-way ANOVA).

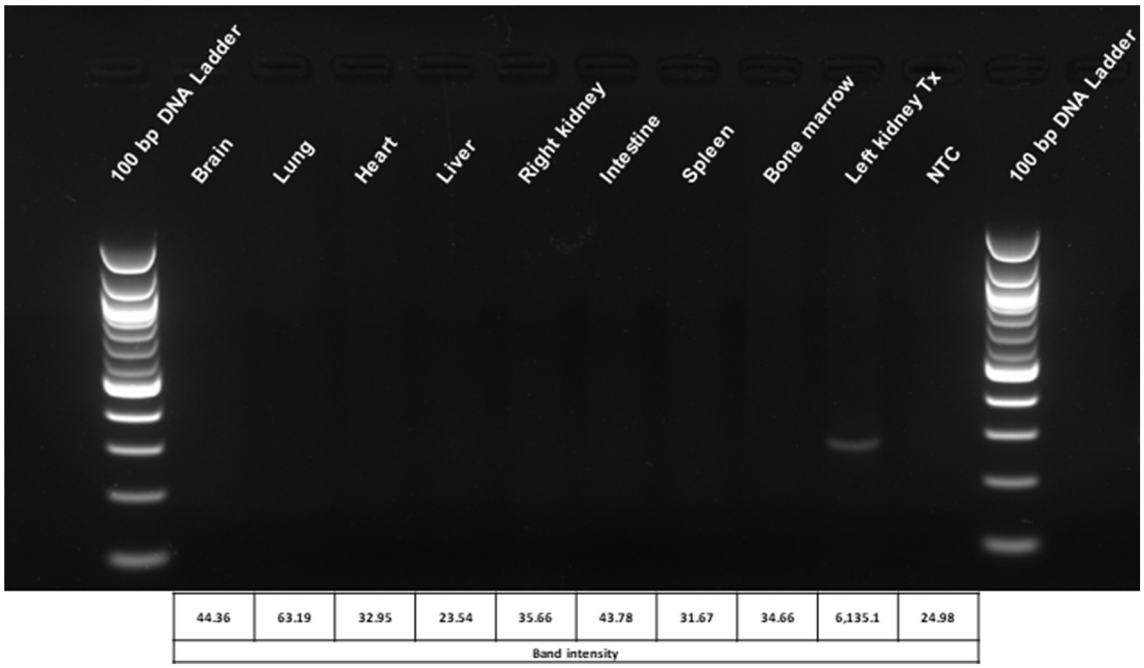

FIGURE 7 | Biodistribution analysis of the lentiviral vector used for genetic modification of the kidneys during *ex vivo* perfusion. The localization of the vector is restricted to the engineered graft. Representative picture of the lentiviral vector biodistribution assay in rat organs and tissues 6 weeks after transplantation with *ex vivo* genetically engineered kidneys. Densitometric analysis of the bands was performed using the BioRad Image Lab 6.0.1 software. Tx, genetically engineered transplanted kidney; NTC, no template control.

kidney graft failure is expected to be triggered by non-HLA-dependent factors. In fact, evidences for the relevance of non-HLA antibodies in leading to kidney transplant dysfunction is increasing. Tissue injury caused by ischemia-reperfusion or vascular injury may favor the upregulation of cryptic autoantigens on the graft endothelial cells such as the angiotensin II type 1 receptor and serve as a target for autoantibodies after transplantation (40, 41). Recently, RNAi has gained plenty of attention in organ transplantation in particular to prevent ischemia reperfusion injury (IRI) by silencing the expression of different genes such as Caspase 3, IKKβ, Fas or RelB. Most of these studies used chemically modified siRNAs or siRNA sequences encoded by plasmid DNA administrated via arterial or venous infusion or injection to prevent IRI. Gene silencing effects using stabilized siRNAs were detectable by periods of 2 to 3 weeks (42–46). In this study, we have selected RNAi as technology to silence MHC class I and II expression and lentiviral vectors to ensure a prolonged expression of the shRNAs. It is well-known that the abrogation of MHC class I expression triggers NK cell cytotoxicity. In previous studies, we have demonstrated that the residual expression of MHC class I molecules is required to prevent NK cell cytotoxicity (22). Thus, we have selected RNAi as the gene regulatory strategy to downregulate MHC expression and not gene editing tools such as CRISPR/Cas9 or TALENs which would generate a complete MHC knockout. In addition, gene regulatory strategies also enable the re-expression of the targeted gene in case of interest by using Tet-ON/OFF promoters. This may be beneficial in case of infections or tumor development. Here, we showed the lentiviral-mediated transduction of the kidney during *ex vivo* perfusion and the sustained transgene expression after ktx as demonstrated by the levels of luminescence in plasma and urine samples of the animals 6 weeks after transplantation (**Figure 2**, **Supplementary Figure 2**). This stable transduction of the kidney grafts is in line with our results in the porcine lung model showing the lentiviral delivery of shRNAs to induce a specific downregulation of MHC class I and MHC class II transcripts (18). We have detected an increase in LDH during perfusion time, however this tendency has also been previously observed in different studies focused in the *ex vivo* recirculating perfusion of organs such as the kidney, liver and lung. This was mainly explained by periods of warm ischemia prior perfusion and by the effect of using pumps and cardiopulmonary bypass during perfusion (18, 47–49). Importantly, we showed that the transduction of the kidney with lentiviral vectors did not cause additional tissue injury or cell death as detected by the LDH levels in the kidney perfusate and renal tissue histological analysis. In order to minimize the risks for cell damage during perfusion, the perfusion solution was oxygenated in our system. Previous studies have indicated the benefits of oxygenating the perfusion solution already during hypothermic perfusion by reducing oxidative stress and supporting the energy status in presence of low metabolic rates (50, 51). During sub-normothermic to normothermic perfusion the increased metabolism demands an appropriate oxygen supply, but recent studies indicate that normothermic perfusion with reduced

perfusate oxygenation for a limited period of time may also be possible without severely compromising renal function or tissue integrity (52).

Cytokines play essential roles in the maintenance of tissue homeostasis and host defense. However, dysregulation of typical cytokine release patterns may trigger detrimental immune cascades after transplantation (25). Previous studies reported that the procedure of organ *ex vivo* perfusion itself elicits an inflammatory reaction with increasing cytokine levels after harvest and placement of organs on the pump-driven perfusion system (53). In this study, we compared cytokine signatures between rat kidneys perfused without and with lentiviral vectors. An increase in the secretion of MIP-1α, MIP-2, IP-10, IL-10, and EGF was detected in the perfusate of organs exposed to lentiviral vectors during perfusion in this model. In contrast, the secretion of cytokines IL-12p70, IL-17, MCP-1, and IFN-γ was lower in genetically engineered organs. The impact of this change in the cytokine secretion pattern in the transplantation outcome needs to be investigated in detail in future experiments. Currently, different approaches to prevent the release of pro-inflammatory cytokines by *ex vivo* perfused organs based on the use of membranes or small molecules are being developed to avoid tissue impairment during perfusion or the activation and polarization of immune responses after transplantation (54, 55).

Safety

Lentiviral vectors are powerful tools for genetic engineering and their use in clinical trials is rising. Nevertheless, the use of lentiviral vectors might be associated with safety concerns such as an increased risk for tumorigenesis (56). In contrast to the *in vivo* application of lentiviral vectors, *ex vivo* organ perfusion allows for the selective genetic modification of the target organ thereby reducing the possibility for off-target and systemic adverse effects. In the transplantation setting an *ex vivo* period of the allograft between transplant procurement and recipient transplantation is inevitable. This inevitable *ex vivo* period of the graft provides a unique opportunity for an *ex vivo* transplant engineering taking advantage of not having to accept systemic off-target effects. In our study, we showed that after transplantation of transduced kidneys the integrated vector DNA was exclusively restricted to the genetically engineered organs. Hence, delivery of the lentiviral vector during *ex vivo* perfusion not only permits the efficient transduction of the organ and stable transgene expression, but simultaneously supports the safety of this procedure.

CONCLUSIONS

In this study, we have demonstrated the feasibility to engineer the kidney during *ex vivo* perfusion over prolonged time periods. Furthermore, levels of MHC class I and II transcripts were also stably downregulated. In future studies, the benefit of invisible of MHC-silenced allografts will need to be investigated toward improvement of allograft survival, function, and reduction of development of donor specific antibodies. Long term goals would be to reduce the amount of immunosuppression and ideally to induce tolerance toward the allograft.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Nidersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (AZ: 17/2476).

AUTHOR CONTRIBUTIONS

CF and RB designed the study. YY, EV, FH, TR, and NW performed experiments. FG and SR performed transplantation experiments. JS, JB, and FG performed histological analyses. DW contributed to the animal experiments. CM contributed with critical advice and to construct the miniature organ perfusion system. CF, RB, FG, NW, YY, and EV analyzed the data and wrote 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/fimmu.2020.00265/full#supplementary-material

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frontiers in Immunology

REVIEW
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Machine Perfusion of Extended Criteria Donor Organs: Immunological Aspects

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Due to higher vulnerability and immunogenicity of extended criteria donor (ECD) organs used for organ transplantation (Tx), the discovery of new treatment strategies, involving tissue allorecognition pathways, is important. The implementation of machine perfusion (MP) led to improved estimation of the organ quality and introduced the possibility to achieve graft reconditioning prior to Tx. A significant number of experimental and clinical trials demonstrated increasing support for MP as a promising method of ECD organ preservation compared to classical static cold storage. MP reduced ischemia–reperfusion injury resulting in the protection from inadequate activation of innate immunity. However, there are no general agreements on MP protocols, and clinical application is limited. The objective of this comprehensive review is to summarize literature on immunological effects of MP of ECD organs based on experimental studies and clinical trials.

Keywords: extended criteria donors, immunological rejection, machine perfusion, marginal organs, transplantation

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INTRODUCTION

The remarkable evolution of solid organ transplantation (Tx) has led to improved overall outcomes for patients with terminal organ dysfunction. However, ischemia–reperfusion injury (IRI) in combination with early immune activation remains a significant challenge limiting the potential of this therapy (1, 2). IRI depends on several factors, including primary condition of the graft and length of cold and warm ischemia time (CIT and WIT). It additionally determines the extent of the inflammatory response and increases immunogenicity and the degree of microcirculatory perfusion failure during reperfusion resulting in early allograft dysfunction or primary non-function (3, 4). As a link between the degree of IRI and activation of innate immunity (5) has been proposed, the discovery of new treatment strategies including tissue allorecognition pathways (**Figure 1**) has gained importance, especially in the era of extended criteria donor (ECD) organ Tx. The direct pathway starts with recipient CD4 and CD8 T cells recognizing endogenous alloantigens presented by donor human leukocyte antigen (HLA) molecules on the surface of donor antigen-presenting cells (APCs) after their migration from the graft to the recipient's lymph nodes. This process is initiated by the massive release of pro-inflammatory cytokines from damaged cells during IRI (4). On the other hand, the indirect allorecognition relies on recipient-derived APCs, which ingest, process, and present alloantigens (typically HLA antigens) in the context of recipient HLA, for self-restricted recognition by recipient T cells (6, 7). In the semi-direct pathway, recipient APCs

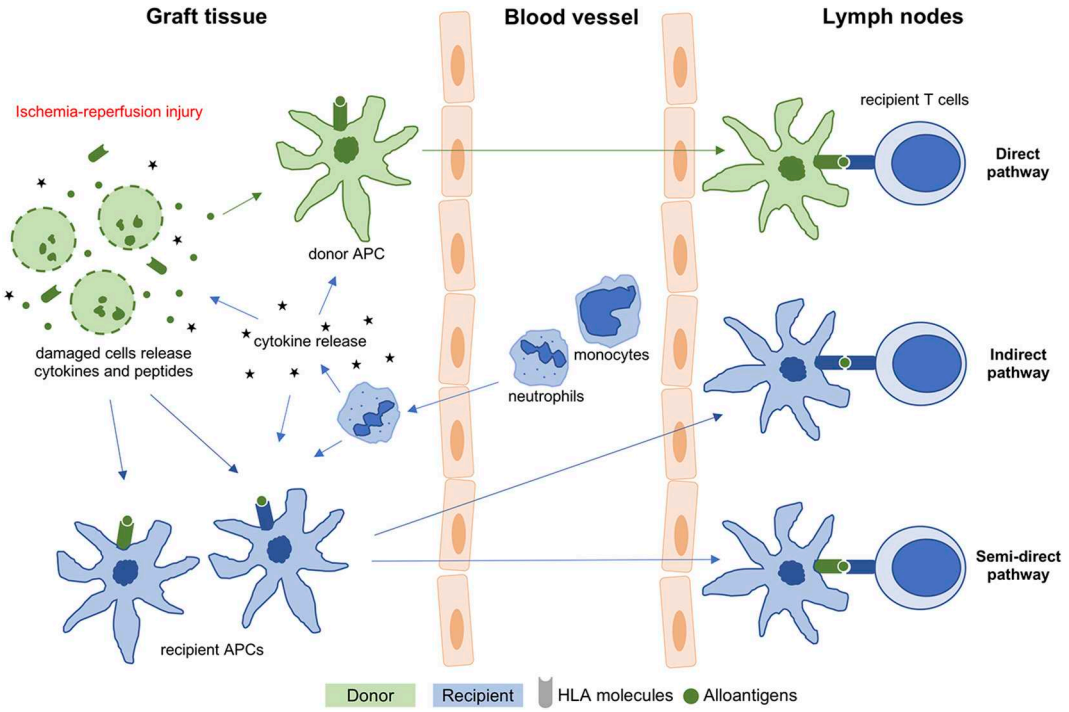

FIGURE 1 | T cell allorecognition pathways in organ transplantation. APC, antigen-presenting cell; HLA, human leukocyte antigen.

acquire donor HLA molecules that present alloantigens directly to recipient T cells (8). Direct allorecognition alone can result in acute rejection, even without indirect mechanisms. Furthermore, depletion of donor immune cells from an organ prior to Tx may prevent rejection (9).

For more than 50 years, static cold storage (SCS) was the gold standard method for organ preservation until the interest in the concept of organ machine perfusion (MP) was renewed (10). To date, a significant number of experimental and clinical trials were published demonstrating increasing support of MP as a more physiologic method of solid organ preservation compared to SCS (11–15).

MP is a promising tool to reduce the gap between organ demand and supply that is resulting in a dramatic prolongation in waiting times and associated with increased morbidity and mortality for patients on the waiting list for Tx (16). In an effort to counter this trend, organ allografts that would have previously been deemed unsuitable are nowadays more frequently used for Tx (12) including donation after circulatory death (DCD) and ECD (aged $\geq$ 60 years or aged 50–59 years with vascular comorbidities) organs (12, 17, 18). Older donor organs have higher immunogenicity, mediated by poorer monocyte clearance of damaged necrotic cells, and therefore recipients may require a more intense immunosuppression in the early period after Tx (19–22). Knowing about the ECD grafts' increased risk for poor function or failure (23–25), implementation of new storage techniques, such as MP, paved the way for better characterization of organ quality and the possibility for graft reconditioning before Tx to improve organ vulnerability and immunogenicity (10, 26). MP reduced IRI in experimental and clinical models of ECD organ Tx resulting in protection from inadequate activation of innate immunity (1, 27–36).

Figure 2 summarizes frequently described MP settings including the underlying mechanisms. Briefly, hypothermic MP (HMP, 4–10°C) is based on the concept that oxidative energy production by mitochondrial electron transport is sustained at reduced rates by keeping low temperatures (10). In contrast, normothermic MP (NMP, 37°C) aims to provide an approximately near physiological environment for organs *ex vivo* (37). Subnormothermic MP (SNMP, ~21°C) is a halfway approach between HMP and NMP, while controlled oxygenated rewarming (COR) is a concept to rescue cold-stored marginal grafts by gentle oxygenated warming up prior to blood reperfusion (38, 39).

Currently, there are no general agreements on MP protocols, and clinical application is limited due to the lack of randomized clinical trials comparing the different MP strategies. The objective of this comprehensive review is to summarize literature on MP of ECD organs and discuss arising immunological aspects based on experimental studies and clinical trials.

MACHINE PERFUSION OF EXTENDED CRITERIA DONOR KIDNEY GRAFTS

It seems that MP for Tx of ECD kidneys is associated with decreased IRI resulting in improved outcome compared to SCS (**Table 1**). Whereas most studies

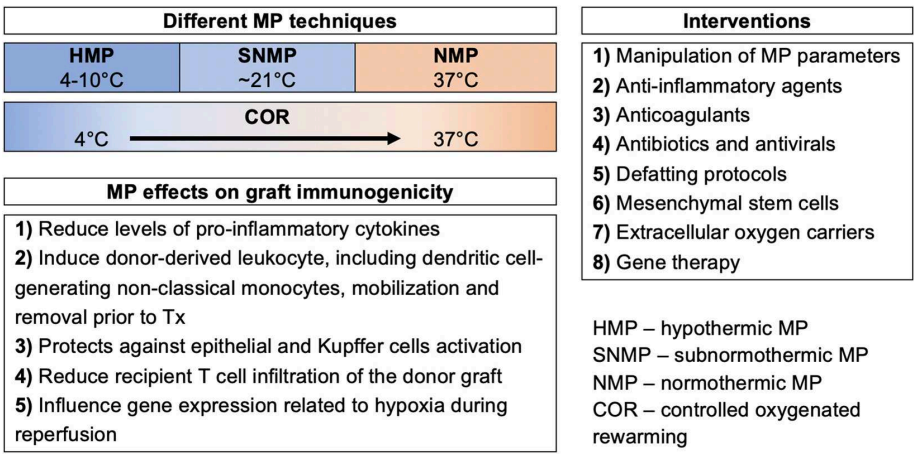

FIGURE 2 | Different machine perfusion strategies of extended criteria donor organs for protection against activation of innate immunity.

on MP in ECD kidneys reported positive effects on the graft, only a few studies reported inconclusive results (40, 48).

Hypothermic Machine Perfusion Techniques

A DCD porcine kidney HMP model demonstrated improved graft outcome (27, 41, 42), particularly concerning the chronic effects of IRI by protecting against chronic immune response by reducing the epithelial to mesenchymal transition (27). Epithelial to mesenchymal transition plays an important role in the genesis of fibroblasts in the course of interstitial fibrosis (27, 52). Furthermore, oxygenated HMP showed superior outcome rates compared to non-oxygenated HMP (41). The significantly reduced occurrence of typical signs for chronic graft loss, like chronic inflammation or interstitial fibrosis, confirmed an improvement in recovery from IRI (41). Lately, the use of an extracellular oxygen transporter was investigated. M101 (hemoglobin of the marine worm) was associated with improved effects of HMP upon recovery and late graft outcome, shown by the nearly absent infiltration of mast cells resulting in reduced levels of fibrosis in the kidney (42). Extracellular oxygen carriers may logistically, rheologically, and immunologically be superior to packed red blood cells, but need further investigation. Studies on human DCD and ECD kidneys supported the superiority of HMP over SCS (32, 43, 45, 46). Reznik et al. (43) found a considerably lower number of complications and negative effects, like acute rejection, correlated with HMP kidneys retrieved from DCD donors. Another study in ECD kidneys (Nyberg Score class C or D) demonstrated an association of HMP with lower levels of early inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-2, and IL-1β] in perfusion solution compared to SCS (32). HMP also affected the expression of hypoxia-related genes [i.e., hypoxia-inducible factor (HIF)-1α] (46). This may limit interstitial fibrosis and tubular atrophy, improving long-term outcomes in kidney Tx. ECD kidneys profited most by application of HMP (46).

Normothermic Machine Perfusion Techniques

In a pig study, reduced graft immunogenicity was achieved by initiating an inflammatory cytokine storm [especially IL-6, interferon leading to a donor-derived leukocyte mobilization and removal prior to kidney Tx (1). The authors proposed that migration of donor leukocytes in conjunction with the secretion of an IL-6, IFN-γ, and CXCL-8 storm leads to direct allorecognition and activates the recipient immune response following Tx (1). Short-term NMP of cold-stored human ECD kidneys did not reduce the incidence of acute rejection, while the rate of delayed graft function improved significantly (5.6 vs. 36.2%) (45). More recently, Weissenbacher et al. (49) was able to maintain the quality of ECD kidneys for up to 24 h, hence buying time for viability assessment, improving the feasibility to exploit this important source of donor organs using the NMP technique.

Although the primary results are encouraging, more research focusing on the reduction of immunogenicity of ECD organs is needed.

MACHINE PERFUSION OF EXTENDED CRITERIA DONOR LIVER GRAFTS

Currently, there is no general consensus on the standardized pretreatment of ECD livers in order to improve Tx outcomes (53). Experimental and clinical studies of MP of ECD livers are summarized in **Table 2**.

Hypothermic Machine Perfusion Techniques

In several studies in DCD rat models, a reduction in IRI in liver tissue was evident after HMP when compared to SCS (28, 29, 54, 56, 57, 61, 62). This finding was confirmed in large domestic animal studies (64, 71). Hypothermic oxygenated

TABLE 1 | Experimental and clinical studies of machine perfusion of extended criteria donor kidney grafts.

Studies	Model	Primary graft condition, *N*	MP time	Results and immunological aspects
ANIMAL STUDIES				
Treckmann et al. (40)	Porcine HMP vs. retrograde oxygen persufflation vs. SCS with autoTx	DCD; *N* = 7/group WIT: 1 h	4 h	Malondialdehyde was dramatically increased in the MP kidneys on day 7, whereas levels in the other two groups were near normal values. The MP kidneys exhibited the most striking histological changes
Vaziri et al. (27)	Porcine HMP with Viaspan UW vs. KPS-1 vs. SCS without Tx	DCD; *N* = 7/group WIT: 1 h	24 h	HMP demonstrated superiority over SCS independently of perfusion solution. Results suggested significant benefits on graft outcome, particularly evident on the chronic effects of IRI with a protection against chronic immune response, epithelial to mesenchymal transition and interstitial fibrosis and tubular atrophy
Thuillier et al. (41)	Porcine HMP ± hyperoxia with Tx	DCD; *N* = 4/group WIT: 1 h	22 h	HMP with oxygen showed signs of higher quality and better function. Furthermore, the typical lesions of chronic graft loss were reduced, confirming improved ability to recover from the IRI
Stone et al. (1)	Porcine NMP without Tx	*N* = 10 CIT: 2 h	6 h	NMP initiated an inflammatory cytokine storm (especially IL-6, IFN-γ, and CXCL-8) and induced donor-derived leukocyte mobilization and removal prior to kidney Tx
Kasil et al. (42)	Porcine HMP ± M101 (2 g/L) ± hyperoxia with autoTx	DCD; *N* = 6/group WIT: 1 h	23 h	The M101 improved the HMP effect upon kidney recovery and late graft outcome. The infiltration of mast-cell leukocyte was nearly absent, leading to reduced fibrosis level in the kidney. Excess supply of oxygen has not improved the results
HUMAN STUDIES				
Reznik et al. (43)	HMP vs. SCS with Tx	Uncontrolled DCD; *N* = 17 vs. 21 WIT: 42.7 ± 1.6	12 h	A considerable number of complications and the negative effects, including acute rejection, correlated with the SCS group of kidneys
Treckmann et al. (44)	HMP vs. SCS with Tx	ECD; *N* = 91/group Median age: 66 y CIT: 13 h	n.d.	HMP preservation clearly reduced the risk of DGF and improved 1-year graft survival and function in ECD kidneys, while acute rejection rate was similar (17 vs. 16%, respectively)
Tozzi et al. (32)	HMP vs. SCS with Tx	Nyberg Score class C or D (donors mean age 67 ± 7 years); *N* = 10 vs. 13 CIT: 70 ± 25 min	12 ± 4 h	The levels of early inflammatory cytokines (TNF-α, IL-2, and IL-1β) were decreased in HMP group in perfusion and preservation liquid; however, there was a non-significant difference comparing sICAM-1
Nicholson et al. (45)	NMP vs. SCS with Tx	ECD; *N* = 10 vs. 47 CIT: ∼11 h	63 ± 16 min	The incidence of acute rejection was similar in both groups (27.7 vs. 23.4%), while the delayed graft function rate was significantly reduced in the NMP group (5.6 vs. 36.2%)
Wszola et al. (46)	HMP vs. SCS	ECD vs. standard criteria donors; *N* = 62	24 h	MP influenced gene expression related to hypoxia during reperfusion and may improve the long-term results of kidney Tx
Wang et al. (47)	HMP vs. SCS with Tx	DCD and ECD; *N* = 24/group	5.86 ± 2.8 h	HMP reduced the incidence of DGF in DCD kidneys, and this effect is greater for ECD kidneys. Acute rejection rate was non-significantly different (4.1 vs. 8.3%, respectively)
Gallinat et al. (48)	End-ischemic HMP vs. SCS alone with Tx	ECD; *N* = 43/group Mean age: 66 vs. 67 years CIT: 13.4 vs. 12.1 years	1.6–12.8 h	PNF and DGF were 0 vs. 9.3% and 11.6 vs. 20.9%. There was no statistically significant difference in 1-year graft survival, while rejection rate within 3 months post Tx was significantly higher in the end-ischemic HMP group (38.5 vs. 10%, respectively)
Weissenbacher et al. (49)	NMP without Tx	DCD and DBD; *N* = 11 WIT: 16.2 ± 10 CIT: ∼35 h	24 h	Demonstrated ability to maintain the condition of donor kidneys of ECD quality for long enough to carry out viability assessment and increase the feasibility to exploit this important source of donor organs
Ruiz-Hernández et al. (50)	Partial vs. total HMP with Tx	ECD; *N* = 119 vs. 74 Median age: 76.9 vs. 69.9 years CIT: 18.4 vs. 16.3 years	>4 h	There is a trend that complete HMP reduces the risk of DGF and improves 1-year graft survival in ECD kidneys
Savoye et al. (51)	HMP vs. SCS with Tx	ECD; *N* = 801 vs. 3,515 Mean age: 63.9 vs. 62.7 years CIT: 16.9 vs. 17.4 h	n.d.	Results confirmed the reduction in DGF occurrence among ECD kidneys preserved by HMP

CIT, cold ischemia time; CXCL, C-X-C motif chemokine ligand; DGF, delayed graft function; ECD, extended criteria donor; DCD, donation after circulatory death; HMP, hypothermic MP; IFN, interferon; IL, interleukin; IRI, ischemia–reperfusion injury; MP, machine perfusion; NMP, normothermic MP; SCS, static cold storage; sICAM, soluble intracellular adhesion molecule; Tx, transplantation; WIT, warm ischemia time; UW, University of Wisconsin solution; PNF, primary graft nonfunction; DBD, donor after brain death.

TABLE 2 | Experimental and clinical studies of machine perfusion of extended criteria donor liver grafts.

Studies	Model	Primary graft condition, *N*	MP time	Results and immunological aspects
ANIMAL STUDIES				
Lee et al. (54)	Rats HMP vs. SCS followed by 1 h machine reperfusion	DCD; N = n.d. WIT: 30 min	10 h	HMP for 10 h improved both function and microcirculation while reducing cellular damage of liver tissue when compared with SCS
Lauschke et al. (55)	Rats HMP with HTK vs. Belzer's solution vs. SCS followed by 45 min machine reperfusion	DCD; N ≥ 5/group WIT: 1 h	24 h	HLA class II antigen expression was detected on post-sinusoidal venular endothelium after SCS of DCD livers, while the antigen was almost absent or markedly reduced after HMP with HTK or Belzer's solution, respectively
Lee et al. (56)	Rats HMP vs. SCS with Tx	DCD; N = 7/group WIT: 30 min	5 h	HMP improved survival and reduced cellular damage of liver tissue that has experienced 30 min of WIT when compared with SCS tissues
Bessems et al. (57)	Rats HMP with Polysol or UW-G vs. SCS followed by 1 h machine reperfusion	DCD; N = 6/group WIT: 30 min	24 h	24 h HMP of DCD rat livers using the newly developed preservation solution Polysol results in less hepatocellular damage and better liver function compared to SCS in UW or HMP using UW-G
Manekeller et al. (58)	Rats HMP vs. SCS followed by 2 h machine reperfusion	DCD; N ≥ 5/group WIT: 30 min CIT: 16	0.5, 1, 2, and 3 h	1 h of post-conditioning after a long time (16 h) of SCS organs improved the viability and sustainability. The significantly higher ATP content and the lack of apoptotic signs in the tissue were observed
Nagrath et al. (59)	Rats NMP ± defatting agent cocktail without Tx	Steatotic livers, N = 7 vs. 5	3 h	Perfusate supplementation with defatting agents significantly reduced the intracellular fat content of perfused livers within a few hours
Olschewski et al. (60)	Rats HMP vs. SNMP vs. SCS without Tx	DCD; N = 5/group WIT: 1 h	6 h	In contrast to preservation at 4 or 12°C MP at 21°C has a beneficial positive effect on the initial organ function, structural integrity of the sinusoidal endothelium, and hepatocellular damage
Stegemann et al. (61, 62)	Rats HMP with different perfusion solutions vs. gaseous oxygen persufflation vs. SCS without Tx	DCD; N = 6/group WIT: 30 min	18 h	The use of Custodiol-N solution led to a significantly decreased release of ALT or LDH during HMP and reperfusion compared with HTK solution and reduced the level of apoptosis. The use of gaseous oxygen persufflation improved the tissue integrity and functional recovery of predamaged livers
Jamieson et al. (63)	Porcine NMP without Tx	Steatotic and normal livers, N = 3 vs. 5 WIT: 16 ± 4 min CIT: 76 ± 11 min	48 h	Steatotic livers can be successfully preserved using NMP for prolonged periods, and NMP facilitates a reduction in hepatic steatosis
Ferrigno et al. (30)	Rats SNMP vs. SCS followed by 2 h machine reperfusion	DCD; N = 5/group WIT: 30 min	6 h	MP preservation at 20°C improves cellular survival reducing the mitochondrial function in livers obtained from DCDs as compared with SCS
Gringeri et al. (31)	Porcine SNMP vs. SCS followed by 2 h machine reperfusion	DCD; N = 5/group WIT: 1 h	6 h	The SNMP group showed better histopathologic results with significantly less hepatic damage compared with SCS
Schlegel et al. (29)	Rats HOPE vs. SCS with Tx	DCD; N = 20/group WIT: 30 min CIT: 4 h	1 h	HOPE treatment significantly decreased IRI of hepatocytes by reducing the activation of Kupffer cells and endothelial cells. Moreover, HOPE-treated DCD livers were protected from activation of the innate immunity according to a decreased IRI
Schlegel et al. (64)	Porcine HMP with different parameters vs. SCS without Tx	DCD; N = 8/group WIT: 1 h CIT: 6 h	1 h	HOPE protected from mitochondrial and nuclear IRI by downregulation of the mitochondrial activity before reperfusion. Cold perfusion itself, under low-pressure conditions, prevented endothelial damage independently of oxygen
Izamis et al. (65)	Rats NMP with Tx	WIT: 0 vs. 1 h N = 11 vs. 7	5 h	MP suppressed lipid oxidation, likely due to the high insulin levels. Perfused livers did not consume all the available oxygen and were hypoxic independent of ischemic injury, suggesting that enhanced microcirculation via vasodilators and anti-thrombolytics might be an effective approach at optimizing the delivery of oxygen to hepatocytes
Minor et al. (38)	Porcine COR vs. HMP vs. SNMP vs. SCS	ECD; N = 6/group CIT: 18 h	1.5 h	COR significantly reduced cellular enzyme loss, gene expression and perfusate activities of TNF-α, radical mediated lipid peroxidation, and increase of portal vascular perfusion resistance upon reperfusion, while HMP or SNMP were less protective
Schlegel et al. (28)	Rats HOPE vs. deoxygenated MP with heterogenic Tx ± immunosuppression	CIT: 30 min	1 h	Study demonstrated that allograft treatment by HOPE not only protects against preservation injury but also impressively downregulates the immune system, blunting the alloimmune response
Bae et al. (33)	Rats HMP with KPS-1 vs. VAS ± VitE vs. SCS without Tx	DCD; N = 5/group WIT: 30 min	8 h	VAS perfusion solution was superior compared with KPS-1, and supplementation of VAS with VitE reduced not only the level of ALT but also levels of inflammatory cytokines (IL-6, TNF-α, and MCP-1) in graft tissue and caspase 3/7 in the circulation

(Continued)

TABLE 2 | Continued

Studies	Model	Primary graft condition, *N*	MP time	Results and immunological aspects
Knaak et al. (39)	Porcine SNMP without Tx	DCD; $N = 5$ WIT: 45 min CIT: 4 h	6 h	SNMP minimized cold ischemic injury and allowed to assess ECD liver grafts prior to Tx
Nassar et al. (66)	Porcine NMP ± vasodilators (prostacyclin or adenosine) without Tx	DCD; $N = 5$/group WIT: 60 min	10 h	Livers perfused with the addition of prostacyclin showed a significantly higher outcome over those perfused by adding adenosine or without vasodilators, indicating the necessity of potent, efficient vasodilation in order to achieve effective preservation of DCD livers during NMP
Nassar et al. (67)	Porcine NMP vs. SNMP vs. SCS followed by 24 h machine reperfusion	DCD; $N = 5$/group WIT: 60 min	10 h	NMP was able to recover DCD livers showing superior hepatocellular integrity, biliary function, and microcirculation compared to SNMP and SCS
Ferrigno et al. (68)	Rats SNMP vs. SCS ± oxygenated washout Rats SNMP vs. SCS Both followed by 2 h machine reperfusion	DCD; $N = 7$/group WIT: 30 min Steatotic livers; $N = 7$/group	6 h	The use of oxygenated washout before SCS reversed liver injury in DCD organs, improving the ATP/ADP ratio; the use of MP did not otherwise prevent liver damage Using dynamic MP, a significantly lower hepatic damage and an increase in bile flow and in the ATP/ADP ratio were found compared with those of the SCS group
Chai et al. (69)	Rats HMP with UW ± metformin (0.165 mg/L) without Tx	Young and aged livers; $N = 6$/group	12 h	The addition of metformin to the UW preservation solution for *ex vivo* HMP reduced liver injury during cold ischemia, with significant protective effects on livers, especially of aged rats
Kron et al. (70)	Rats HOPE vs. SCS with Tx	Steatotic livers (≥60% macrosteatosis); $N = 12$/group CIT: 12 h	1 h	HOPE after cold storage of severely fatty livers significantly prevented reperfusion injury (less oxidative stress, nuclear injury, Kupffer and endothelial cell activation, as well as less fibrosis within 1 week after Tx) and improved graft function
Compagnon et al. (71)	Porcine HMP vs. SCS with Tx	DCD; $N = 6$/group WIT: 1 h	4 h	HMP-preserved livers functioned better and showed less hepatocellular and endothelial cell injury. In addition to improved energy metabolism, this protective effect was associated with an attenuation of inflammatory response, oxidative load, endoplasmic reticulum stress, mitochondrial damage, and apoptosis
Kakizaki et al. (72)	Porcine SNMP vs. SCS with Tx	DCD vs. DBD; $N = 5$/group WIT: 20 min CIT: 4 h	30 min	SNMP before Tx provided some recovery from IR injury in DCD liver grafts and significantly improved the survival rate
Nostedt et al. (73)	Porcine NMP after initial flush with different solutions and temperatures without Tx	DCD; $N = 4$/group WIT: 1 h	12 h	Avoiding initial hypothermia does not improve liver graft quality in a porcine DCD model of NMP
HUMAN STUDIES				
Henry et al. (34)	HMP vs. SCS with Tx	$N = 18$ vs. 15 WIT: 45.1 ± 6.3 min CIT: 9.3 ± 2.2 h	4.2 ± 0.9 h	HMP significantly reduced pro-inflammatory cytokine expression, relieving the downstream activation of adhesion molecules (ICAM-1) and migration of leukocytes, including neutrophils and macrophages, leading to improved overall outcomes
Bruinsma et al. (74)	SNMP without Tx	High-risk DCD and DBD; $N = 7$ WIT: ∼28 min CIT: ∼11.5 h	3 h	SNMP effectively maintained liver function with minimal injury and sustained or improved various hepatobiliary parameters post-ischemia
Dutkowski et al. (75)	HOPE vs. SCS with Tx	DCD; $N = 50$ vs. 25 WIT: ∼35 min CIT: ∼6.5 h	∼2 h	HOPE protected extended DCD livers from initial reperfusion injury, leading to a better graft function and the prevention of intrahepatic biliary complications. Acute rejection rate was similar (16 vs. 12%)
Vogel et al. (76)	NMP without Tx	DCD (69%); $N = 13$ Mean age: 61.9 ± 11.3 years WIT: 11.3 ± 4 min CIT: 9.5 ± 3.7 h	24 h	They demonstrated the possibility to perfuse high-risk livers consistently for 24 h. The neutrophil infiltrate in grafts was eliminated after prolonged NMP
Laing et al. (77)	NMP with Hemopure* vs. RBC-based solution (matched) without Tx	High-risk (80% DCD); $N = 5$/group CIT: 7.5 h	6 h	Hemopure-based perfusion fluid is a feasible alternative to the blood-based solution currently used for liver NMP and may be logistically, rheologically, and immunologically superior to packed RBCs

(Continued)

TABLE 2 | Continued

Studies	Model	Primary graft condition, *N*	MP time	Results and immunological aspects
Nasralla et al. (78)	NMP vs. SCS with Tx	DBD and DCD (∼36%); *N* = 121 vs. 101	∼9 h	NMP was associated with a 50% lower level of graft injury, measured by hepatocellular enzyme release, despite a 50% lower rate of organ discard and a 54% longer mean preservation time. There was no significant difference in bile duct complications, graft survival, or survival of the patient

ALT, alanine aminotransaminase; CIT, cold ischemia time; COR, controlled oxygenated rewarming; ECD, extended criteria donor; DCD, donation after circulatory death; HLA, human leukocyte antigen; HMP, hypothermic MP; HOPE, hypothermic oxygenated perfusion; ICAM, intercellular adhesion molecule; IL, interleukin; IRI, ischemia–reperfusion injury; LDH, lactate dehydrogenase; MCP, monocyte chemoattractant protein; MP, machine perfusion; NMP, normothermic MP; RBC, red blood cell; SCS, static cold storage; SNMP, subnormothermic MP; TNF, tumor necrosis factor; Tx, transplantation; WIT, warm ischemia time; HTK, histidine-tryptophan-ketoglutarate solution; VAS, vasosol solution; DBD, donor after brain death.

perfusion (HOPE) treatment of DCD and severely fatty livers significantly decreased IRI of hepatocytes by reducing the activation of Kupffer and endothelial cells (29, 70). Moreover, HOPE successfully suppressed the recipient's immune system, blunting the alloimmune pathway (28, 29). This was evident by decreased Kupffer and endothelial cell activation induced by initial anti-oxidative effects and damage-associated molecular pattern (DAMP) release as a consequence of HOPE treatment and liver Tx (28). Furthermore, T cell infiltration in liver grafts as well as blood levels of circulating activated T cells decreased (28). A short time (1 h) of reconditioning of DCD rat and porcine livers using HMP after up to 16 h of SCS showed improvements in organ quality (58, 64). Long-term (24 h) HMP of DCD rat livers markedly reduced HLA class II antigen expression on post-sinusoidal venular endothelium compared to SCS (55). Bae et al. (33) found that supplementation of HMP perfusion solution with the antioxidant, vitamin E, reduced inflammatory cytokine levels [IL-6, TNF-α, and monocyte chemoattractant protein (MCP)-1], involved in alloimmune response, in graft tissue. The addition of metformin to HMP preservation solution reduced liver IRI, with significant protective effects on livers, especially in aged rats (69). Furthermore, HMP significantly reduced pro-inflammatory cytokine expression (TNF-α, IL-1β, and IL-8) (34). The attenuation of those cytokines affects many downstream pathways, including a reduced expression of chemokines and adhesion molecules such as intercellular adhesion molecule (ICAM)-1, MCP-1, P-selectin, and others. This effect subsequently decreases the level of neutrophil activation and inevitable leukocyte migration to stressed cell sites, leading to improved overall outcome rates in human livers (34). In another study, HOPE protected DCD livers from initial IRI, leading to improved graft function preventing intrahepatic biliary complications; however, acute rejection rate remained similar (16 vs. 12%) when compared to SCS (75).

Subnormothermic/Normothermic Machine Perfusion Techniques

SNMP and NMP significantly ameliorated hepatic damage in DCD livers compared to SCS in animal models (31, 39, 60, 65, 68, 72). In a porcine model of liver MP, prolonged periods of NMP facilitate a reduction in hepatic steatosis (63), while the supplementation of perfusate with defatting agents significantly reduced the intracellular fat content of perfused rat livers within a few hours (59). Efficient vasodilation was found to be important in order to improve the effectiveness in the preservation of DCD livers during NMP (66). Olschewski et al. (60) compared HMP to SNMP and SCS, demonstrating beneficial effects on the initial organ function, structural integrity of the sinusoidal endothelium, and hepatocellular damage when DCD rat livers were perfused using SNMP. Furthermore, SNMP was associated with lower IRI when compared to SCS (74), while prolonged NMP additionally eliminated the neutrophil infiltrate in grafts (76). Another study of ECD livers showed superiority of COR over HMP, SNMP, and SCS (38). When comparing NMP to SNMP and SCS, NMP was most efficient in terms of recovery of DCD livers (67). Avoiding initial hypothermia did not improve liver graft quality in a porcine DCD model of NMP (73). Recently, the first randomized controlled trial showed a 50% reduction in liver graft injury, despite a 50% decrease in the number of discarded organs and a 54% increased mean preservation time after a period of NMP compared to SCS (∼36% of grafts were DCD). However, they found no significant difference in bile duct complications, graft, or patient survival (78).

The currently ongoing VITTAL trial aims to improve the suitability of non-transplantable livers in the UK by monitoring their function during NMP followed by Tx of the sufficiently improved graft (79, 80). We expect that the results of this novel approach could improve consistency and increase the usage of ECD liver grafts without compromising recipient safety.

MACHINE PERFUSION OF EXTENDED CRITERIA DONOR LUNG GRAFTS

Experimental and clinical studies of ECD lungs and MP are compiled in **Table 3**.

Hypothermic Machine Perfusion Techniques

Short-term HMP could resuscitate ischemically damaged DCD lungs and ameliorate IRI. In a canine model of MP, HMP improved the ATP production by the mitochondrial electron transport chain, leading to a significant decrease in oxidative damage and production of pro-inflammatory cytokines (IL-6 and TNF-α) after reperfusion compared to SCS (81). Moreover, short-term HMP washed out residual microthrombi in the donor lungs.

TABLE 3 | Experimental and clinical studies of machine perfusion of extended criteria donor lung grafts.

Studies	Model	Primary graft condition, *N*	MP time (h)	Results and immunological aspects
ANIMAL STUDIES				
Nakajima et al. (81)	Canine HMP after SCS vs. SCS alone followed by 4 h machine reperfusion	DCD; $N = 5$/group WIT: 4 h CIT: 12 vs. 14 h	2	Short-term HMP could resuscitate ischemically damaged DCD lungs and ameliorate IRI. HMP significantly decreased oxidative damage and the production of pro-inflammatory cytokines after reperfusion compared with SCS
Mulloy et al. (82)	Porcine NMP vs. SCS vs. SCS + NMP with Tx. Perfusate supplemented with adenosine A2A receptor agonist	DCD; $N = 5$/group WIT: 60 min CIT: 4 h (SCS group)	4	The adenosine A2A receptor agonist exerts anti-inflammatory effects and reduces IRI when administered to DCD donor lungs during MP
Stone et al. (83)	Mice NMP ± A2A receptor agonist vs. SCS without Tx	DCD; $N = 10$–12/group WIT: 1 h CIT: 1 h	1	MP modulates pro-inflammatory genes and reduces pulmonary dysfunction, edema, pro-inflammatory cytokines, and neutrophil numbers in DCD lungs, which are further reduced by A2A receptor agonism
Stone et al. (9)	Porcine NMP vs. SCS with Tx	DCD; $N = 12$ WIT: 65 min CIT: 2 h	3	NMP resulted in reduction of donor leukocyte transfer into the recipient, and recipient T cell infiltration of the donor lung was significantly diminished
HUMAN STUDIES				
Stone et al. (36)	NMP without Tx	DCD; $N = 7$ WIT: 65 min CIT: 3 h	2	NMP showed the capacity to remove donor dendritic cell generating non-classical monocytes from graft
Nakajima et al. (35)	NMP ± broad-spectrum antibiotic without Tx	DBD with clinically diagnosed lung infection; $N = 15$ CIT: ∼10 h	12	The results demonstrated that treatment with antibiotics significantly reduced bronchoalveolar lavage bacterial counts and inflammatory injury by decreasing endotoxin levels and key inflammatory mediators (TNF-α, IL-1β, MIP-1α, MIP-1β)
Nakajima et al. (84)	NMP ± MSCs with Tx	$N = 6$/group CIT: 24 h	12	The administration of MSCs ameliorated ischemic injury in donor lungs during NMP and attenuated the subsequent IRI after Tx

CIT, cold ischemia time; ECD, extended criteria donor; DCD, donation after circulatory death; HMP, hypothermic MP; IL, interleukin; IRI, ischemia–reperfusion injury; MIP, macrophage inflammatory protein; MP, machine perfusion; MSCs, mesenchymal stromal cells; NMP, normothermic MP; SCS, static cold storage; TNF, tumor necrosis factor; Tx, transplantation; WIT, warm ischemia time; DBD, donor after brain death.

All of those factors are important for Tx outcomes, including the reduction of the immunological rejection rate.

Normothermic Machine Perfusion Techniques

NMP was able to modulate pro-inflammatory gene expression and reduce pulmonary dysfunction, edema, pro-inflammatory cytokines, and the number of neutrophils in animal DCD lungs (82, 83). Moreover, NMP resulted in reduced donor leukocyte transfer into the recipient by inducing mobilization of donor leukocytes into the perfusate and allowing their removal via the leukocyte filter prior to Tx (9). Therefore, reduced donor leukocyte migration to recipient lymph nodes resulted in a reduction of direct allorecognition and T cell priming, diminishing recipient T cell infiltration, the hallmark of acute rejection (9). In a clinical study, NMP showed the capacity to remove donor dendritic cells generating non-classical monocytes, which are directly involved in immune surveillance, from the graft (36). NMP of donor after brain death (DBD) lungs with clinically diagnosed infection significantly reduced bacterial counts in the fluid of the bronchoalveolar lavage and inflammatory injury by decreasing endotoxin levels and key inflammatory mediators [TNF-α, IL-1β, macrophage inflammatory protein (MIP)-1α, MIP-1β] when combined with broad-spectrum antibiotic treatment (35). The administration of mesenchymal stromal cells (MSCs) ameliorated ischemic injury in donor lungs during *ex vivo* NMP and attenuated the subsequent IRI after Tx (84).

The use of MP in reconditioning of ECD donor lungs for Tx is currently under investigation in clinical trials (85, 86), with results being expected soon.

MACHINE PERFUSION OF EXTENDED CRITERIA DONOR HEART GRAFTS

Currently, clinical evidence of MP in ECD heart grafts is limited (**Table 4**). HMP improved the preservation of DCD heart grafts compared to SCS proven by superior post-reperfusion contractility. The underlying mechanisms could include enhanced preservation of the energetic states and superior cellular integrity (87). Recently, Korkmaz-Icöz et al. (88) demonstrated that HMP of aged donor hearts with MSCs protected against myocardial IRI in a rat model.

MACHINE PERFUSION OF EXTENDED CRITERIA DONOR PANCREAS GRAFTS

There is a limited number of studies evaluating the safety and feasibility of *ex situ* MP for ECD pancreas graft for whole-organ Tx (**Table 5**). HMP of porcine DCD pancreas was associated

TABLE 4 | Experimental and clinical studies of machine perfusion of extended criteria donor heart grafts.

Studies	Model	Primary graft condition, *N*	MP time (h)	Results and immunological aspects
ANIMAL STUDIES				
Van Caenegem et al. (87)	Porcine HMP vs. SCS followed by 1 h machine reperfusion	DCD; $N = 4$/group WIT: 8–44 min	4	HMP improved the preservation of the heart grafts of DCD donors compared with SCS. This was proved by superior post-reperfusion contractility. The underlying mechanisms could include improved preservation of the energetic states and superior cellular integrity
Korkmaz-Icöz t al. (88)	Rats HMP ± MSCs with Tx	Aged donors; $N = 6$–9/group	5	HMP of donor hearts with MSCs protects against myocardial IRI in aged rats

ECD, extended criteria donor; DCD, donation after circulatory death; HMP, hypothermic MP; IRI, ischemia–reperfusion injury; MP, machine perfusion; MSCs, mesenchymal stromal cells; SCS, static cold storage; Tx, transplantation; WIT, warm ischemia time.

TABLE 5 | Experimental and clinical studies of machine perfusion of extended criteria donor pancreas grafts.

Studies	Model	Primary graft condition, *N*	MP time (h)	Results and immunological aspects
ANIMAL STUDIES				
Karcz et al. (89)	Porcine HMP without Tx	DCD; $N = 15$ WIT: 25 min CIT: ∼2.5 h	5:25	There was significant post-perfusion reduction in islet and acinar cell damage after HMP
Hamaoui et al. (90)	Porcine HMP after SCS vs. SCS alone followed by 2 h machine reperfusion	DCD; $N = 3$/group WIT: 30 min CIT: ∼26.5 h	5	HMP-subjected grafts were associated with stable perfusion dynamics and minimal edematous weight change as well as potentially better endocrine viability and functionality
HUMAN STUDIES				
Leemkuil et al. (91)	HMP vs. SCS without Tx	Declined (DCD and DBD); $N = 20$ WIT: ∼20 min CIT: ∼4 h	6	This study indicated that especially the more injured DCD pancreas benefits more from oxygenated HMP compared with SCS alone
Branchereau et al. (92)	HMP vs. SCS without Tx	Rejected for organ or islet Tx; $N = 7$ vs. 2 WIT: n.d. CIT: n.d.	24	24 h of HMP of ECD human pancreas–duodenum organs was feasible with no deleterious parenchymal effect

CIT, cold ischemia time; ECD, extended criteria donor; DCD, donation after circulatory death; HMP, hypothermic MP; MP, machine perfusion; SCS, static cold storage; Tx, transplantation; WIT, warm ischemia time; DBD, donor after brain death.

with a reduction in islet and acinar cell damage, stable perfusion dynamics, and minimal edematous weight change as well as potentially ameliorated endocrine viability and functionality after preservation (89, 90). More recent studies in the human pancreas indicated that especially DCD pancreas benefits more from oxygenated HMP compared to SCS alone (91). Even 24 h of HMP of ECD human pancreas–duodenum organs was feasible resulting in no deleterious parenchymal effects (92). Since those studies focused on the results after MP without following Tx, currently, there are no data available about clinical outcomes in this context.

CONCLUSION

MP allows successful utilization of more vulnerable and immunogenic otherwise discarded ECD organs. It has been shown that MP not only reduces the levels of pro-inflammatory cytokines and positively influences gene expression related to hypoxia during reperfusion but also induces donor-derived leukocytes, including dendritic cell-generating non-classical monocytes, mobilization, and removal prior to Tx. Moreover, MP was able to protect against epithelial and Kupffer cell activation and to reduce recipient T cell infiltration of the donor graft. More recently, novel methods such as viral vector delivery during MP to allografts are under investigation (93). This biological modification of the graft prior to Tx may be a future therapeutic strategy to suppress the immune response against the allograft leading to Tx without or at least reduced dose of the systemic immunosuppression that carries the additional risk of infection and malignancy. Many studies have already shown superiority of ECD organ MP over the current standard SCS. However, there are no general agreements on MP protocols, and wider clinical application is limited due to the lack of randomized controlled trials. More trials focusing on immunological pathways in the different MP settings with respect to every single organ are mandatory to get detailed

mechanistic insights. This knowledge about various pathways will help us to optimize organ quality after MP of ECD organs and therefore improve Tx outcomes as well as graft and patient survival.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CASE REPORT
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Iloprost in Acute Post-kidney Transplant Atheroembolism: A Case Report of Two Successful Treatments

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Cholesterol embolization (CE) is a rare and alarming post-transplant complication, responsible for primary non-function (PNF) or delayed graft function (DGF). Its incidence is expected to rise due to increasingly old donors and recipients and the extended criteria for donation. Therapy with statins and steroids has not been shown to be effective, while agonism of prostaglandin I_2 has been reported to be useful in systemic CE. We report two cases of acute post-transplant CE in which intravenous iloprost (0.05 mg/kg/day) was added to standard statin and steroid therapy. In the first instance, CE was due to embolization from the kidney artery resulting in embolization of the small vessels; after a long DGF and 15 days of iloprost therapy, renal function recovered. The second instance is a case of embolization from the iliac artery of the recipient, where CE manifested as a partial renal infarction. After 5 days of iloprost administration, creatinine levels improved. Iloprost acts on vasodilation and on different inflammatory pathways, improving the anti-inflammatory profile. Post-transplant CE is difficult to diagnose and, if not treated, can lead to loss of function. Iloprost added to standard therapy could be beneficial in accelerating renal function recovery immediately after transplant.

Keywords: cholesterol embolism, kidney transplant, prostaglandin agonism, delayed graft function, extended criteria donors

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INTRODUCTION

Cholesterol embolization (CE) is a rare but alarming complication in renal allograft. Its reported frequency is roughly 0.4% (1–3) and, when it presents acutely after transplant, is recognized as one of the causes of primary non-function (PNF) and delayed graft function (DGF) (2, 4, 5).

Considering the increase in transplants from extended criteria donors (ECDs), from donation after circulatory death (DCD), and the tendency for recipients to be older, the possibility of embolization arising from either donor or recipient vessels is expected to increase (6–10). Moreover, since embolization leads to focal and patchy damage, diagnosis is difficult, and injury severity may be underestimated (2, 11–13).

In the absence of a standard and effective therapy, strategies usually aim at stabilizing the plaque by using statins associated with steroids if the disease is recurring and systemic. Reports describe the effectiveness of iloprost, a synthetic analog of prostaglandin I_2, as a rescue therapy in systemic CE (2, 11, 14–16). Moreover, in the coronary angiography setting, where ischemic damage to renal tissue is the leading pathogenic mechanism, a reduction in the incidence of contrast-induced

nephropathy has been reported in patients with baseline renal insufficiency undergoing coronary intervention (17).

To the best of our knowledge, there are no recent reports on the use of iloprost in CE after kidney transplantation (2, 16, 18).

Here we report two cases of acute post-transplant CE in which the addition of iloprost to the standard care helped accelerate the recovery of kidney function.

Written informed consent was obtained from the participants for the publication of these case reports.

CASE REPORT

Case 1

A 44-year-old man received a kidney transplant from a brain-dead donor (DBD). The donor was 59 years old, had died from cerebral hemorrhage, his Kidney Donor Profile Index (KDPI) was 83%, Karpinsky's score was 3, and he had been a smoker with a past history of prostate cancer for which he was in regular follow-up (19). The surgeon described atheromatous plaques in the renal artery that were particularly evident at the confluence with the aorta and were partially removed before implantation. Immunosuppressive therapy consisted of basiliximab, steroids, and tacrolimus. Owing to persistent oligo-anuria, a kidney biopsy was performed on the eighth post-operative day (POD). Histology showed severe acute tubular necrosis (ATN), diffuse cholesterol embolism in the arterioles, inflammatory mixed infiltrate, and interstitial edema. A borderline cellular rejection was diagnosed, and thymoglobulin (ATG) therapy at a dose of 3 mg/kg was administered. Because of the persistence of DGF, the kidney biopsy was repeated on POD 16. The sample showed regression of the interstitial infiltrate, with persistence of ATN and diffuse CE (**Figure 1**). Therefore, we started rescue therapy with intravenous iloprost at a dose of 0.05 mg/kg/day for 15 days. We observed a slow but progressive recovery of kidney function. No peripheral signs of embolism were observed on physical examination. After 3 months, creatinine was 3 mg/dl; at the 1 year of follow-up, it had improved to 2 mg/dl (**Table 1**). In this case, the probable source of embolization was the donor renal artery, which presented as a severe atherosclerotic plaque at retrieval.

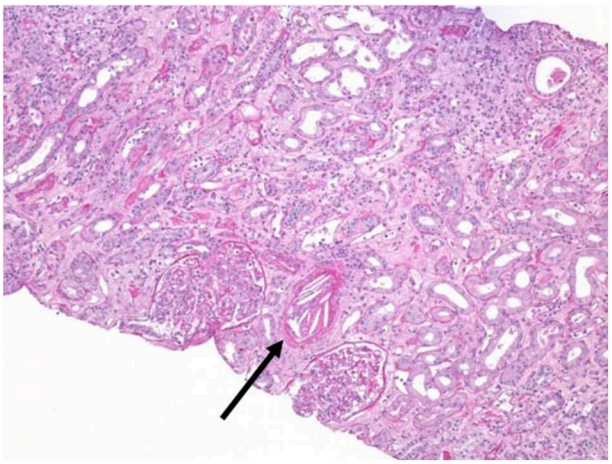

FIGURE 1 | Kidney biopsy at post-operative day 16. Periodic acid–Schiff (PAS) staining, magnification 20×. Arrow indicates a massive cholesterol embolization occluding the arteriolar lumen.

Case 2

A 71-year-old hypertense woman underwent a DBD double kidney transplant. The iliac vessels of the recipient, a smoker, presented with severe atheromatous plaques such that it was difficult to find a suitable vessel to perform the arterial anastomoses; some plaques were fixed to the walls of the vessel with 6-0 prolene. The 81-year-old donor had died from a cerebral hemorrhage, had a KDPI of 99%, and a Karpinsky's score of 4 in both kidneys. Immunosuppressive therapy consisted of ATG, steroids, and tacrolimus. The graft function was prompt, with creatinine levels of 1.7 mg/dl on POD 4, and routine ultrasounds were normal. On POD 13, we observed an abrupt rise in creatinine (2.4 mg/dl), lactate dehydrogenase (LDH) 1,100 U/l, and a slight decrease in diuresis. A contrast-enhanced ultrasonography showed a lack of vascularization in the upper pole of one of the kidneys compatible with a partial infarction. Intravenous iloprost at a dose of 0.05 mg/kg/day was administered as a rescue therapy for 5 days. After 3 days, we started to see progressive recovery of kidney function; after 3 months, the creatinine level was 1.5 mg/dl (**Table 1**). No peripheral signs of embolism were observed on physical examination. In this case, the most likely source of embolization was the recipient's iliac artery.

DISCUSSION

Atheroembolic renal disease in kidney transplantation is recognized as a possible cause of graft loss. It can occur in the early days post-transplant as well as in the late phases of transplant follow-up (2, 4, 5).

When presenting acutely post-transplant, CE usually occurs due to an acute embolization from either the aorta or the renal artery of the donor during organ harvesting or from the vascular axis of the recipient during surgery.

As a result of the increasing number of ECD and of the aging of both donor and recipient population, atherosclerosis of the vascular axis of the graft and of the recipient is becoming a serious challenge in the field of organ transplantation (20–23).

In our first case, we described the embolization of the donor artery in which plaque disruption probably occurred at harvesting or during the preliminary vascular manipulation made before implantation. In the second instance, the likely cause the acute deterioration of function was crystal embolization from the recipient iliac artery. Our final diagnosis was difficult to prove since no peripheral or systemic signs of CE were present, no other causes of acute kidney injury were identified, and an ischemic area was clearly identified by contrast-enhanced ultrasound. We were aware that the patient was severely atherosclerotic from the results of multiple computed tomography-angiographies performed during the time spent on the waiting list. The surgeon, due to our experience in high atherosclerotic patients, defined her

TABLE 1 | Clinical course of both cases.

	Case 1				Case 2			
	Post-surgery	**Pre-iloprost**	**During iloprost**	**After iloprost**	**Post-surgery**	**Pre-iloprost**	**During iloprost**	**After iloprost**
Blood pressure (mmHg)		130/65	130/80	120/80	110/70	140/85	130/70	140/80
Urine volume (ml/day)	0	0	1,200	2,000	1,500	1,000	1,200	1,200
Creatinine (mg/dl)	8	8.2	8	3	1.4	2.4	2	1.6
Urea (mg/dl)	118	169	157	90	54	78	81	73
Eosinophils 10^9/L	0.14	0	0.15	0.07	0.06	0.16	0.22	0.09
LDH U/L	357	302	349	250	370	1193	540	402

to be suitable for transplant; her condition, however, was found to be worse than predicted.

When the plaque disrupts, microemboli spray downstream, and occlude the vascular lumen of small arteries. The ensuing damage is a combination of tissue ischemia, direct cytotoxic effects of crystals, and necrosis due to the local inflammatory reaction. Soon after embolization, the first damage occurs to endothelium mitochondria (24). Then, because of the large dimensions of cholesterol crystals (1 μm−1 mm), macrophages are not able to digest them completely; this "*frustrated phagocytosis*" triggers an intracellular danger signal mediated by damage-associated molecular patterns, interleukin (IL)-1α, IL-1β, and nuclear factor (NF)-κB (24–27). The vicious cycle of necroinflammation eventually leads to necrosis (28, 29). Moreover, it has been demonstrated that cholesterol crystals also activate the complement-dependent inflammasome and cytokines (30, 31). Overall, the ischemic and inflammatory pathways activated by this phenomenon increase the already high cardiovascular and inflammatory risk profile of transplant recipients (32).

Since the damage caused by CE is patchy, it is well-known that histologic diagnosis is difficult and often underestimated; this also occurs in native kidneys (12, 13). Moreover, cholesterol crystals are not always present in the sample, and the only lesions seen are ATN and inflammatory infiltrates (2). In light of this, the mild interstitial mixed infiltrate already present in the biopsy of our first case could be explained as related more to an inflammatory reaction to the severe and diffuse embolism rather than to cellular rejection, especially considering the ischemic lesions present in the sample.

Given the key role of inflammation in CE, therapies have always been based on adding steroids to statins, although there is no clear evidence of its effectiveness (2). There are also very few reports showing positive results in the use of the synthetic prostacyclin iloprost as a rescue therapy in systemic CE (2, 11, 14–16, 33, 34). Recently, prophylactic intravenous iloprost therapy has shown some effectiveness in reducing the incidence of contrast-induced nephropathy in the coronary angiography setting in patients with baseline renal insufficiency undergoing coronary intervention, a setting in which toxic ischemic damage is the leading pathogenic event to renal cells (17).

In the 80s and 90s, scientific literature put great emphasis on the prostacyclin system and on the use of prostacyclin analogs in kidney disease (14, 35, 36). The main application field was ischemic injury, but there are also some reported experiences in the field of transplantation. In fact, pretransplant graft perfusion or administration of iloprost in the early days post-transplant led to some benefits in cases of DGF and of cyclosporin-induced toxicity (18, 35, 37–40).

Regarding CE in kidney transplantation, there are no reports exploring the effectiveness of PGI_2 agonism.

Iloprost is an analog to PGI_2 that exerts different effects both on the vascular wall and blood cells. Acting directly on endothelial cells, smooth muscle, and adventitia, it stimulates angiogenesis, endothelial cell integrity, and relaxation of smooth muscle cells. Moreover, PGI_2 has inhibitory effects on the activation of endothelial cells and on the proliferation and migration of smooth muscle cells (41–43). PGI_2 acts on leukocytes stimulating the production of anti-inflammatory cytokines and inhibiting the release of IL-1, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. PGI_2 also regulates macrophage functions, promoting their anti-inflammatory profile (44, 45). Effects on the inhibition of platelet aggregation have also been described (20, 43).

The acute continuous iloprost therapy we administered to our patients may have partially counteracted the necroinflammation and vasoconstriction caused by the emboli through vasodilatation, the inhibition of IL-1 and TNF, and the production of other cytokines; in combination with high steroid doses commonly used in the early post-transplant phases, iloprost may have strengthened the positive effects that the reduction of oxidative damage exerts on the outcome of the transplant (46).

Our cases are a good example of increasingly common complications related to detrimental vascular characteristics of grafts and recipients. Moreover, in the case of transplantation, this phenomenon could be restricted to the graft, without the occurrence of peripheral or systemic lesions. Since the embolization could be patchy, the pathognomonic lesion could be invisible in the histologic sample, making the final diagnosis even more difficult. It is important to note that the only lesion seen at biopsy could be ATN associated with an inflammatory infiltrate, easily attributable to cellular rejection (2).

CONCLUSIONS

Acute post-transplant CE seems to be increasingly diagnosed in patients with severe atherosclerosis and ECD donors. In the

context of transplantation, diagnosis can be difficult since CE can be limited to the graft and the histology can be confused with cellular rejection. As prompt treatment can help in reducing the risk of PNF and in the recovery of function, CE should always be suspected in cases of persistent DGF or acute cellular rejection not responding to therapy. Iloprost, with its vasodilator and anti-inflammatory effects, could potentially act on the molecular pathways activated by cholesterol crystals; it is our opinion that prompt intravenous therapy with iloprost, added to statins and steroids, has accelerated the good outcome of the two patients whose cases we have described in this report.

Of course, the effectiveness of iloprost infusion in hindering the inflammatory and ischemic cascades induced by CE in the immediate post-transplant setting should be investigated in depth, especially considering that prompt intervention is essential. Larger case control studies and clinical trials are needed to prove the causality between iloprost administration and the improvement of kidney function and investigate when prompt intervention is essential.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VCo and GC contributed to conception, design of the work, analysis, and interpretation of data. MR, VA, FO, AA, IC, and VCu contributed to the acquisition of data for the work. GL revising it critically for important intellectual content. All the authors provide approval for publication of the content.

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REVIEW
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Transplanting the Elderly: Mandatory Age- and Minimal Histocompatibility Matching

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Worldwide over 40% of patients receiving renal replacement therapy (RRT) are aged 65 years or older, a number that is still increasing. Renal transplantation is the preferred RRT, providing substantial survival benefit over those remaining on dialysis, including the elderly. Only 3% of patients aged 65 years or older accepted on the waiting list actually received a kidney transplant offer within the Eurotransplant allocation region. To increase the chance for elderly to receive a timely kidney transplant, the Eurotransplant Senior Program was introduced. The ESP supports local allocation of older kidneys to older donors in order to decrease cold ischemia time, while disregarding former exchange principles based on matching for HLA antigens. As a consequence, more elderly received a kidney transplant and a relative higher incidence of acute rejection resulted in additional courses of high steroids and/or depleting antibody therapy. Since death with a functioning graft due to infections is the dominant reason of graft loss in elderly, more intense clinical immunosuppression to prevent or treat acute rejection is not a very attractive option. Therefore in elderly kidney transplant candidates, we advocate reintroduction of minimal histocompatibility criteria (i.e., HLA-DR matching) followed by age-matching with mandatory local/regional allocation to also facilitate short cold ischemia.

Keywords: kidney transplantation, elderly, allocation, histocompatibility, HLA, old-for-old program

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INTRODUCTION

End stage renal disease (ESRD) is a rapidly becoming a critical problem worldwide. In The Netherlands, for instance, the prevalence of patients receiving renal replacement therapy (RRT) was 17531 or 1020 per million population (pmp) in the year 2017, an increase of almost 35% in the last decade (1). In countries contributing to the ERA-EDTA registry the prevalence of RRT was 592779 (854 pmp) in 2012, a number that also increased almost 30% in recent annual reports (2). In the US even greater numbers have been documented with a prevalence of 726331 patients (2206 pmp) on RRT in 2016, a number that almost doubled since 2000 [(3) USRDS annual data report: Epidemiology of kidney disease in the United States National Institutes of Health]. With increasing numbers of senior citizens and associated health care challenges, even more elderly with chronic kidney disease and need for RRT can be anticipated. Nowadays, according to the Dutch National Renal Replacement Database (Renine) and the ERA-EDTA registry, already 45% of patients on RRT are 65 years of age or older (2). Likewise, in the US 41% of the patients with ESRD is over the age of 65 years.

It is known that patients aged 65 years or older, and especially those over 75 year, constitute a separate group with different views and needs regarding health care issues. This is also reflected in choice of RRT. Although kidney transplantation is generally the preferred treatment option when it comes to survival benefit across all ages (4–7), only 3% of the patients between 65 and 74 years were actually transplanted in 2017 and virtually none of the patients receiving a kidney transplant were over the age of 75 years (2). The majority of older transplant candidates are likely to die while on the waiting list before they get a transplant offer according to the Dutch Renine data. Mortality early after transplantation is also higher in elderly with a large French registry study reporting a 3-fold higher mortality risk in the first 3 postoperative months as compared to waitlisted counterparts (8).

Another important cause of death after kidney transplantation is failure of the kidney allograft, which is an important independent risk factor of mortality. After graft loss, the risk of mortality in those relisted for a repeat transplant is also higher as compared to patients with a functioning graft or those listed for their first transplant (9–11). Since all-cause mortality increases with age, the longevity of the first kidney graft, even allowing less optimal renal function, is of critical importance.

In this paper we reconsider the relative importance and causes of graft failure in the elderly as well as the challenges, hurdles and potential different approaches to prolong survival. We focus on the elderly and need for carefully balanced strategies in this vulnerable group of patients with ESRD.

THE ELDERLY WITH A FAILING KIDNEY TRANSPLANT

Overall, kidney graft survival improved significantly in the past decades and mainly due to the prevention of early acute rejection by the use of more potent immunosuppressive drugs like tacrolimus, mycophenolate and more frequent use of poly- or monoclonal antibodies as induction therapy. The improvement in short-term and long-term graft survival has not improved concomitantly and long-term graft survival more or less stabilized between 1988 and 2005 (12). This trend continued beyond 2005 with an approximate 5% annual loss of kidney allografts after the 1st year (13). An analysis of the Collaborative Transplant Study (CTS) registry confirmed that also in Europe graft survival has improved mainly due to short term outcome parameters. Since the year 2000, however, there has been a lack of improvement in short- and long-term graft survival, even after taking into account the changing donor and recipient demographics and donor or organ quality characteristics. This observation probably reflects a lack of further innovation in the management and treatment options around the kidney transplant procedure (14).

Older kidney transplant recipients probably require different allocation and/or treatment strategies as compared to their younger counterparts. A critical first consideration is the notion that the elderly that actually do get a transplant offer are a highly selected subgroup of elderly patients with ESRD. First, these patients are generally rigorously medically selected before acceptance on the active kidney waiting list. Secondly with increasing age, only very low proportions of these patients actually reach the end of the cue, while significant numbers of patients are removed from the list due to comorbidities and the majority die while waiting for a kidney transplant offer. Taken together, there is an important selection based on cardiovascular and/or oncological exclusion criteria. Where these issues are important long-term topics for younger transplant recipients, in elderly transplant candidates or recipients these are not the main reason for graft loss and therefore may require other strategies.

The Elderly and Mortality

In elderly the most important reason for late allograft loss is death with a functioning graft (DWFG) (15, 16). In a large registry using UNOS and USRDS data, Ojo et al. (17) found that 42,5% of graft loss was due to DWFG. Age at transplantation was an obvious, strong and independent risk factor. When compared to those aged 18–29 years, recipients aged over 65 years had a 7-fold increased risk to die with a functioning graft. Besides age, ESRD caused by systemic vascular diseases such as hypertension or diabetes mellitus was an independent risk factor for premature death (17). This was confirmed in a study of El-Zoghby et al. (18) where 43.4% of the grafts were lost due to death. Interestingly, the most important cause of death more than 5 years after transplantation was due to infectious causes. Transplant recipients who died due to infections were older (64.6 vs. 59.4 years) as compared to those due to cardiovascular diseases. This observation supports the fact that elderly kidney transplant recipients are an already highly selected group with excess infectious comorbidity and mortality, the downside of current potent clinical immunosuppressive drugs, and therefore a key consideration comparable to the increased cardiovascular risk in younger recipients (18).

Death due to infectious causes is the consequence of clinical overimmunosuppression, especially in elderly with an already immunosenescent immune system in the context of aging (19). In the light of solid organ transplantation, the consequence of the aging immune system has been documented in a shift from naïve T-cells toward relatively more memory T-cells resulting in decreased immune reactivity (20–22). Indeed the incidence and severity of infections parallels the increase in age in patients (23). Regarding renal transplant recipients, several studies have reported that older patients have more infectious problems and that older recipients die more often due to infectious causes (24, 25). Recently, Lemoine et al. (16) studied renal recipients over 70 years of age and confirmed the mortality risk in elderly due to infections. In a total of 171 recipients death-censored 1-year graft survival was 82.6 and 9.9% of included patients died after the 1st year with infectious causes in 58.5% of cases (16).

Kidney Transplant Failure

The Elderly and Rejection

It has become widely accepted that older transplant recipients may encounter less acute rejection episodes after transplantation as compared to younger recipients due to immunosenescence (26, 27). However, if they do experience acute rejection, this episode is more likely to compromise graft- and/or patient

survival, the latter in particular due to additional excessive immunosuppression (28). In a large registry, 5-year death-censored graft survival after rejection was 59.9% in recipients aged 65 years or older as compared to 82.1% in recipients aged 18 to 35 years (29).

Overall the frequency of rejection declines within subsequent age categories, but a higher donor age is significantly associated with higher rejection rates (30–32). A study in 2016 among 244 elderly also showed that although older recipient age was protective for the occurrence of acute rejection, this was clearly outweighed by the dominant negative effect of donor age and increased immunogenicity of the organ reflected by more rejection and more donor specific antibody (DSA) formation with increased HLA-DR mismatch (33). This study shows that 1 or 2 HLA-DR mismatches give a higher chance on TCMR and the development of DSAs which both results in decreased allograft survival (66% for TCMR and 63% for dnDSA compared to 82 and 80% resp). They also show a graded effect, patients with 2 HLA-DR mismatches had worse graft survival rates after 3 and 7 years after transplantation compared to 0 or 1 HLA-DR mismatch (80%, 76% and 73% for 0, 1 and 2 HLA-DR mismatches 7 years after transplantation). This complex interaction of risks for increased rejection incidences was confirmed by a Dutch group in 2017 where elderly recipients with an older DCD (donation after circulatory death) kidney had a 2.78 times higher risk of delayed graft function and rejection compared to elderly receiving a young DBD kidney (34). This increased immunogenicity in recipients of a more vulnerable kidney allograft could be due to more endothelial activation in the context of ischemia-reperfusion injury, bacterial and viral infections resulting in a more pro-inflammatory cytokine environment, increased expression of HLA molecules and/or recruitment of antigen-presenting cells (35). These data are especially relevant regarding renal transplantation in elderly, since expansion of the donor pool with older, high-risk kidney donors, is a key strategic policy for this subgroup of renal recipients. Especially in view of rejection treatment and higher risk on infections cumulating in the most important cause of death in elderly. Therefore, the optimal strategy to decrease rejection risks while still allowing a timely transplant in the elderly is of critical importance.

Donor Specific Antibodies

When addressing transplant failure, *de novo* donor specific antibodies against HLA antigens (dnDSAs) after transplantation gained more and more interest. Overall Lachmann et al. (36) reported significant a lower 10-year graft survival being 49% versus 83% in patient with and those without DSA, respectively. A recent study showed that DSAs in combination with other risk factors can be even more detrimental for graft function. In this study, DSAs were associated with an increased incidence of T cell mediated rejection (TCMR) and led to a three-fold increase in graft loss (37). Lemoine et al. (16) showed that anti HLA antibodies are an independent risk factor for patient death and graft loss within the 1st year in patients older than 70 years. In elderly their role was recently debated by von Moos et al. (38) since elderly have a lower risk in developing DSAs than pediatric patients. However, they found more dnDSA in patients treated with cyclosporine as compared to tacrolimus so regarding immunosuppressive protocols for elderly, their role is still important in long term graft survival.

Multiple studies have been performed to address the prevalence, risk factors and consequences of dnDSA. Most studies report a prevalence of dnDSA of 10–19% after kidney transplantation and most are formed in the 1st year after transplantation with an annual incidence of 5% thereafter (39–43). There are several risk factors for the formation of DSA and not surprisingly, non-adherence or lowering immunosuppressive drugs for clinical reasons play a crucial role (44–48). However, one can only form antibodies if there is a foreign HLA molecule and therefore the main risk factor is the degree of HLA mismatch between the recipient and the donor (49). Several studies show that HLA class II mismatch, in particular HLA-DQ, is most important (40, 41, 50). Other described risk factors for the formation of dnDSA are kidneys of deceased donors and younger age of the recipient.

Despite the current knowledge there is still no clearly defined clinical advice regarding DSAs and the prevention of formation. Guidelines from the Transplantation Society, the sensitization in transplantation: assessment of risk (STAR) working group and the Heidelberg algorithm, based on the CTS and data from the Heidelberg Transplant Center, all advise to test post-transplantation in pre-specified patient groups. All agree that patients most at risk are patients with a pre-activated immune system, measured by pre-existing antibodies or soluble CD30, in combination with periods of under-immunosuppression and should be monitored closely (51–53).

HLA compatibility between donor and recipient is currently assessed by the number of HLA mismatches on serologic level although HLA antibodies recognize accessible polymorphic sequences of amino acids rather than whole HLA antigens. These polymorphic sequences, so called epitopes, can be shared between HLA antigens so the true mismatch is much more complicated than serologic level shows. Therefore, the question can be raised whether current matching principles are reliable enough to reduce or minimize the risk of dnDSA formation.

Using the original HLA Matchmaker algorithm (54), Wiebe et al. (55) evaluated the development of *de novo* class-II DSAs in 286 kidney transplant recipients. Epitope mismatches were significantly more frequent in the patients who developed dnDSAs. In this study the optimal threshold for development of antibodies against HLA-DR was 10 mismatched epitopes and for HLA-DQ 17 mismatched epitopes (55). In a second study they investigated the interaction between medication adherence and degree of epitope mismatch. In this study in 596 renal recipients the optimal threshold for development of class II dnDSAs was 11 epitope mismatches for both HLA-DR and HLA-DQ. The combination of a high alloimmune risk (> 11 epitope mismatches) and tacrolimus trough levels below 5 ng/ml led to development of dnDSAs whereas patients with less than 11 epitope mismatches tolerated low tacrolimus trough levels (56). Recently they published the result of a study in 664 renal recipients. This study confirmed that the risk of dnDSAs was more strongly correlated to epitope mismatches as compared

to conventional HLA mismatch. However, the threshold in this study was 7 epitope mismatches for HLA-DR and 9 for HLA-DQ (57). Also, Snanoudj et al. (58) investigated the epitope mismatch load by using HLA Matchmaker in 89 renal recipients. They found that epitope load was more strongly associated with dnDSAs compared to the number of serologic HLA mismatches. Of note, in this study the optimal threshold was 27 epitope mismatches (58).

So, one can easily appreciate potential pitfalls in these newer matching methods that were developed based on the epitope level. Although more accurate in predicting dnDSA development than conventional matching, defining a reliable threshold as a risk factor is difficult and needs to be solved. To identify patients at risk, or maybe equally relevant those with a lower risk and safer option to adjust immunosuppressive load, there is an urgent need for well-defined risk factors to guide clinical decision making.

THE ELDERLY AND AGE-MATCHING: THE EUROTRANSPLANT SENIOR PROGRAM

Organ shortage and the continuously growing waiting list, demands a progressive expansion of the potential kidney donor pool. Therefore, boundaries of organ quality criteria are continuously stretched and more and more older donors with or without comorbid conditions are accepted for renal transplantation (8). With the acceptance of older donors, the proportion of what was historically called extended criteria donors (ECD) also increased significantly. Since 2015 donors in the US have been assessed by the so-called Kidney Donor Profile Index (KDPI) score, which is associated with the life expectancy of the graft. Kidneys with a KDPI > 85%, or high risk kidneys, are expected to function for more than 5.5 years and are therefore considered to be comparable to the previous so-called ECD kidneys (59).

It is well known that graft survival decreases with increasing donor age and decreasing organ quality, but also that the elderly still benefited from a successful kidney transplant using high risk kidneys in terms of life expectancy as compared to their waitlisted counterparts (60). Recipients of a high-risk kidney had a significantly lower mortality risk (RR 0.75; 95% CI 0.65-0.86), results confirmed by several studies (6, 60, 61).

It is widely accepted that each kidney should be allocated to the recipients in whom is it expected to survive the longest to improve the match between life expectancy of donor and recipient. Since older transplant recipients are more likely to die with a functioning graft and younger recipients have a higher chance on re-transplantation later in life, it seems logical to allocate older kidneys, with an increased chance of graft failure, to older recipients.

Therefore, in 1999 the Eurotransplant Senior Program (ESP) was implemented to shorten the waiting time for older transplant candidates and improve the perspective on patient survival with ESRD. In this program kidneys from donors > 65 years are allocated to recipients > 65 years with preferred local allocation in order to shorten cold ischemia times (CIT) and the likelihood of delayed graft function and/or rejection. To reach these goals, HLA matching was neglected, obviously resulting in a higher HLA mismatch rates in 'old for old' transplant programs. In 2008 the 5 years results were published and main goals were reached, waiting time decreased and CIT went down to 11.9 h compared to > 17 h in the regular ETKAS allocation program (62). However, there was a 5 to 10% higher rejection rate within ESP (29.1%) as compared to regular allocation. As mentioned in the study of Halleck et al. (33), this could be due to a higher HLA mismatch, especially HLA-DR, which led to significantly impaired graft survival. Indeed, in the ESP 92.9% of the recipients had 2 HLA-DR mismatches compared to 54.9% ≥ 1 HLA-DR mismatch in the normal allocation scheme.

CHANGING THE STRATEGY FOR OLDER TRANSPLANT CANDIDATES

At the moment only a minority of selected elderly transplant candidates actually receive a kidney transplant and the mortality rate among this patient group is relatively high, especially the 1st year after transplantation or in case complications occur such as an acute rejection episode. In order to increase transplant rates, more older donors are accepted and preferably for older recipients, which in turn leads to more acute rejection episodes and rejection treatments.

In younger transplant recipients, the increased risk of acute rejection with the use of older donors could possibly be overcome with induction therapy or more potent maintenance therapy. In elderly the complex interplay between immunosuppression on the one hand and immune defense on the other hand is even more challenging due to pre-existing comorbidities, changes in pharmacokinetics of immunosuppressive drugs, polypharmacy and the immunosenescence mentioned earlier. Probably more balanced immunosuppressive protocols and more advanced immunological monitoring strategies are needed to balance this critical equipoise. A second, more feasible and practical strategy could be to change the allocation protocol to decrease the risk of acute rejection and/or DSA formation without the need of more clinical immunosuppression.

Adjusting Maintenance Immunosuppressive Protocols

Calcineurin Inhibitors

Calcineurin inhibitors (CNI) remain the most potent immunosuppressive drugs in preventing acute rejection and have been critical to improve short-term graft survival. Due to the nephrotoxic potential of CNIs on long-term graft failure, there has been an overall shift toward reduction or CNI-withdrawal preferably later after transplantation. Since elderly are more susceptible to infections and the other side effects of immunosuppressive drugs and older kidney grafts are more vulnerable to CNI induced vasoconstriction and/or nephrotoxicity, several studies have suggested that especially elderly could benefit from CNI withdrawal or avoidance (63). In

addition, the pharmacokinetics of CNIs change with increasing age and Staatz et al. (64) concluded in their review that especially maintenance therapy in older patients potentially needs more frequent monitoring and adjustments. Jacobson et al. (65) reported in a clinical trial that elderly (> 65 year) yielded similar trough levels with lower CNI dose and that dose-normalized trough levels were more than 50% higher in older patients.

Various studies have indicated that CNI-withdrawal in the regular population of renal transplant recipients may not be successful (66–71). The results of reduction of tacrolimus differ in literature, but the CTS study showed that graft survival is compromised below a trough level of 5 ng/ml and in those with high intra-patient variability (48). Several studies have reported an increase in dnDSA formation below a certain trough level. Gatault et al. (72) found only dnDSAs in the group with a mean tacrolimus trough level of 4.1 ng/ml. Recently Davis et al. (73) reported a 4-fold risk of dnDSAs for patients with a mean tacrolimus through level of 4-6 ng/ml as compared to ≥ 8 ng/ml in the 1st year after kidney transplantation. As mentioned before, the Winnipeg group addressed the risk of minimization of calcineurin inhibitors and the development of dnDSA in relation to epitope mismatch load. Both studies confirmed that patients with a higher epitope load were at risk for dnDSAs after minimizing immunosuppression (56, 58).

In elderly, Arbogast et al. (74) used an CNI free protocol after ATG induction followed by mycophenolate mofetil (MMF) and prednisone. Cumulative 5-year patient and allograft survival was 88 and 70%, respectively. And although these results are in itself excellent for older renal transplant recipients, the acute rejection rate was more than 25% and these patients returned to a regimen with a CNI after the rejection treatment which underlines the importance of a tailormade strategy rather than a standard protocol regarding immunosuppression (74).

mTOR Inhibitors

In order to reduce CNI exposure, also the mammalian target of rapamycin inhibitors (mTORi) have been introduced and positioned. Several randomized trials have been performed of which the most recent one is the large TRANSFORM study. In this study 2037 renal recipients were randomized to standard dose CNI + MMF or reduced CNI + mTORi. The latter proved to be non-inferior regarding a binary endpoint of BPAR or glomerular filtration rate (GFR) < 50ml/min/1.73m^2. Benefits of the regimen with mTORi were a significantly reduced incidence of viral infections, which could be direct clinical benefit in the elderly. Although elderly were not excluded from this trial, mean age was 49.3 year (75). Recently the results from the SENATOR trial were reported. In this trial renal recipients participating in the Eurotransplant Senior Program (ESP) were included and 7-weeks after transplantation randomized to standard therapy with CNI + MMF or converted to MMF + everolimus and basiliximab at weeks 7 and 12. The patients who were converted and remained on everolimus had comparable kidney function and comparable rates of BPAR. Only 37.2% of the patients were actually randomized, identifying elderly as a vulnerable study population. From the patients who were randomized, 27.8% discontinued everolimus due to adverse events. This study underscores the challenge of randomized studies in elderly transplant recipients and general need for tailored treatment in this group.

Allocation With Prospective HLA Matching

As expected, the ESP program achieved the goal to minimize cold ischemia time and also the anticipated reduction in the rate of delayed graft function. The higher incidence of acute rejection was not expected and this could suggest a greater role of immunogenicity in the context of less histocompatibility. One could overcome this increased rejection risk by increasing clinical immunosuppression. The elderly, however, already have a compromised immune system and are more vulnerable for infectious complications. In addition, marginal donors are more vulnerable for the nephrotoxic side effects of immunosuppressive drugs. Therefore, at least in theory, this may be a suitable strategy in a proportion of younger patients receiving older and more immunogenic kidneys but may not be the best options for the older transplant recipients.

A different strategy is to require a minimal degree of histocompatibility between donor and recipient while maintaining the shorter CIT and therefore the benefit of less delayed graft function. Due to a high degree of linkage between HLA-DR and HLA-DQ antigens, matching for HLA DR frequently results in matching on HLA-DQ (76). As previous studies proved, graft survival is worse even with 1 HLA-DR mismatch. Therefore, prospective HLA-DR matching with zero mismatches would be a potentially elegant strategy to improve rejection free survival without the need of excessive immunosuppression.

CONCLUDING REMARKS

Given the inherent limited life expectancy of older patients, their best option when encountering ESRD would be the option of a kidney transplant as soon as possible. In order to reach this goal age matching is a suitable strategy and most patients will receive a kidney from an older deceased donor.

Even with a timely kidney transplant offer from an age-matched donor, there are other issues to consider in elderly recipients. Recipients of older kidneys are more susceptible to acute rejection with HLA class-II mismatch being a potentially preventable key risk factor also for the subsequent formation of DSAs. The mere fact that the older recipient has an older immune system as compared to adolescents, does not overcome the dominant effect of donor age over recipient age.

We therefore underlined the importance of prospective HLA matching in the allocation algorithm of older kidneys to older kidney transplant candidates. Since most DSAs are directed against HLA class II antigens, HLA-DR matching is likely to reduce the need for more intense clinical immunosuppression and/or additional acute rejection treatments, ensuing reduction

of excess infectious cause morbidity and mortality while delivering the prospect of prolonged life expectancy.

To reintroduce prospective matching for HLA class-II antigens, the Eurotransplant Senior DR-compatible Program (ESDP) study was designed and the results will be important to guide future clinical practice.

AUTHOR CONTRIBUTIONS

GD wrote the first draft of the manuscript. JF wrote sections of the manuscript and revised the manuscript. Both authors contributed to the final manuscript revision, read and approved the submitted version.

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Perirenal Adipose Tissue Displays an Age-Dependent Inflammatory Signature Associated With Early Graft Dysfunction of Marginal Kidney Transplants

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Background: Better understanding of the contribution of donor aging and comorbidity factors of expanded criteria donors (ECD) to the clinical outcome of a transplant is a challenge in kidney transplantation. We investigated whether the features of donor-derived stromal vascular fraction of perirenal adipose tissue (PRAT-SVF) could be indicative of the deleterious impact of the ECD microenvironment on a renal transplant.

Methods: A comparative analysis of cellular components, transcriptomic and vasculogenic profiles was performed in PRAT-SVF obtained from 22 optimal donors and 31 ECD deceased donors. We then investigated whether these parameters could be associated with donor aging and early allograft dysfunction.

Results: When compared with the PRAT-SVF of non-ECD donors, ECD PRAT-SVF displayed a lower proportion of stromal cells, a higher proportion of inflammatory NK cells. The global RNA sequencing approach indicated a differential molecular signature in the PRAT-SVF of ECD donors characterized by the over-expression of CXCL1 and IL1-β inflammatory transcripts. The vasculogenic activity of PRAT-SVF was highly variable but was not significantly affected in marginal donors. Periorgan recruitment of monocytes/macrophages and NK cells in PRAT-SVF was associated with donor aging. The presence of NK cell infiltrates was associated with lower PRAT-SVF angiogenic activity and with early allograft dysfunction evaluated on day 7 and at 1 month post-transplant.

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Conclusions: Our results indicate that human NK cell subsets are differentially recruited in the periorgan environment of aging kidney transplants. We provide novel evidence that PRAT-SVF represents a non-invasive and timely source of donor material with potential value to assess inflammatory features that impact organ quality and function.

Keywords: marginal kidney donors, kidney transplantation, natural killer cells, endothelial inflammation, perirenal adipose tissue, kidney allograft dysfunction

INTRODUCTION

Increasing recipient demand combined with inadequate organ supply has led to the use of suboptimal marginal kidneys from expanded criteria donors (ECD) with cardiovascular risk factors (1–3). While the use of ECD kidney transplants enables more patients to benefit from renal transplantation, various studies have reported that marginal transplants from elderly donors are associated with an increased incidence of delayed graft function (DGF), slow graft function recovery (SGF) (3–6) and poorer long-term graft outcome (5, 7–9).

The underlying mechanisms which associate donor age and cardiovascular risk factors with a worsened outcome of these marginal transplants are not completely understood. Since graft endothelial cells constitute the critical interface between the donor and the recipient, pre-existing endothelial dysfunction of the donor could be considered as an initial checkpoint leading to deleterious recipient immune responses and vascular rejection (10–13). Several studies have highlighted the "higher immunological risk" of transplants from marginal donors resulting in stress-induced senescence mechanisms (14, 15) and induction of endothelial adhesion and inflammatory molecules (16–19).

The current challenge is therefore to delineate the donor-related features that determine the capacity of transplant endothelium to resist further exposure to ischemia, oxidative, uremic and alloimmune inflammatory stresses associated with the transplant procedure. There is a lack of models that identify donor-related features that reflect the endothelial quality of aging ECD transplants (16).

Our study is based on the hypothesis that perirenal adipose tissue (PRAT), systematically discarded during the surgical preparation of a renal transplant, represents an easily accessible source of donor-derived material allowing assessment of the quantitative and functional features that characterize exposure of donor cells to the ECD microenvironment.

Indeed, adipose tissue (AT) can be enzymatically processed to yield stromal vascular fraction (SVF), a heterogeneous cellular mixture devoid of adipocytes that recapitulates the variety of cells that constitute the vasculature such as mesenchymal stem cells, pericytes, endothelial progenitor cells and leucocytes. Endothelial progenitor cells, also called endothelial colony forming cells (ECFC), have been recently identified in the vessel wall and SVF (20–22). We and others have reported that the phenotypic and angiogenic activity of ECFC can be altered in deceased contexts associated with cardiovascular risk factors or genetic or epigenetic determinants (23–25). Furthermore, it was recently demonstrated that donor age and comorbidities can alter the angiogenic and paracrine immunosuppressive properties of human bone marrow-derived stromal cells (BM-SC) obtained after *in vitro* cell culture expansion (26). It is thus likely that the ECD microenvironment can also alter the vascular potential of the various types of PRAT SVF-resident cells.

Based on this knowledge, we postulated that donor PRAT-SVF could represent a relevant and non-invasive model to evaluate the ECD microenvironment factors that could contribute to the alteration of renal transplant quality. This study aimed to (1) provide a comprehensive view of cellular, transcriptomic, and angiogenic profiles that could characterize the peri-organ SVF obtained from marginal kidney donors, and (2) analyze whether the features of PRAT-SVF could be indicative of the deleterious impact of donor aging and cardiovascular risk factors on early kidney allograft dysfunction.

MATERIALS AND METHODS

Patients and Sample Collection

We conducted a monocentric prospective study involving 53 renal transplantation procedures performed in the Department of Urology and Renal transplantation, La Conception University Hospital in Marseille, France from 2016 to 2018. For each renal transplant, the stromal vascular fraction (PRAT-SVF) was isolated from the perirenal AT collected during kidney procurement and submitted to analysis of cellular components, transcriptomic profile and vasculogenic activity. The study was approved by the National Ethics Committee of the Agence de la Biomédecine (ABM), the National Ministry of Research and adhered to the Jardé Law on human investigation. All procedures were conducted in compliance with the Declarations of Helsinki and Istanbul. Data were prospectively and anonymously collected in a dedicated database for the exclusive access of the authorized authors.

Clinical Variables

The following demographic data were recorded for donors and recipients: sex, age, body mass index, blood group, serum creatinine, cardiovascular risk factors (history of smoking, hypertension, dyslipidemia, diabetes mellitus, coronary heart disease. Renal function (serum creatinine, glomerular filtration

Abbreviations: ABM, Agence de la Biomédecine; DGF, delayed graft function; ECD, expanded criteria donors; ECFC, endothelial colony forming cells; GFR, glomerular filtration rate; LD, living donor; NK, Natural Killer cells; Non-ECD, non-expanded criteria donors; PRAT, perirenal adipose tissue; SGF, slow graft function; SVF, stromal vascular fraction.

rate) were recorded at D7, M1, and M12 during renal transplantation follow-up. The CKD EPI formula was used to evaluate renal function in adults and the Schwartz formula was used in younger recipients (<18 years) (27).

Definition of Endpoints

ECD kidney transplants were defined as those from donors aged ≥60 years or 50 to 59 years with 2 of the following comorbidities: hypertension, serum creatinine >1.5 mg/dl, or death following cerebrovascular accident.

Delayed graft function (DGF) was defined as the use of dialysis within 7 days of the transplant (28). Slow graft function (SGF) was defined by serum creatinine > 250 umol/L (3.0 mg/dL) on postoperative day 7 (29).

Identification of Anti-HLA Antibodies

The detection of HLA-specific antibodies was performed using standard techniques. The presence of allograft- specific antibodies was screened through Luminex screening assays (LAScreen® mixed, One Lambda, Canoga Park, CA, USA) using Luminex flow beads (LAScan™ 100, Luminex, Austin, TX, USA). To determine their antibody specificity, all samples with a positive screening result were further evaluated using single-antigen flow bead assays according to the manufacturer's recommended protocol (LAScreen® Single Antigen class I or LAScreen® Single Antigen class II, One Lambda, Canoga Park, CA, USA). The percentage of HLA sensitization for the single-antigen assays were calculated according to the manufacturer's instructions as the percentage of positive bead reactions among the 99 class I beads and 97 class II beads.

Isolation of the Stromal Vascular Fraction From Donor Perirenal AT

Perirenal adipose tissue (at least 30 g) was collected under aseptic conditions during the multi-organ retrieval for cadaveric donor renal transplantation. Excised fat was manually sliced with scissors into units of ∼3 × 3 × 3 mm. Enzymatic digestion was performed using 0.25UI/mL collagenase NB4 (Serva, Heidelberg, Germany) for 1 h at 37°C under constant agitation. Three cycles of wash/centrifugation were performed to eliminate adipose and red blood cells. Freshly isolated PRAT-SVF was used for flow cytometry analysis and RNA extraction and cryopreserved at −180°c for delayed angiogenic assays performed on thawed samples.

Phenotypic Characterization of PRAT- SVF Cell Subsets

Multiparameter flow cytometry analysis was performed to compare the quantitative distribution of the CD45- and CD45+ cell subsets in the donor-derived PRAT-SVF in the non ECD and ECD groups.

Half a million cells per tube were suspended in 100 µL of phosphate-buffered saline (PBS, Gibco®, Life Technologies), stained 20 min at room temperature and protected from light with the DRAQ5 nuclear marker, the NucBlue viability marker and two pre-prepared antibody mixes or corresponding isotype controls in matched concentrations. The first monoclonal antibody mix contained the following surface markers: CD146, CD34, and CD45, labeled respectively with the following fluorochromes: PE, ECD, and PC5. The second mix was composed of the following surface markers: CD14, CD34, CD45, CD56, CD3 labeled respectively with FITC, ECD, PC5, PC7 and APC-Alexa Fluor 750 fluorochromes (References in **Supplementary Table 1**). Flow cytometry was performed with a NAVIOS instrument (Beckman Coulter, Brea, California, USA). Data files were analyzed using Kaluza software (Beckman Coulter, Brea, California, USA). The gating strategy used to identify the various cell subsets is summarized in **Supplementary Figure 1**.

RNA Purification and RNAseq Gene Expression Analysis

Total RNA was isolated from PRAT-SVF using RNeasy mini kits (QIAGEN Inc., Valencia, CA, USA) including a DNase I digestion step removing genomic DNA. RNAseq analysis of SVF profiles in ECD vs. non-ECD kidney donors was performed by HalioDX. Briefly, the purity and concentration of the samples were estimated by spectrophotometer. The integrity of the RNA (RIN > 8) was evaluated on an RNA 6000 Nano LabChiprun Agilent 2100 Bioanalyzer (Agilent technologies, Germany). Generation of libraries was performed using PerklinElmer technologies with the NEXTflex qRNA-Seq kit v2 after total RNA enrichment by NEXTflex Poly(A) beads following manufacturer's recommendations (PerkinElmer). RNA-seq library were sequenced on Illumina Nextseq sequencer. The generated reads were single-end and of 76-nt length. FastQC (version 0.11.5) was used to examine the read quality. Trimming of reads was performed using Trimmomatic (version 0.33) on the base of an average phred quality of 20. The raw single-ends reads were then mapped against the human genome (GRCh38.90) from the Ensembl database using STAR (version 2.5.3a) sequence mapper. The resulting BAM files were examined by Qualimap (version 2.2). Duplicates reads were removed using the function MarkDuplicates of picard tools (version 2.9.0). Unduplicated reads were used to count reads per gene with FfeatureCounts (version 1.5.2). Raw counts are converted in reads per Million (RPKM) and log transformed (log base 2) in order to help with distributional assumptions, linearity and consistency with PCR based methods for calculating the Fold Change. Genes of interest were filtered using a mean RPKM >25 and a coefficient of variation >50% (1183 genes passed out).

R/Bioconductor packages including DESeq2 were used for gene expression analysis. Finally, we selected differentially expressed genes with a *P*-value <0.05 and a Fold Change (FC) of a least 1.5.

Genes up or downregulated were separately subjected to functional annotation analysis using the Database for Annotation Visualization and Integrated Discovery (DAVID, david.ncifcrf.gov/) online tool to find significantly enriched genes biological functions and associated pathways. Gene Ontology: Biological Process and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was performed with a

cut-off criteria for the threshold of EASE score< 0.05 (modified Fisher Exact *P*-value).

Real Time PCR Analysis of Transcripts

Total RNA (5 μg) was converted to cDNA using 200U of M-MLV reverse transcriptase (Invitrogen). Real-time PCR amplification was performed with the Light Cycler 480 SYBR Green I Master kit (Roche). Cycling conditions were 10 min at 95°C (hot-start PCR), followed by 40 cycles, 10 s at 95°C (denaturation), 15 s at 62°C (annealing) and 20 s at 72°C (elongation). Melting curve analysis was performed to check the specificity of amplification. Reported values are relative numbers of specific transcripts detected per 10^6 *GAPDH* transcripts. The primers used for gene-specific amplification are described in **Supplementary Table 2**.

Tube Formation Assay

Tube formation assay was analyzed *in vitro* using PRAT-SVF in Matrigel™ (6 mg/ml) (Corning® Matrigel® Basement Membrane Matrix Growth Factor Reduced, Phenol Red Free, 356231) as described by Zakhari et al. (30). The PRAT-SVF from ECD and non-ECD donors were loaded at a density of 20,000 cells/well in a μ-slide angiogenesis (81506, IBIDI) system coated with 10 μl of growth factor-reduced Matrigel™ (6 mg/ml) (Corning® Matrigel® Basement Membrane Matrix Growth Factor Reduced, Phenol Red Free, 356231), previously polymerized for 30 min, and were maintained in endothelial basal cell culture medium-2 (EBM2) supplemented with MV SingleQuots (EGM2-MV) (Lonza, Clonetics, Walkersville, MD, USA) at 37°C with 5% CO_2. Capillary-like structures were recorded after 72 h using a Leica DMI8 video-imaging inverted microscope equipped with an Incubator I8 at 5X magnification and were captured using Leica Application Suite X software (Las X 3.0.2.16120). As previously described (30), various parameters that reflect the relevant steps of SVF vasculogenic/angiogenic capacity *in vitro*, were quantified: the number of clusters, indicative of the capacity of the plated cells to self-assemble; the number of clusters with tip cells, indicative of the ability of cells to undergo specialization into cells able to migrate away from the cluster and initiate sprouting; the number of clusters with stalk cells that represent the capacity of cells to proliferate and elongate neovessels; and the number of branching points that provide information on the capacity of cells to develop as complex vascular networks. Cell clusters were automatically counted using Fiji software under cellular analysis with minimum object size set at 500 μm and a maximum object size set at 3,000 μm. These counts along with manual counts of tip cells, stalk cells and branch points were taken using still frames of both groups. Each experiment was performed in triplicate.

Spheroid-Based Sprouting Assay

Angiogenic sprouting was analyzed *in vitro* using PRAT-SVF in a collagen gel matrix as previously described by Korff et al. (31). The images were then analyzed using the Sprout Analysis plug-in developed by Eglinger et al. (32) in the Fiji distribution of ImageJ, to evaluate the different vascular parameters, such as sprout length and branch points.

PRAT-SVF from ECD, non-ECD deceased donors and living donors were suspended in culture medium containing 0.2% (wt/vol) carboxymethylcellulose (M0512, Sigma, Munich, Germany), which was then seeded in non-adherent round-bottom 96-well plates (82.1582.001, Sartstedt), leading to the formation of spheroids with a defined cell number. After 72 h, the spheroids were collected and embedded in collagen gels (354236, Corning® Collagen I, Rat Tail). The spheroid containing gel was rapidly transferred into pre-warmed Labtek II slides (NUNC 54534, ThermoFisher) and allowed to polymerize (30 min), then 100 μl of EGM2-MV medium were added on the top of the gel. Following 24 h of culture in EGM2-MV medium, the spheroids were fixed for 30 min in 4% paraformaldehyde at room temperature. After washing and permeabilization 2 h at 4°C with PBS containing 0.1% Triton X-100 and 1% BSA, the spheroids were immunolabeled overnight at 4°C with phalloidin coupled with Alexa-647 (A22287, ThermoFisher Scientific) (1/100), and nuclei were stained with 6-diamidino-2-phenylindole (DAPI) (1/5000) diluted in PBS 1% BSA. After washing, we then captured a fluorescent optical image stack along the z-axis at 20X magnification using two lasers in sequential mode under a Leica DMI8 microscope (at least n = 10 spheroids per condition). Las X software was used during all image acquisition procedures. Image processing prior to image measurements was performed with Huygens Essential deconvolution software (Scientific Volume Imaging,) using up to 40 iterations of the classical maximum likelihood estimation algorithm, with a theoretical PSF and automatic background correction. The images were then analyzed using the Sprout Analysis plug-in developed by Eglinger et al. (32) in the Fiji distribution of ImageJ, to evaluate the different vascular parameters, such as sprout length and branch points.

Statistical Analysis

Statistical analysis comparing continuous variables in 2 groups was performed using non-parametric Wilcoxon–Mann–Whitney test and categorical variables with Chi2 test using Xlstat® version 2018.5 (Addinsoft, Paris, France) and Graphpad Prism® version 7 (GraphPad Software, California, USA). Categorical variables were presented as frequencies and continuous variables as the mean ± standard deviation (SD) or median and 10–90 or 25–75 percentile according to the test of normal distribution using the Kolmogorov-Smirnov test. Spearman rank correlation was used to evaluate the associations between quantitative parameters analyzed in PRAT-SVF and age or creatinine or CKD evaluated in the recipient at D7 and M1 post-transplant. Only variables with $P < 0.20$ were considered. Significant differences were considered when the *P*-value was < 0.05. Univariate and multivariate logistic regression analyses were performed to evaluate whether the PRAT-SVF parameters evaluated could discriminate the effect of aging (<59 years) or transplants that had good functional recovery from those with impaired graft function (based on the use of the 60 and 45 ml/ min per 1.73m^2 CKD eGFR cut-off values corresponding to moderate or mild CKD at M1). The AUC of the receiver operating characteristic (ROC) curve was used to define the threshold of quantitative variables that best predicted early dysfunction of the transplant at M1 post-transplant.

RESULTS

Donor and Recipient Characteristics

Fifty-three donors were included: 31 (49%) expanded criteria donors (ECD) and 22 (35%) non-expanded criteria donors (non-ECD). Donor characteristics are summarized in **Table 1**. Compared with non-ECD, the ECD group presented with higher age (71 vs. 42 years, $P < 0.01$) and a higher prevalence of cardiovascular risk factors (hypertension, dyslipidemia, vasculopathy, all $P < 0.05$) except for the prevalence of diabetes that did not reach significance Second transplant concerned 7% of the analyzed cohort. All the patients received the same induction therapy except for one patient in the non ECD donor group that was treated with anti IL2. Rabbit antithymocyte globulin (rATG) were administered on day 0 (1.25 g/kg/day) for 8 days. Prednisolone was administered on day 0 (initially 1 mg/kg/day), with subsequent tapering to achieve a targeted mean maintenance dose of 0.25 mg/kg/day at day 30 after transplant. As previously described (33), maintenance immunosuppressive therapy associated Tacrolimus/mycophenolate mofetil (FK/MMF, 64%) or ciclosporine/azathioprine (CSA/Aza, 36%) immunosuppressive combination. All recipients transplanted with an optimal kidney (non ECD) were treated with FK/MMF maintenance therapy, while the CSA/Aza immunosuppressive combination was used in 62 % of the recipients transplanted with an ECD kidney (**Table 1**).

The recipients of ECD kidney grafts were significantly older than the non-ECD recipients (median 67 vs. 39 years, $P < 0.01$). However, the prevalence and duration of dialysis before kidney transplantation were comparable in both groups. While most patients were negative for anti HLA panel reactive antibodies (PRA) evaluated before graft (63 %), HLA class I PRA sensitization was observed in 29% of the patients, anti HLA class II sensitization without Class I immunization in 2% of the patients and combined immunization against class I and Class II in 5% of kidney transplant recipients. None of the anti HLA antibodies detected before transplant were Donor specific antibodies (DSA) and transplant recipients analyzed in the study cohort did not develop *de novo* DSA during the first 3 months following transplant. The incidence of slow/delayed graft function (SDGF) was significantly higher in the recipients of ECD allografts (45% vs. 14%, $P = 0.02$). The average glomerular filtration rate (eGFR) of ECD donor kidney grafts was significantly lower when compared to non-ECD kidney grafts on day 7 (D7) and at 1 month (M1) post-transplant (with eGFR of 25 vs. 67 ml/min/1.73m^2 on D7 and 41 vs. 82 mL/min/1.73m^2 at M1, all $P < 0.05$). Mean follow-up post-transplantation period was 12.7 months.

TABLE 1 | Donors and recipients characteristics.

	ECD	Non-ECD	*P*-value
	n = 31	*n* = 22	
DONOR BASELINE CHARACTERISTICS			
Gender (M/F) %	55/45 %	59/41%	0.79
Age, years (median, 25–75 IR)	71 [65–78]	42 [30–53]	<0.01
BMI, kg/m^2 (median, 25–75 IR)	27 [24–29]	24 [22–27]	0.03
DONORS RENAL FUNCTION			
Serum creatinine (micromoles/L)	78 [60–98]	67 [48–100]	0.39
Proteinuria, g/L	0.2 [0.1–0.5]	0.2 [0.1–0.5]	0.65
DONOR MEDICAL HISTORY			
Smoking %	7%	50%	< 0.01
Hypertension %	61%	0%	< 0.01
Dyslipidemia %	26%	0%	0.02
Diabetes mellitus %	10%	5%	0.63
Vasculopathy %	29%	0%	0.01
Cold ischemia time, hours	12 [9–15]	13 [10–17]	0.33
Side (Left/Right), %	89/11%	80/20%	0.63
RECIPIENT BASELINE CHARACTERISTICS			
Gender (M/F) %	58/42%	82/18%	0.57
Age, years (median, 25–75 IR)	67 [61–73]	39 [28–52]	<0.01
BMI, kg/m^2 (median, 25–75 IR)	25 [22–27]	23 [19–26]	0.07
RECIPIENT MEDICAL HISTORY			
Hemodialysis %	84%	85%	0.99
Dialysis duration, months	35 [17–47]	32 [2–53]	0.64
Smoking %	33%	10%	0.08
Hypertension %	92%	70%	0.11
Dyslipidemia %	25%	20%	0.73
Diabetes mellitus %	25%	5%	0.11
Coronary heart disease %	29%	10%	0.15
PRETRANSPLANT ASSESSMENT			
HLA class I and/or class II sensitization (%)	35%	39%	0.79
% of HLA class I positive beads	5% [5–7]	5% [5–5]	0.99
% of HLA class II positive beads	26% [13–39]	6% [6–6]	0.26
Rank of renal transplantation >1	9%	5%	0.63
MAINTENANCE IMMUNOSUPRESSIVE TREATMENT			
Steroids/Tacrolimus/Mycofenolate Mofetil	38.5%	100%	<0.01
Steroids/Ciclosporin/Azathioprine	61.5%	0%	
RENAL GRAFT OUTCOME			
Slow/delayed graft function %	45%	14%	0.02
Graft function day 7 (D7)			
Serum creatinine (micromoles/L)	374 [146–573]	171 [66–178]	<0.01
eGFR (mL/min/1.73m^2)	25 [6–40]	67 [38–111]	<0.01
Graft function at month 1 (M1)			
Serum creatinine (micromoles/L)	162 [111–193]	103 [72–129]	<0.01
eGFR (mL/min/1.73m^2)	41 [23–50]	82 [68–120]	<0.01
Mean time Follow-up, months	13.5	11.6	0.62

Values are reported as % or median [25–75 interquartile ranges].

The Cell Subset Distribution of Perirenal SVF Is Altered in ECD Donors

High inter-individual variability in the distribution of SVF cell subsets was observed among the donors (**Figure 1**). The CD45+ leucocyte population was the most prevalent subset (median 61 %, 25–75 percentile range 47–74) and tended to be higher in the ECD donors (**Figure 1A**). The median percentage of stromal cells (13%, 25–75 percentile: 7–30%) was significantly lower in the ECD donors (9 %) when compared with the non-ECD donors (18%, $p = 0.03$) (**Figure 1B**). The quantitative distribution of endothelial cells (median 8.5 %, 25–75 percentile:

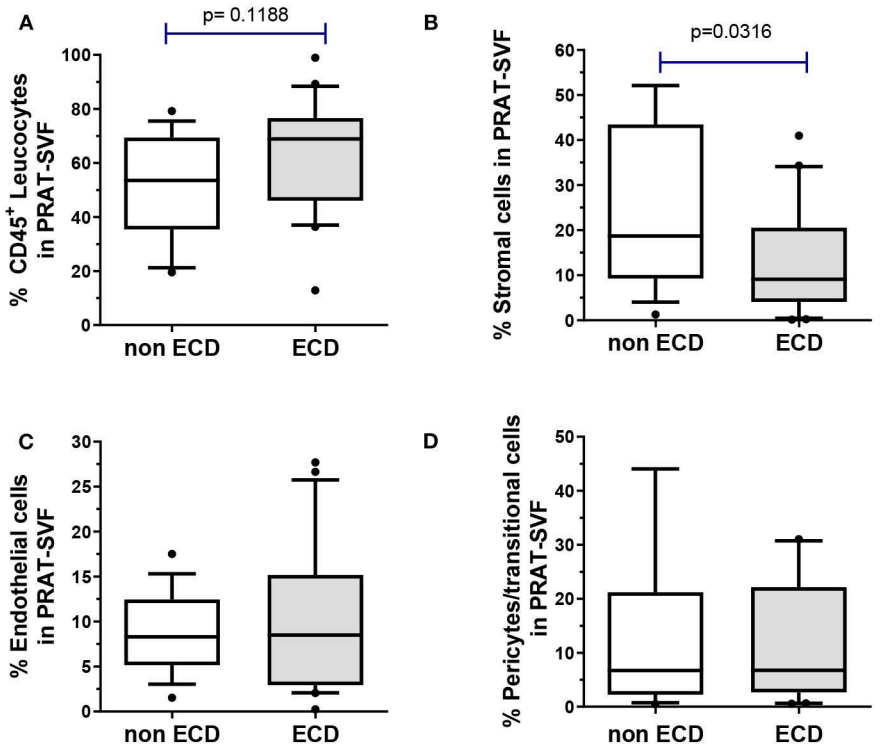

FIGURE 1 | The distribution of cell subsets composing the PRAT-SVF was determined using flow cytometry and compared between the non-ECD and ECD donors: **(A)** $CD45^+$ leukocytes were comparable in the two groups **(B)** mesenchymal stem/stromal cells were significantly lowered in the ECD vs. non-ECD group **(C)** pericytes and transitional cells **(D)** and endothelial cells were not statistically different between the two groups. ECD, extended criteria donors. Non ECD, non-extended criteria donors. Results on the graphs are reported as box and whiskers plots representative of median values, and 25–75 interquartile ranges (Boxes) and error bars indicative of 10-90 percentile ranges. Dots indicates values out of the 10–90% quartile range.

4–13%, **Figure 1C**) and pericytes (median 7%, 25–75 percentile: 2.4–22.1, **Figure 1D**) were comparable in PRAT-SVF from the two groups of kidney donors.

Comparative Analysis of PRAT-SVF the Angiogenic Activity of ECD and Non-ECD Donors

The SVF-dependent formation of capillary-like structures, evaluated in an *in vitro* Matrigel^{TM} assay (**Figure 2A**) as the number of clusters, was similar in the ECD and non-ECD donors (**Figure 2B**). A trend toward a decrease in the tip cell ($P = 0.07$) as well as stalk cell ($P = 0.07$) percentages was observed for ECD PRAT-SVF, but did not reach significance (**Figure 2B**). In addition, the vessel complexity characterized by the Matrigel branching points was also preserved in the ECD donor PRAT-SVF (**Figure 2B**). In a 3D spheroid assay (**Figure 2C**), the sprout formation, total network length and the average number of junctions formed by sprouts presented a trend toward a decrease in ECD PRAT-SVF, when analyzed in reference to the non-ECD donor PRAT-SVF (**Figure 2D** and **Supplementary Figure 2**).

Taken together, these data suggest a high inter-individual heterogeneity in the angiogenic potential of donor PRAT-SVF but does not identify a significant impairment of the median angiogenic activity of PRAT-SVF derived from ECD donors, when compared to ECD donors.

Transcriptomic Analysis of PRAT-SVF Identified Inflammatory Profiles Specific to ECD Donors

A comparative RNAseq transcriptomic analysis was performed to compare the PRAT-SVF molecular transcripts in ECD and non-ECD donors (**Supplementary Table 3**). Volcano plot distinguished a significant differential gene expression profile based on the comparison of ECD and non-ECD patients (**Figure 3A**). Overall, differential expression analysis revealed 245 genes showing fold change (FC) values ≥ 1.5 (111 genes overexpressed in ECD vs. non-ECD, **Supplementary Table 4**) and FC ≤ -1.5 (134 genes under-expressed in ECD vs. non-ECD) with $P \leq 0.05$ (**Supplementary Table 5**). To provide a cohesive view of the biological functions associated with the changes in the ECD-SVF gene expression profile, we conducted a gene ontology analysis using the DAVID database. The up-regulated genes showed a strong association with the inflammatory response and cytokine secretion as well

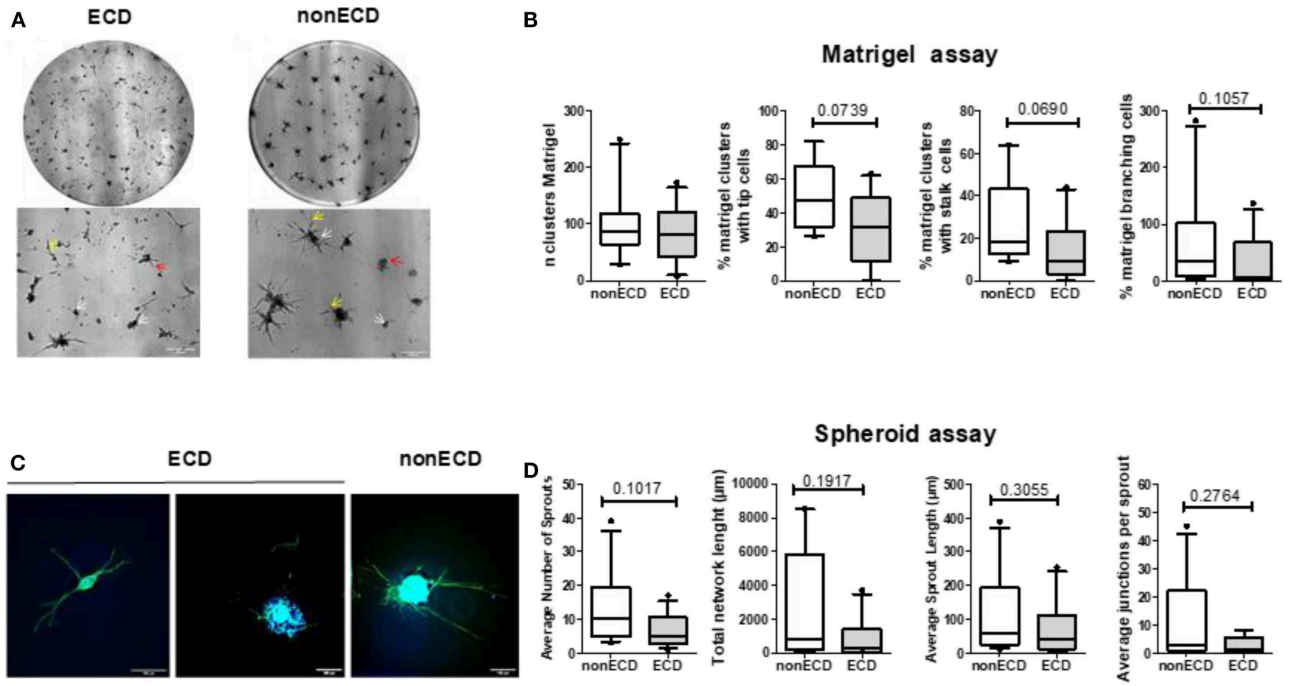

FIGURE 2 | (A) Representative experiment of capillary tube formation by SVF from ECD or non-ECD. A total of 20,000 cells/well were seeded on growth factor reduced Matrigel. Images were recorded at 72 h with a phase-contrast microscope. Original magnification x5; upper panel (scale bars, 300 μm) correspond to the total image of the well while the lower panels were zooms of previous images (scale bars, 200 μm). White arrows identify initial cell clustering; red arrows marked the tip cells while yellow arrows identified branching. **(B)** Quantitative analysis of number of clusters, percentage of clusters with tip and stalk cells, number of branching points. Data are expressed as means ± SEM of independent experiments performed in triplicate using PRAT-SVF obtained in 14 ECD and 10 non-ECD donors **(C)** Representative experiment of 3D *in vitro* angiogenic assay with collagen gel-embedded spheroids of SVF from ECD or non-ECD (original magnification x20; scale bars, 100 μm). Imaging of Vascular sprouts was obtained after merging of actin staining (phalloidin in green) and nuclei staining (DAPI, blue) as detailed in **Supplementary Figure 2 (D)** Quantitative analysis of number of sprouts, branch points, and total network length per spheroid as well as average sprout length was compared in PRAT-SVF from the ECD and non ECD donors. For each experiment, at least 10 spheroids were analyzed.

as circulatory system development **Supplementary Table 6**, whereas the categories enriched among the down-regulated genes were associated with the regulation of metabolic processes and the regulation of the circulatory system development (**Figure 3B** and **Supplementary Table 7**). Moreover, KEGG pathway analysis revealed differential inflammatory pathways, as "chemokine pathway," "NF-kappa B pathway" or "TNF signaling pathway," as well as "Graft-versus-host disease" (**Figure 3C** and **Supplementary Tables 8**, **9**).

Based on their previous involvement in graft rejection or angiogenesis and the FC in their differential expression between ECD and non-ECD patients, five genes were selected (*CXCL1, VWA7, CCL4, IL1-β, IFN-γ*) for further quantitative RT-PCR (qPCR) validation in the PRAT-SVF samples used to perform transcriptomic analysis (**Supplementary Table 10**). Analysis of an extended number of PRAT-SVF samples derived from 12 non-ECD and 13 ECD additional PRAT-SVF samples showed highly variable transcript expression among donors and confirmed the enhanced levels of CXCL1, IL1-β transcripts in ECD donor PRAT-SVF (**Figure 3D**). Relative transcript levels of CCL4 tended to be higher in ECD PRAT-SVF ($p = 0.07$, **Figure 3D**). Thus, these data identified an enrichment of genes involved in the control of inflammatory responses.

Donor Aging Is Associated With Inflammatory Profile in PRAT-SVF

Transcriptomic data prompted further analysis of the distribution of inflammatory cells within CD45+ cell compartment of PRAT-SVF. While the distribution of CD45+CD14+ macrophages/monocytes (**Figure 4A**), CD45+CD14- neutrophils (**Figure 4B**), and CD45+CD3+ T lymphocyte subsets (**Figure 4C**) was comparable among the two groups, the percentage of CD45+CD3-CD56+ NK cells was significantly higher in ECD PRAT-SVF (median value: 2.8%, 25–75 percentile: 1.3–5.1%) compared to non ECD PRAT-SVF (0.97%, 0.4–2.1%, $p = 0.01$) (**Figure 4D**). Interestingly, the percentage of NK cells was further associated with the level of transcripts encoding INF-γ inflammatory cytokines and the activating NKG2D receptor (**Table 2**). Enhanced levels of NK cell infiltrates were also associated with parameters indicative of endothelial dysfunction such as lowered angiogenesis scores and FGFR2 transcript levels (**Table 2**).

Donor age was also statistically associated with an inflammatory profile characterized by a significantly higher percentage of NK cells in PRAT-SVF. Stratification of donors according to the 59-year median value observed in the cohort confirmed the increased percentage of NK and T cell lymphocytes in the PRAT-SVF of donors ≥59 years (**Figure 5**). Donor-related

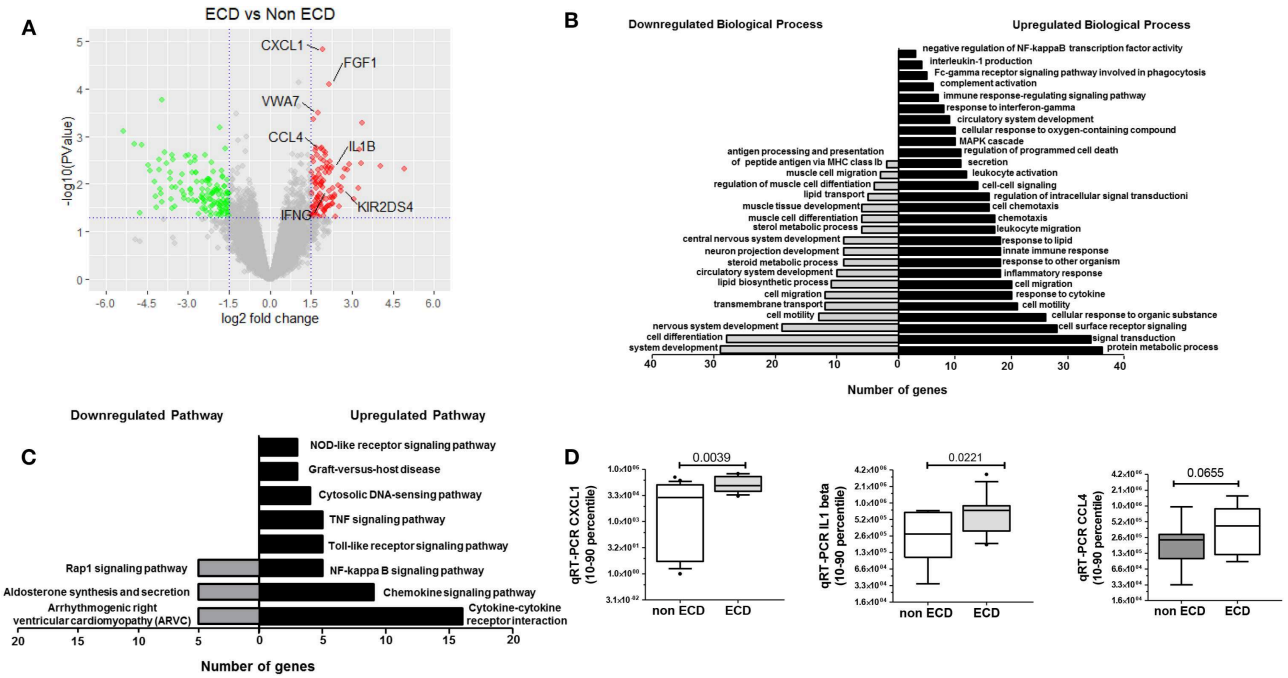

FIGURE 3 | Comparative Transcriptomic analysis of SVF from ECD ($n = 5$) and non-ECD ($n = 5$) patients. **(A)** Volcano plot of differentially expressed genes from SVF from ECD and non-ECD. Log2 Fold Change value obtained by RNAseq plotted against the –log10 of *P*-value. Genes with a fold change > |1.5| and $P < 0.05$ were deemed to be differentially expressed. $P = 0.05$ is indicated by horizontal lines. Positive and negative fold change values are reflective of down-regulated (green) or up-regulated (red) genes compared with non-ECD condition, respectively. **(B)** Fold enrichment over chance for the Gene Ontology Biological process of the Down (gray) and Up (black) gene lists using DAVID (fold change > |1.5| and $P < 0.01$). **(C)** Fold enrichment over chance for the KEGG Pathway of the Down (gray) and Up (black) gene lists using DAVID (fold change > |1.5| and $P < 0.01$). **(D)** qRT-PCR validation of selected genes expressed a relative levels of specific transcripts detected per 10^6 GAPDH transcripts [median (25–75% quartile)].

factors other than age could not be significantly associated with PRAT-SVF inflammatory profile.

The NK Inflammatory Profile of PRAT SVF Is Associated With Early Allograft Dysfunction

We then investigated whether parameters evaluated in PRAT-SVF could relate to allograft dysfunction during the first month following transplantation. Creatinine levels on D7 and eGFR at M1 post-transplant were significantly correlated with donor age, but did not correlate with cold ischemia time in the studied cohort (**Table 3**). Early graft dysfunction, as defined by creatinine levels at day 7 and values of eGFR<45 mL/min at one-month (M1) after transplantation, were also correlated with the proportion of PRAT-SVF NK inflammatory cells and monocytes/macrophages (**Table 3** and **Figure 6**) in univariate analysis. We used ROC curve analysis to set a 1.5% NK cell threshold in PRAT-SVF associated with lower CKD at M1 (area under ROC curve = 0.82, sensitivity 100%, specificity 75%). Interestingly, logistic regression models further showed that a percentage of NK cells > to this 1.5 threshold value of NK cells observed in pre-transplant donor PRAT-SVF, was an independent factor associated with lowered graft function recovery (eGFR< 45 or 60 at 1-month post-transplant), regardless of the HTA status of the donor (**Table 4**).

DISCUSSION

Taking advantage of the accessibility of donor perirenal adipose tissue, our study is the first to evidence significant changes in the cellular and molecular features that characterize the PRAT-SVF of marginal donors. Compared with optimal donors, marginal donors exhibited significant alterations in the cellular composition of PRAT-SVF that notably comprised an increase in immune-cell infiltrates and levels of transcripts encoding inflammatory chemokines/cytokines. This molecular inflammatory signature was impacted by donor age and could be further associated with early graft dysfunction.

The cell subset distribution in PRAT-SVF of ECD transplants indicated an imbalance between pro and anti-inflammatory cells. ECD donor PRAT-SVF showed a lowered proportion of stromal/mesenchymal cells, recently identified as an immunomodulatory cell compartment of the perirenal adipose tissue (34), and a higher proportion of immune NK lymphocyte infiltrates. Interestingly, enhanced representation of NK and T cells in PRAT-SVF was identified as an age-related specific feature. These results are in line with experimental studies suggesting that kidneys from older donors are more immunogenic and induce increased T-cell responses than kidneys from young donors (24).

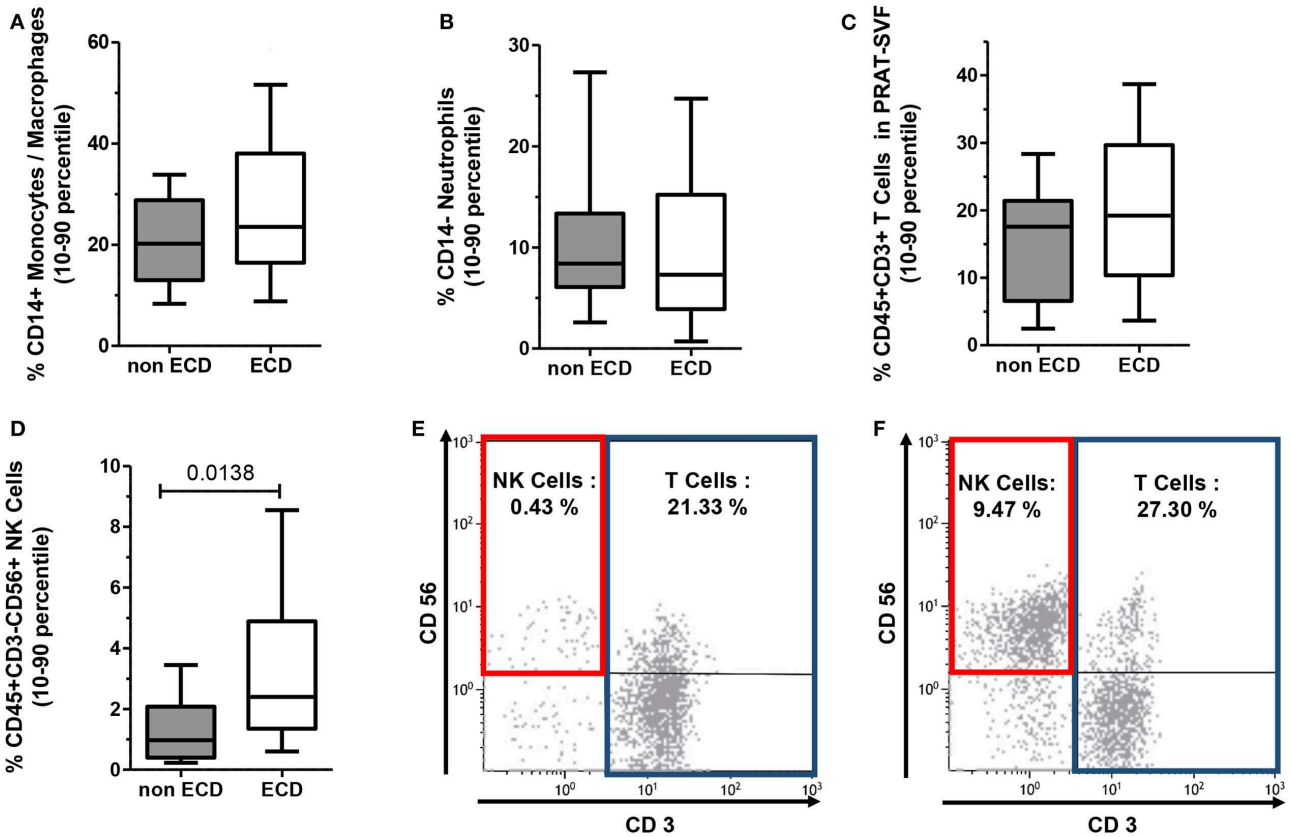

FIGURE 4 | The distribution of CD45$^+$ hematopoietic cells composing the PRAT-SVF of ECD and non-ECD donors was analyzed using flow cytometry according to the gating strategy illustrated in **Supplementary Figure 1**. Results on the graphs are reported as box and whiskers plots representative of median values, and 25–75 interquartile ranges (Boxes) and error bars indicative of 10–90 percentile ranges. **(A)** CD14+ Monocyte/Macrophage subset. **(B)** CD14-neutrophil subset. **(C)** CD3+ T lymphocytes. **(D)** CD3−CD56+ NK cells. **(E)** Histograms illustrate the gating and % of CD3+ T cells and CD3-CD56+ NK Cells observed when analyzing PRAT-SVF of a non-ECD donor (0.43% of NK Cells) or **(F)** PRAT-SVF from an ECD donor (9.47% of NK cells).

Consistently, major changes in the transcriptomic signature of ECD donors were found to be related to upregulation of inflammatory pathways. Among the most up-regulated genes was CXCL1, which is also known as GROα. CXCL1 is a pro-inflammatory chemokine that binds to CXCR2 to promote neutrophil chemotaxis. CXCL1-dependent neutrophil accumulation in a kidney transplant after reperfusion is an important predictor of delayed graft function (35, 36). CXCL1 has also been associated with various inflammatory kidney diseases such acute kidney ischemia and glomerulonephritis (37) and progression of chronic kidney disease (38). Inhibition of CXCR2 prevents kidney graft function deterioration owing to ischemia/reperfusion (39). Our transcriptional analysis also demonstrated that upregulation of the CCL4 chemokine and IL1-beta were associated with the ECD profile. This upregulation was consistent with an enhanced proportion of NK cells infiltrating ECD donor PRAT-SVF and previous data reporting the activation of a CCL4 and IFN-γ dependent pathway in patients with kidney graft rejection (40). Although extrapolation of these observations in donor PRAT-SVF could not be matched with those occurring in the pre-transplant biopsy, these results corroborate previous findings that identify the NKG2D activating receptor as a candidate marker of kidney graft quality in pre-transplant biopsy specimens from donors over 55 years (41).

In kidney transplant recipients, innate NK cells have recently been identified as a key effector mechanism regulating the level of endothelial lesion and repair as well as vascular rejection and allograft vasculopathy (11, 25, 42–44). NK cells have also been reported to contribute to immune senescence in kidney transplant candidates (45). Our observations suggest that PRAT-SVF recruitment of donor NK cells could also promote pro-inflammatory signals that affect the vascular homeostasis of a marginal transplant prior transplantation. While the quantitative distribution of PRAT-SVF endothelial cells was comparable in ECD and non-ECD donors, we observed a high inter-individual variability in PRAT-SVF angiogenic activity, that did not reach significance between the ECD and non-ECD groups analyzed in the present study. Such heterogeneity among donors has already been reported for mesenchymal stem cells (26). However, in line with this result, the study by Aird et al. did not evidence major changes in the angiogenic potential of AT-SVF with aging except a delayed phase of neovessels maturation *in vivo* (46). However,

TABLE 2 | Analysis of the link between quantitative parameters evaluated in PRAT-SVF and % of PRAT-SVF NK cells.

% CD3- CD56+ NK cell PRAT-SVF	Spearman r	*P*-value
DONOR CHARACTERISTICS		
Donor age	0.6228	0.0007***
PRAT SVF IMMUNE CELLS		
% CD3+ T cell PRAT-SVF	0.4845	0.0121*
% CD14+ Mono/macro PRAT-SVF	0.4216	0.0319*
PRAT-SVF TRANSCRIPTS		
PRAT-SVF NKG2D transcript Levels	0.676	0.0021**
PRAT-SVF FGFR2 transcript levels	−0.624	0.0098**
PRAT-SVF IFN-γ transcript levels	0.5611	0.0101*
ANGIOGENESIS		
Spheroid total network length	−0.5368	0.0178*
Spheroid average junction per sprout	−0.5035	0.028*
Spheroid number of sprouts	−0.4906	0.033*
Matrigel number of clusters	−0.4719	0.0413*

*BMI, Body Mass Index; PRAT, PeriRenal Adipose Tissue; SVF, Stromal Vascular Fraction. *P < 0.05, **P < 0.01, ***P < 0.001.*

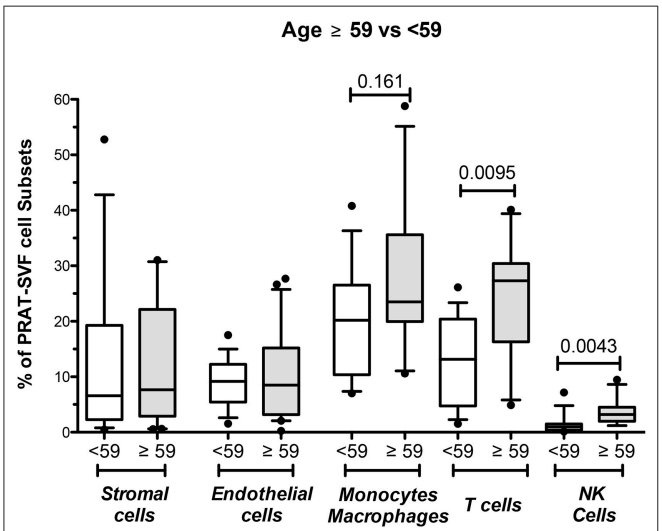

FIGURE 5 | Analysis of perirenal adipose tissue stromal vascular fraction (PRAT-SVF) in young vs. aged donors. PRAT-SVF was analyzed according to donor age when stratified in aging donors (≥59 years, $n = 29$) and younger donors (<59 years, $n = 24$). Percentages in stromal and endothelial cells were not different between the aging and younger donors. However, the aging donors presented a trend for increased representation of the CD45+ CD14+ monocyte macrophage cell subset and a significantly higher percentage of T and NK cells.

we observed that the percentage of NK cells in PRAT-SVF was inversely correlated with the angiogenic potential, suggesting that, at an individual level, donor-dependent NK cell activation could also provide an inflammatory environment that favors endothelial vulnerability prior to transplantation.

Importantly, among the parameters analyzed with PRAT-SVF, the proportion of NK cells was identified as associated to graft dysfunction evaluated at 7 days and 1-month post transplantation, indicating a potential impact on the clinical

TABLE 3 | Analysis of parameters correlating with early graft function at Day 7 (D7) and 1 month (M1) post-transplant.

	Serum creatinine Day 7		eGFR M1	
PRAT-SVF donor	**Spearman r**	***P*-value**	**Spearman r**	***P*-value**
DONOR CHARACTERISTICS				
Donor Age	0.3725	0.006	−0.5371	0.0001
Cold Ischemia time (hours)	0.2666	0.0802	ns	ns
PRAT-SVF IMMUNE CELLS INFILTRATION				
% CD45+CD14+ Monocytes/ macrophages	0.4461	0.0289	−0.4874	0.0214
% CD3-CD56+ NK cells	0.3991	0.0533	−0.4485	0.0363
% CD3+ T cells	0.34	0.104	ns	ns

PRAT, PeriRenal Adipose Tissue; SVF, Stromal Vascular Fraction; GFR, Glomerular Filtration Rate; Ns, Non-significant.

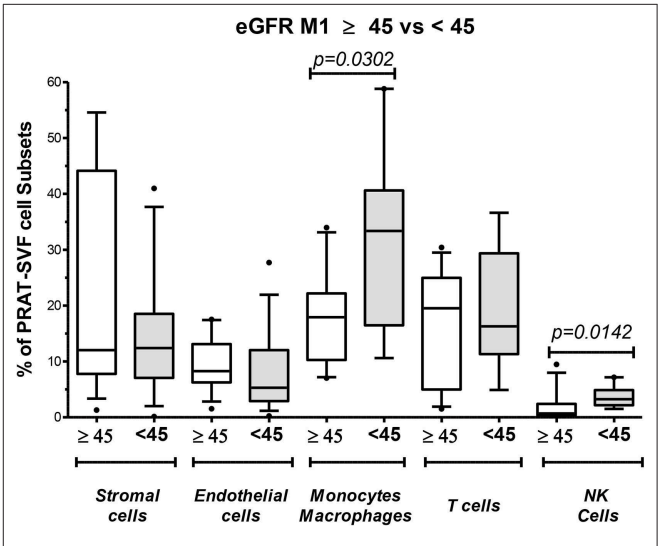

FIGURE 6 | Analysis of perirenal adipose tissue stromal vascular fraction (PRAT-SVF) with lower recovery of graft persisting at M1 post-transplant. Donors were split into two groups according to graft recovery persisting at M1 post-transplant: lower recovery (eGFR M1 < 45) and normal recovery (eGFR > 45). The proportion of cell subsets was analyzed according to this splitting. The eGFR M1 < 45 presented a significantly higher percentage of monocyte/macrophage subset and NK cells. Other cells were similarly distributed between the two groups.

outcome of marginal transplants from aging donors. These findings make it possible to speculate that the heterogeneity of inflammatory cytokine overexpression and age-dependent NK cell activation in ECD transplants could contribute to shaping allograft immunogenicity by perpetuating immune cell recruitment and activation, thereby rendering the endothelial cells of the graft more vulnerable to further exposure to ischemia/reperfusion, uremic, and alloimmune inflammatory stresses. These markers could thus be regarded as potential molecular targets for strategies enabling to reduce inflammation in ECD transplants.

Our study presents limitations since the unavailability of pre-transplant renal biopsies did not enable evaluation of the specific features that characterized PRAT-SVF in marginal donors which

TABLE 4 | Logistic regression models linking the presence of PRAT-SVF NK cell infiltrates to lowered allograft function evaluated 1-month (M1) post-transplantation.

	Odds Ratio	Std. Err.	z	*p*	[95% Conf. Interval]
Predictive factors of CKD <45 mL/min M1					
NK SVF ≥1.5%	25.6	33.5	2.48	0.01	[1.9–332.4]
Donor HTA	6.7	10.1	1.26	0.21	[0.3–130.2]
Predictive factors of CKDM1 <60 mL/min M1					
NK SVF ≥ 1.5%	10.6	11.2	2.2	0.03	[1.3–84.8]
Donor HTA	4.4	5.9	1.1	0.27	[0.3–61.7]

could be extended to the renal parenchyma (47). However, these data provided evidence that tissue recruitment of donor NK cells may *per se* participate in the pre-conditioning of transplant vulnerability and quality and prompt further investigation of the clinical relevance of such biomarkers in larger cohorts. This work could introduce PRAT-SVF as an innovative and less invasive approach with added value in terms of feasibility compared with pre-implantation biopsies and also document the specific features that characterize perirenal fat (47, 48). Another limitation is that the RNAseq and the functional analysis of the angiogenic capacity of donor-derived cells were performed on the whole PRAT-SVF and not on individual SVF sorted cell types. This experimental design allowed to integrate the cellular crosstalk between SVF cell subsets and prevented a potential bias associated with cell subset isolation and expansion *in vitro*. However, it did not allow to define if the observed changes resulted from alteration of PRAT-SVF composition or from a specific imprint of the ECD microenvironment on a given cell type. These data call for a more in-depth analysis using a single cell approach characterizing the transcriptomic profile of PRAT-SVF specific to the microenvironment of the ECD donor and the specific study of mesenchymal and endothelial cells purified from perirenal SVF. Future single cell analysis approaches and comparative analyses of purified endothelial and mesenchymal cells isolated from PRAT-SVF ECD and optimal donors would be of value to provide additional mechanistic clues.

Although the immediate implications of PRAT-SVF are not compatible with the current clinical setting of transplantation, our work may open perspectives to target inflammatory pathways in order to reduce donor-related inflammation before transplantation during the dynamic hypothermic machine perfusion process with the aim to optimize transplant quality.

CONCLUSION

Our results argue in favor of a donor-dependent inflammation-driven alteration of pre-transplant allograft quality and identify NK cells as potential effectors of pro-inflammatory remodeling mechanisms that can affect the function of marginal elderly transplants.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher. The RNAseq data has been submitted to the GEO repository under accession numbers GSE140122.

ETHICS STATEMENT

The study was approved by the National Ethics Committee of the Agence de Biomédecine (ABM), the National Ministry of Research and adhered to the Jardé Law on human investigation. All procedures were conducted in compliance with the Declarations of Helsinki and Istanbul. Data were prospectively and anonymously collected in a dedicated database for the exclusive access of the authorized authors.

AUTHOR CONTRIBUTIONS

RB, PP, GK, EL, and FS contributed to the conception and design of the study. RB, GK, MM, EL, BG, TL, and SB enrolled subjects into the study, collected primary data. LL, PF, BG, SS, JM, LG, and LA performed the experiments. PP reviewed the statistical analysis and wrote the manuscript. FD and FS wrote sections of the manuscript. All authors contributed to manuscript revision and 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/fimmu.2020.00445/full#supplementary-material

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Molecular Fingerprints of Borderline Changes in Kidney Allografts Are Influenced by Donor Category

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The fate of transplanted kidneys is substantially influenced by graft quality, with transplantation of kidneys from elderly and expanded criteria donors (ECDs) associated with higher occurrence of delayed graft function, rejection, and inferior long-term outcomes. However, little is known about early molecular fingerprints of these events in different donor categories. Borderline changes represent the most frequent histological finding early after kidney transplantation. Therefore, we examined outcomes and transcriptomic profiles of early-case biopsies diagnosed as borderline changes in different donor categories. In this single-center, retrospective, observational study, we compared midterm outcomes of kidney transplant recipients with early borderline changes as a first pathology between ECD ($n = 109$), standard criteria donor (SCDs, $n = 109$), and living donor (LD, $n = 51$) cohorts. Intragraft gene expression profiling by microarray was performed in part of these ECD, SCD, and LD cohorts. Although 5 year graft survival in patients with borderline changes in early-case biopsies was not influenced by donor category (log-rank $P = 0.293$), impaired kidney graft function (estimated glomerular filtration rate by Chronic Kidney Disease Epidemiology Collaboration equation) at M3, 1, 2, and 3 years was observed in the ECD cohort ($P < 0.001$). Graft biopsies from ECD donors had higher vascular intimal fibrosis and arteriolar hyalinosis compared to SCD and LD ($P < 0.001$), suggesting chronic vascular changes. Increased transcripts typical for ECD, as compared to both LD and SCD, showed enrichment of the inflammatory, defense, and wounding responses and the ECM–receptor interaction pathway. Additionally, increased transcripts in ECD vs. LD showed activation of complement and coagulation and cytokine–cytokine receptor pathways along with platelet activation and cell cycle regulation. Comparative gene expression overlaps of ECD, SCD, and LD using Venn diagrams found 64 up- and 16 down-regulated genes in ECD compared to both LD and SCD. Shared increased transcripts in ECD vs. both SCD and LD included thrombospondin-2 (*THBS2*), angiopoietin-like 4 (*ANGPTL4*), collagens (*COL6A3, COL1A1*), chemokine *CCL13*, and interleukin *IL11*, and most significantly,

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down-regulated transcripts included proline-rich 35 (*PRR35*) and fibroblast growth factor 9. Early borderline changes in ECD kidney transplantation are characterized by increased regulation of inflammation, extracellular matrix remodeling, and acute kidney injury transcripts in comparison with both LD and SCD grafts.

Keywords: marginal donor, borderline changes, kidney transplantation, gene expression, microarray

INTRODUCTION

The association of aging with chronic and functional kidney changes has long been acknowledged (1). Kidney recipients from expanded criteria donors (ECDs) are supposed to have inferior midterm renal function and graft survival outcomes (2, 3). In addition to decreasing numbers of functional nephrons, deteriorating alloimmune mechanisms contribute to worse graft outcomes in marginal donors.

Increased transcriptional activation of acute-phase proteins, complement components, and chemokines has been observed during implantation biopsy of grafts from deceased donors vs. living donors (LDs) (4). These underlying molecular mechanisms thus reflect donor organ quality. After transplantation, ischemia/reperfusion injury leads to the up-regulation of inflammation- and apoptosis-related genes due to increased intragraft infiltration of immunocompetent cells (5). This may further aggravate existing injury in ECD grafts.

In recent years, there has been an increase in the use of ECD kidneys [reaching 42–65% (6)] toward meeting demand from patients with end-stage renal disease potentially benefitting from transplantation. Based on midterm follow-up data, marginal donors are associated with inferior renal graft function, higher incidence of delayed graft function (DGF), and infectious complications, despite incidence of acute rejections and long-term graft function being similar to standard criteria donors (SCDs) (2, 7).

In indication biopsies performed early after transplantation, a wide spectrum of diverse diagnoses can be observed, ranging from acute tubular necrosis to T cell– or antibody-mediated rejection. Some of the most frequent findings in early indication biopsies are borderline changes, despite their clinical significance being the subject of debate. Previously, we showed that early borderline changes (BL) biopsies are associated with increased expression patterns of immunity- and inflammation-related genes. Higher donor age as well as some inflammation-related genes additionally contributed to late graft dysfunction (8). Although the transcriptome of kidney graft biopsies in the early period reflects the early alloimmune response, it can also be influenced by ischemia/reperfusion injury and transferred chronic histological changes. While previous study (8) focused on outcomes of BL, in this study on another patient cohort and using different platform, we focus on molecular assessment of various donor categories. The aims of this single-center study were to evaluate renal transcripts associated with donor category in a cohort exhibiting early borderline changes and to identify organ quality-specific patterns, thus limiting any potential bias associated with different histological categories.

Abbreviations: AKI, Acute kidney injury; CI, Confidence interval; DGF, Delayed graft function; ECD, Expanded criteria donor; eGFR, Estimated glomerular filtration rate; HLA, Human leukocyte antigen; LD, Living donor; rATG, Rabbit polyclonal antithymocyte globulin; PRA, Panel-reactive antibody; SCD, Standard criteria donor; HR, Hazard ratio.

MATERIALS AND METHODS

Study Design and Population

To study the effect of donor category (deceased vs. living, ECDs vs. SCDs), we carried out a retrospective, single-center, observational, cohort analysis of patients with borderline changes early after transplantation. Of 6,197 kidney recipients transplanted at our center between January 2005 and January 2017, all borderline changes were retrospectively identified (12.6%). Only patients with BL from case biopsies performed early after transplantation [median 9 days (min 4, max 60)] were enrolled in our study cohort ($n = 338$) (**Figure 1**). To obtain a cohort of borderline changes as a first pathology, all cases with prior episodes of rejection or thrombotic microangiopathy were excluded. To determine pure BL pathology, cases with concurrent presence of antibody-mediated rejection (ABMR), thrombotic microangiopathy (TMA), recurrent glomerulonephritis, glomerulitis >1, and BK virus (BKV) nephropathy were excluded. Furthermore, patients with primary graft dysfunction were deemed ineligible to participate in the study. A final cohort of 269 patients with early BL biopsies as a first and sole pathology was formed, with midterm outcomes compared between ECD ($n = 109$), SCD ($n = 109$), and LD ($n = 51$) categories. Expanded criteria donor kidneys were obtained from deceased donors either aged ≥ 60 years or 50–59 years meeting at least two of the following conditions: serum creatinine >1.5 mg/dL (132.5 μmol/L), cerebrovascular accident as a cause of death, or history of hypertension (9). Standard criteria donors are all deceased donors who failed to meet the criteria for ECD (10). Living donor kidney transplantation was performed between ABO-compatible genetically related or unrelated relatives or friends or with non-directed donors when kidney paired donation was performed. All kidney transplant recipients were treated according to standard center protocol, receiving no induction, T cell–non-depletive (basiliximab, daclizumab) induction, or T cell–depletive induction (rATG or infliximab) followed by a standard triple immunosuppression regimen based on a combination of tacrolimus/cyclosporine, mycophenolate mofetil (MMF)/mycophenolic acid (MPA), and steroids.

For the purpose of the transcriptomic study, we analyzed only patients receiving no induction or non-depletive induction therapy to eliminate the effect of different posttransplant immunosuppression on expression profiles. Furthermore,

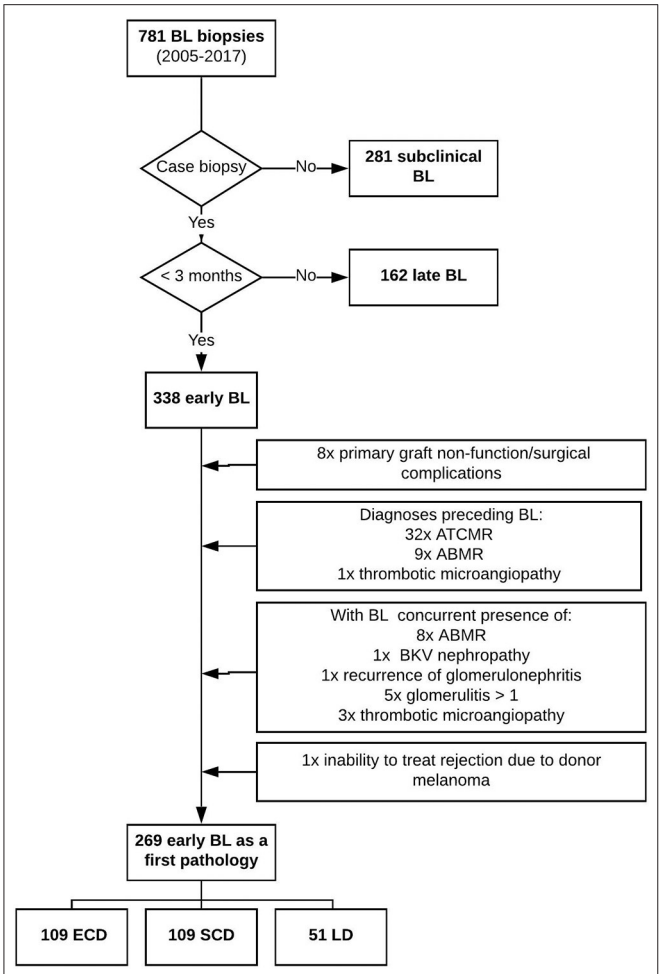

FIGURE 1 | Flowchart of the study.

TABLE 1 | Demographics of patient groups analyzed by microarray.

	SCD ($n = 4$)	ECD ($n = 9$)	LD ($n = 8$)	*P*
Recipient age, years	44 [38, 60]	49 [31, 65]	50 [21, 53]	0.567
Recipient gender, male, *n* (%)	3 (75%)	8 (88.9%)	7 (87.5%)	0.791
Donor age, years	35 [4, 53]	58 [4, 67]	49 [30, 63]	0.044
Donor gender, male, *n* (%)	0 (0%)	5 (55.6%)	2 (25%)	0.119
Dialysis vintage, months	10 [6, 56]	13 [1.6, 20]	7 [0, 31]	0.401
HLA mismatch	3 [3, 4]	4 [1, 5]	5 [2, 5]	0.478
Peak PRA	1 [0, 4]	2 [0, 12]	0 [0, 3]	0.144
DGF, *n* (%)	0	2 (22.2%)	1 (12.5%)	0.563
Cold ischemia, h	17 [9, 18]	17 [11, 22]	0.7 [0, 1.5]	0.001
Induction treatment				0.037
No	0	4 (44.4%)	0	
Basiliximab	4 (100%)	5 (55.6%)	8 (100%)	
Creatinine at biopsy, μmol/L	179 [169, 213]	397 [175, 651]	185 [126, 486]	0.016
Biopsy post-operative day (POD), days	8 [6, 13]	10 [6, 12]	6.5 [5, 13]	0.432

only biopsies performed within the first 14 days after transplantation were analyzed to reduce time-dependent changes in transcriptional profiles. Thus, the final cohort for molecular analysis consisted of 21 patients across 3 donor categories: ECD, SCD, and LD. Demographics of the microarray cohort are given in **Table 1**.

The study was approved by ethics committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital With Multi-center Competence under number G-16-06-09.

Microarray Analysis

Total RNA was isolated from renal biopsies using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA quality and integrity were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA integrity number of <6 were excluded from the analysis. RNA concentration was determined using a Qubit® fluorometer with Qubit® RNA BR Assay (ThermoFisher Scientific, Waltham, MA, USA).

A total of 150 ng RNA served as a template for the amplification and generation of Cy3 fluorescent cRNA using the Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies), according to the manufacturer's instructions. Labeling efficiency, yield, and purity of cRNA were determined using a NanoDrop spectrophotometer. Labeled cRNA (700 ng with specific activity >10.0 pmol Cy3/μg cRNA) was hybridized to Agilent SurePrintG3 Human Gene Expression v3 8×60K Array at 65°C for 17 h in a rotating hybridization oven at a speed of 10 rounds per minute. After hybridization, microarrays were washed sequentially for 1 min in wash buffer 1, for 1 min with prewarmed (37°C) wash buffer 2 (Agilent Technologies) and then immediately dried and scanned. Scanning was performed on the Agilent C Microarray Scanner, with data extraction and quality control performed using Agilent Feature Extraction Software (version 10.7.3.1). The resulting text files were analyzed using R software. The R software Lumi package was used to process raw data obtained from microarray analysis, with the quantile method used for normalization. Raw data sets used in the study were deposited at the Gene Expression Omnibus database (11) under ID GSE134386. When comparing particular donor subgroups, only two genes, *PRR35* and *CD163L1*, differentially expressed between ECD and LD, remained significant after multiple corrections [(false discovery rate (FDR) $P < 0.05$, fold change >2]. Therefore, in further analysis, differentially expressed genes were chosen as those with a fold change >2 and an unadjusted $P < 0.05$. Affected genes were functionally annotated, with deregulated pathways identified using the David database (http://david.abcc.ncifcrf.gov). In order to compare lists of deregulated genes, we availed of an interactive online tool for Venn diagrams (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Statistics

Data normality was tested using the Kolmogorov–Smirnov test. As most variables exhibited non-normal distribution, we

compared two groups using the two-tailed Mann-Whitney *U*-test and three groups using the Kruskal–Wallis test, followed by the *post hoc* Dunn multiple-comparisons test. Categorical data were compared using the χ^2 or Fisher exact test. Differences in kidney graft function between SCD, ECD, and LD were calculated using the General Linear Model (GLM) repeated-measures model. Graft survival was compared using Kaplan–Meier estimates and the log-rank test. Two-sided $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of Donor Category on Graft Outcomes

We compared midterm outcomes of kidney transplant recipients with borderline changes as a first pathology diagnosed at a median of 9 days after transplantation between ECD ($n = 109$), SCD ($n = 109$), and LD ($n = 51$) kidney transplantation cohorts. The ECD group had not only older donors but also higher recipients age ($P = 0.029$) and longer cold ischemia times than did the SCD group ($P = 0.020$). The LD group had the lowest recipient ages, cold ischemia times, and panel-reactive antibody levels and also the shortest dialysis spans ($P < 0.001$). The LD group contained a significantly higher proportion of female donors ($P < 0.001$) (**Supplemental Table 1**). The highest incidence of DGF was in the ECD group (37%) followed by SCD (32%), with prevalence of DGF only 6% in the LD group ($P < 0.001$).

The effect of donor category on individual Banff indication biopsy scores with borderline change findings showed the greatest chronic changes in the ECD group (**Table 2**). Graft biopsies from ECD donors revealed significantly higher vascular intimal fibrosis (cv) and arteriolar hyalinosis (ah) compared to both SCD and LD ($P < 0.001$), pointing to chronic vascular changes as well as higher tubular atrophy scores (ct) in grafts from marginal donors. On the contrary, biopsies from the LD group had significantly lower arteriolar hyaline thickening (aah) scores than those from the ECD group ($P < 0.001$) and SCD group ($P < 0.05$).

Patients from the ECD group had significantly worse renal graft function at biopsy, at 3 months, and in the first, second, and third years after biopsy [medians of estimated glomerular filtration rate (eGFR): 0.29, 0.59, 0.64, 0.67, 0.65 mL/s] compared to patients in the SCD (medians of eGFR 0.39, 0.79, 0.83, 0.84, 0.85 mL/s) and LD groups (medians of eGFR: 0.55, 0.79, 0.87, 0.85, 0.94 mL/s) ($P < 0.001$). The renal function of patients who received grafts from LD was better at biopsy compared to the SCD group ($P < 0.001$), despite no differences being found thereafter (**Supplemental Figure 1**).

Neither 5 year graft survival nor rejection-free intervals significantly differed among recipients with early borderline changes based on donor category (log-rank $P = 0.293$ and 0.219, respectively) (**Supplemental Figure 2**).

TABLE 2 | Histological findings in indication biopsies with BL performed early after transplantation stratified according to donor type.

Banff score	SCD ($n = 109$)	ECD ($n = 109$)	LD ($n = 51$)	*P* (ANOVA)
Glomerulitis (g)	0.12 ± 0.33	0.11 ± 0.31	0.17 ± 0.4	0.592
Chronic glomerulopathy (cg)	0.01 ± 0.1	0 ± 0	0 ± 0	0.482
Interstitial inflammation (i)	0.52 ± 0.53	0.49 ± 0.54	0.45 ± 0.58	0.754
Tubulitis (t)	1.22 ± 0.59	1.23 ± 0.61	1.22 ± 0.61	0.989
Total inflammation (ti)	0.51 ± 0.6[1]	0.49 ± 0.57	0.31 ± 0.55	0.121
Tubular atrophy (ct/TA)	0.72 ± 0.54[1]	0.86 ± 0.54	0.67 ± 0.52	**0.047**
Interstitial fibrosis (ci/IF)	0.51 ± 0.59[1]	0.54 ± 0.63	0.45 ± 0.54	0.669
Vascular intimal fibrosis (cv)	0.83 ± 0.76[5]	1.34 ± 0.85	0.95 ± 0.72	**<0.001**[a,d]
Arteriolar hyalinosis (ah)	0.98 ± 0.75	1.35 ± 0.78	0.82 ± 0.72	**<0.001**[b,c]
Arteriolar hyaline thickening (aah)	0.35 ± 0.65	0.39 ± 0.75	0.14 ± 0.40	0.061[c,e]
Peritubular capillaritis (ptc)	0.11 ± 0.44[2]	0.09 ± 0.35	0.06 ± 0.31	0.713

Dunnett post hoc test confirmed significant differences between SCD and ECD at [a]P < 0.001 or [b]P < 0.01; ECD and LD at [c]P < 0.001 and [d]P < 0.05; SCD and LD at [e]P < 0.05. Significant p values are in bold. The numbers in superscript indicate missing data. ANOVA, analysis of variance.

Effect of Donor Category on the Intragraft Transcriptional Profile of Early Borderline Changes

The effect of donor category on the intragraft transcriptional profile was studied in sections of the ECD ($n = 9$), SCD ($n = 4$), and LD ($n = 8$) cohorts. All biopsies were clinically indicated at a median of 9 days after transplantation (min 5, max 13 days) and diagnosed as borderline changes. There was no difference in the follow-up to biopsy among the ECD, SCD, and LD cohorts ($P = 0.432$). All patients had received their first transplants, had low levels of panel-reactive antibodies, and therefore received no induction or basiliximab. The demographics of this microarray set of patients are given in **Table 1**. Differences between groups, such as older donors ($P = 0.044$) in the ECD group or shorter cold ischemia times ($P = 0.001$) in the LD group, reflect particular donor category definitions. In addition, patients from the ECD group had the worst renal function at biopsy (median of creatinine was 397 μmol/L for the ECD group, 177 μmol/L for the SCD group, and 185 μmol/L for the LD group, $P = 0.016$).

Similar to our analysis of the larger clinical cohort (**Table 2**) also in microarray-analyzed biopsies, patients from the ECD group had significantly higher vascular intimal fibrosis (cv) ($P = 0.028$, **Supplemental Table 2**).

In the ECD group, microarray revealed higher expression of 244 transcripts compared to SCD and 437 compared to LD. Compared to both SCD and LD, gene annotation analysis of transcripts with increased expression in ECD grafts showed enrichment of the inflammatory response ($P = 0.013$, $P = 7.4 \times 10^{-8}$, respectively), the response to wounding ($P = 0.001$, 1.3×10^{-12}, respectively), the defense response ($P = 0.005$, $P = 5.5 \times 10^{-7}$, respectively), and the ECM–receptor interaction pathway ($P = 0.043$, $P = 0.004$, respectively) (**Table 3**). Additionally, annotation analysis of increased transcripts in

TABLE 3 | Biological processes and KEGG pathway–enriched case biopsies with borderline changes in ECD compared to SCD, in ECD compared to LD, and in SCD compared to LD.

Enriched biological processes and KEGG pathways	Transcripts increased in ECD vs. LD	Fold enrichment	Benjamini *P*
hsa04610: complement and coagulation cascades	*F12, FGG, FGA, C3, CFB, FGB, F13A1, CFH*	7.37	0.00390
hsa04512: ECM–receptor interaction	*COL6A3, COL3A1, ITGB6, LAMC2, SV2B, ITGB3, COL1A1, THBS2, SPP1*	6.81	0.00370
hsa04060: cytokine–cytokine receptor interaction	*LIF, INHBB, INHBA, TNFSF10, CCL13, IL6, IL2RA, OSMR, CXCL2, TNFRSF18, CCL18, IL11, CCL17*	3.15	0.01960
GO: platelet activation	*FGG, IL6, SAA2, FGA, SAA1, FGB, COL3A1, IL11*	18.28	0.00010
GO: regulation of nuclear division, regulation of mitosis	*NEK2, DLGAP5, BUB1, CENPF, IGF1, CENPE, CDC25C, CD28*	10.45	0.00100
GO: regulation of inflammatory response	*FCER1A, F12, IL6, IL2RA, SAA2, SERPINF1, C3, OSMR, SAA1*	8.66	0.00100
GO: regulation of cell cycle process	*NEK2, DLGAP5, CENPF, IGF1, CENPE, ANLN, BIRC5, UBE2C, CDC25C, GTSE1, LIF, BUB1, CD28*	8.34	0.00000
GO: coagulation, blood coagulation	*F12, FGG, IL6, SAA2, FGA, SAA1, FGB, F13A1, COL3A1, ITGB3, IL11*	7.89	0.00020
GO: acute inflammatory response	*F12, IL6, SAA2, C3, SAA1, CFB, CLU, CFH, SERPINA3, CD163*	7.46	0.00090
GO: hemostasis	*F12, FGG, IL6, SAA2, FGA, SAA1, FGB, F13A1, COL3A1, ITGB3, IL11*	7.45	0.00030
GO: regulation of mitotic cell cycle	*NEK2, DLGAP5, CENPF, IGF1, CENPE, ANLN, BIRC5, UBE2C, CDC25C, GTSE1, BUB1, MYC, CD28*	6.25	0.00020
GO: regulation of body fluid levels	*F12, FGG, IL6, SAA2, FGA, SAA1, FGB, F13A1, COL3A1, ITGB3, AGR2, IL11*	6.22	0.00050
GO: wound healing	*F12, IL6, F13A1, COL3A1, IGF1, ITGB3, CDH3, IL11, FGG, FGA, SAA2, FGB, SAA1, HMOX1, TM4SF4*	5.74	0.00010
GO: inflammatory response	*NFKBIZ, F12, IL6, IL2RA, ELF3, CFB, C3, CLU, CXCL2, GAL, CCL18, CCL17, CD163, FOS, CCL13, SAA2, SAA1, STAB1, HMOX1, ITGB6, SERPINA3, CFH, PTX3, SPP1*	5.40	0.00000
GO: response to wounding	*NRP1, ELF3, C3, F13A1, CLU, CXCL2, COL3A1, ITGB3, CDH3, IL11, FOS, FGG, SAA2, FGA, FGB, SAA1, HMOX1, ITGB6, CFH, SERPINA3, PTX3, SPP1, F12, NFKBIZ, IL6, IL2RA, CFB, IGF1, GAL, CCL18, CD163, CCL17, CCL13, LYVE1, STAB1, VCAN, TM4SF4*	5.10	0.00000
GO: defense response	*ELF3, C3, CLU, CXCL2, HP, FOS, SAA2, SAA1, HMOX1, ITGB6, CFH, SERPINA3, LTF, PTX3, SPP1, F12, NFKBIZ, IL6, IL2RA, CFB, GAL, CCL18, HPR, CD163, CCL17, INHBB, INHBA, CCL13, CD19, LILRB5, STAB1, CTSG*	3.69	0.00000
GO: cell adhesion, biological adhesion	*OLFM4, NRP1, MYBPC2, NELL2, COL3A1, POSTN, ITGB3, SOX9, CDH3, CDH6, VCAM1, COL7A1, COL6A3, ITGB6, SPON2, LOXL2, THBS2, SPP1, COL15A1, CDHR4, LYVE1, STAB1, CD209, CPXM1, CLDN1, VCAN, LAMC2, ADAM12, HABP2*	3.03	0.00010
GO: immune response	*C3, CLU, CXCL2, LIF, CFH, LTF, SPON2, PTX3, CD28, F12, TCF7, IL6, IL2RA, CFB, FOXJ1, RELB, IGJ, CCL18, CCL17, CCL13, TNFSF10, LILRB5, FCGR2B, CD209, CTSC, CTSG*	2.76	0.00090
	Transcripts increased in ECD vs. SCD	**Fold enrichment**	**Benjamini *P***
hsa04512: ECM–receptor interaction	*TNC, COMP, COL6A3, SV2B, COL1A1, THBS2*	8.30	0.04300
GO: inflammatory response	*CCL11, C1QB, FOS, CCL13, HIF1A, CEBPB, ADORA3, CCL8, C1S, GPR68, GAL, VSIG4, CHST1*	4.40	0.01300
GO: response to wounding	*CEBPB, ADORA3, TNC, CCL8, GPR68, C1S, GAL, IL11, PLAUR, CHST1, CCL11, PCSK1, FOS, C1QB, CCL13, SLC1A3, HIF1A, SERPINE1, VSIG4*	3.60	0.00100
GO: defense response	*ADORA3, CEBPB, KLRC3, CCL8, CD300C, COLEC12, GPR68, C1S, GAL, CHST1, CCL11, INHBA, FOS, C1QB, CCL13, HIF1A, LILRB5, TFF3, VSIG4*	3.40	0.00500
GO: cell adhesion	*TNC, EMILIN2, SIGLEC14, COL16A1, CLDN14, ITGBL1, CCL11, NLGN4Y, COMP, CD33, SIGLEC7, COL6A3, MFAP4, ADAM12, THBS2, COL8A2, NTM, CDH11, SPON1*	3.00	0.01300
GO: biological adhesion	*TNC, EMILIN2, SIGLEC14, COL16A1, CLDN14, ITGBL1, CCL11, NLGN4Y, COMP, CD33, SIGLEC7, COL6A3, MFAP4, ADAM12, THBS2, COL8A2, NTM, CDH11, SPON1*	3.00	0.01100
	Transcripts increased in SCD vs. LD	**Fold enrichment**	**Benjamini *P***
hsa02010: ABC transporters	*ABCA8, ABCB1, CFTR, ABCB4*	17.12	0.03431
hsa04610: complement and coagulation cascades	*C8A, F12, FGG, CR2, F13A1*	13.65	0.01873
GO: regulation of lipid transport	*APOA2, APOA1, APOC3, PON1*	32.79	0.04672
GO: mitosis, nuclear division	*CCNB2, DLGAP5, CENPF, BIRC5, PBK, UBE2C, ASPM*	7.82	0.0386
GO: response to wounding	*C8A, F12, APOA2, FGG, CR2, F13A1, ITGB3, IGFBP1, CDH3, ADORA1, ORM2, SPP1*	5.57	0.00526

Only the most significant GO terms associated with biological processes are shown.

ECD vs. LD showed activation of complement and coagulation cascades ($P = 0.0039$), cytokine–cytokine receptor interaction pathways ($P = 0.02$), and other Gene Ontology (GO) terms such as regulation of the cell cycle process ($P = 1.9 \times 10^{-6}$) and platelet activation ($P = 0.0001$) (**Table 3**). Interestingly, GO term response to wounding was more activated in ECD kidneys in comparison with SCD ($P = 0.001$) and LD kidneys (1.3×10^{-12}), and similarly, it was higher in SCD kidneys compared with LD ones ($P = 0.005$). Activation of the KEGG complement and coagulation cascades pathway was observed in both deceased donor categories (ECD and SCD) in comparison with LD ($P = 0.039$ and $P = 0.019$, respectively). Moreover, higher regulation of lipid transport was observed in SCD vs. LD (**Table 3**).

Comparative gene expression overlaps of differentially expressed genes between ECD vs. SCD and ECD vs. LD using Venn diagrams (**Figure 2**) found 64 up- and 16 down-regulated genes in ECD compared to both LD and SCD. Shared increased transcripts in ECD vs. both SCD and LD included thrombospondin 2 (*THBS2*), synaptic vesicle glycoprotein

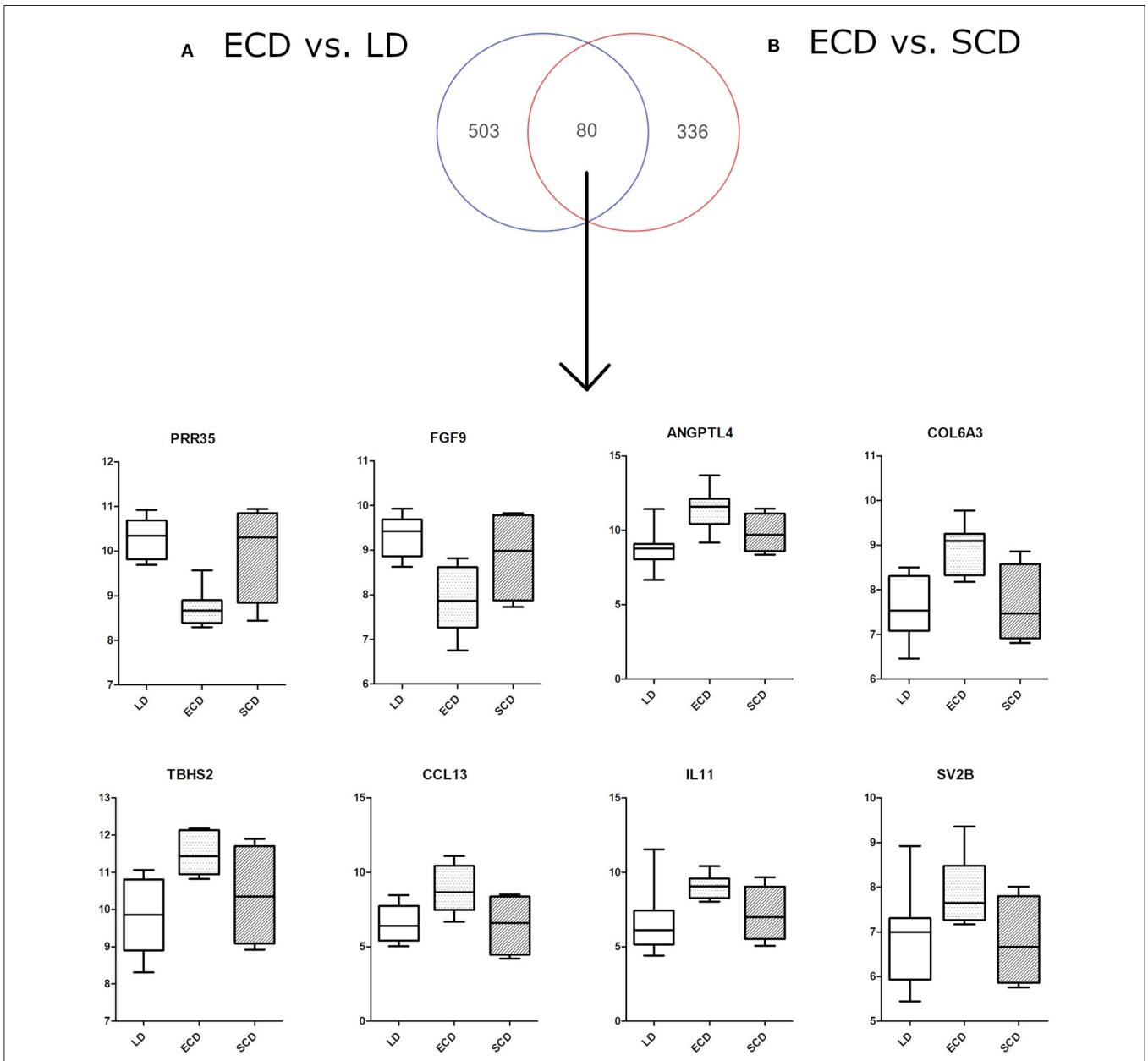

FIGURE 2 | Venn diagram showing overlap of deregulated genes (80 transcripts) for particular comparisons of donor categories: **(A)** ECD vs. LD (583 deregulated transcripts), **(B)** ECD vs. SCD (416 deregulated transcripts). The overlap of some genes shows differential expression of ECD compared with SCD and LD. PRR35, proline-rich 35 protein; FGF9, fibroblast growth factor 9; ANGPTL4, angiopoietin-like 4; COL6A3, collagen, type VI, alpha 3; TBHS2, thrombospondin 2; CCL13, chemokine (C-C motif) ligand 13; IL11, interleukin 11; SV2B, synaptic vesicle glycoprotein 2B.

(*SV2B*), angiopoietin-like 4 (*ANGPTL4*), collagens (*COL6A3, COL1A1*), chemokines *CCL13*, and interleukin *IL11* and, most significantly, down-regulated transcripts including proline-rich 35 (*PRR35*) and fibroblast growth factor 9 (*FGF9*).

From 30 injury-repaired associated transcripts related to acute kidney injury (AKI) described by Famulski et al. (12), 28 were measured on the chip in our study, and 19 (64%) of those transcripts were significantly up-regulated in ECD compared to LD donors (**Figure 3**). Five of those transcripts, *LCN2,* lipocalin 2; *LTF*, lactotransferrin; *VCAN*, versican; *ITGB6*, integrin beta 6; and *SERPINA3*, serpin peptidase inhibitor, were among top ranked significant transcripts that differentiated ECD from LD. Of note, three of 19 AKI transcripts (*LTF*, *LCN2*, and *SERPINA3*) were more than 10 times more regulated in ECD as compared to LDs (fold change >10).

DISCUSSION

Donor kidney quality significantly affects kidney transplantation outcomes. It is widely accepted that transplantation of kidneys from elderly marginal donors results in inferior renal function and limited graft life. Expanded criteria donor kidney graft recipients typically suffer from more frequent DGF and acute rejection. In this study, however, the DGF rate was similar between ECD and SCD cohort but much higher than in the LD group (37, 32, and 6%, respectively). Similarly, higher DGF rate was found in respective groups when analyzing a larger cohort of 254 late biopsies (22% in SCD, 18% in ECD, and 4.5% in LD), which may reflect different therapy used in the ECD group. A recent multicenter study reported DGF frequency to be 2.24× higher in ECD compared to SCD recipients ($P = 0.02$) (13). The reason seems to be associated with aggravated alloimmune response and fibrogenesis in already-injured organs. Apart from conventional histological assessment, little is known about molecular pathways typical of marginal kidney grafts. Increasing knowledge in this area may lead to improvements in predicting premature graft loss and adapting therapy appropriately.

To the best of our knowledge, our study is the first to compare intragraft transcriptional profiles from different donor categories in the early posttransplant period, in fact within first 14 posttransplant days. Previous studies have compared preimplant donor biopsies (14) and 0 h (4, 15–17) or postreperfusion

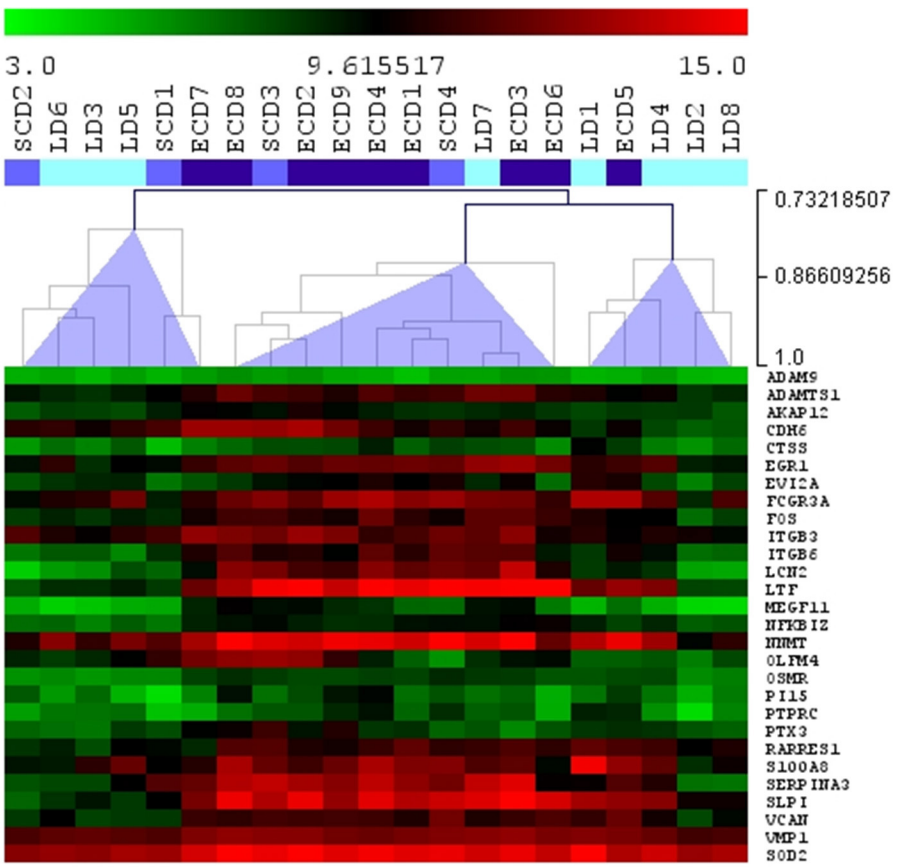

FIGURE 3 | Hierarchical clustering (Spearman rank correlation) for 28 injury-repaired associated transcripts related to acute kidney injury measured in early indication biopsies with borderline changes in different donor categories using Agilent microarray. Light blue: LD, living donor; intermediate blue: SCD, standard criteria donor; dark blue: ECD, expanded criteria donor. Most acute kidney injury transcripts were increased in the second cluster, formed in 70% by grafts from ECD donors.

(18) graft biopsies from different donor categories. Because all of the indication biopsies we analyzed were diagnosed as borderline changes with no previous pathology, modifications in the transcriptome among donor categories could not have been influenced by different underlying pathological processes. Using microarray, we found higher expression of inflammation- and extracellular matrix remodeling–associated transcripts in the kidney allografts of ECD donors compared with other donor categories.

Compared to the ideal LD group, we observed increased transcripts associated with inflammatory, wounding, and defense responses; complement and coagulation cascades; and cytokine–cytokine receptor interaction pathways in the ECD cohort. This observation seems to be in line with a study by Mueller describing up-regulation of acute phase proteins, complement components, and chemokines in postreperfusion implant biopsies obtained from deceased (compared to living) donors (4). Collectively, this suggests that early transcriptional activations persist at least up to 14 days posttransplant, during which time the biopsies in our study were performed.

In our study, ECD-derived biopsies exhibited increases in several transcripts associated with extracellular matrix remodeling such as thrombospondin 2 (*THBS2*), collagens (*COL6A3*, *COL1A1*), synaptic vesicle glycoprotein (*SV2B*), and interleukin 11 (*IL11*). Increased expression of *THBS2*, which plays a role in extracellular matrix remodeling, was previously detected in kidney allografts suffering from acute rejection (19). The dominant profibrotic role of *IL11* in the heart and kidneys was recently described (20). An experimental study found increased expression of *ANGPTL4* to be an early biomarker of podocyte injury in a minimal change disease rat model (21). It's up-regulation preceded heavy proteinuria and increased urinary ANGPTL4 protein levels.

Next, in our study, proline-rich 35 (*PRR35*) and *FGF9* were significantly down-regulated in biopsies from ECD donors compared to other cohorts. In another study, expression of FGF9 in biopsies with AKI was lower than in biopsies with primary graft function (22).

Interestingly, *PRR35* and *CD163L* were the most significantly deregulated genes in ECD and LD cohorts, with *PRR35* gene transcripts nearly three times lower in biopsies from ECD donors compared to LD. PRR35 is a protein-coding gene of unknown function. *CD163L*, a macrophage scavenger receptor associated with the anti-inflammatory response and tissue remodeling, has been shown to exhibit three times higher expression in ECD donors (23).

Most importantly, we found significant expression of AKI-related transcripts in ECD kidney grafts. This information is in line with previous "0 h" biopsies study (17). Thus, higher AKI transcripts reflect parenchymal injury associated with donor age and ischemia time and sustain at least 14 days after transplantation, the most critical time period for generation of initial alloimmune response.

In our study, patients with ECD grafts experienced worse renal function at 3 years (median eGFR, 0.65 mL/s) compared to SCD (median eGFR, 0.85 mL/s) and LD grafts (median, 0.94 mL/s). This suggests a higher risk of premature graft loss, although in our study we found no differences in 5 year graft survival between donor categories, which is perhaps unsurprising given the inconclusive results of other studies (2, 7, 24–26). Although the effect of marginal kidneys on graft outcomes has been previously described, it has not been evaluated in a well-defined cohort of patients with the same first pathology of "mild rejection" during the early posttransplant period.

In our early biopsies of ECD patients with borderline changes, the transmission of chronic histological changes was more common, represented by vascular intimal fibrosis (cv), arteriolar hyalinosis (ah), and tubular atrophy (ct) Banff scores compared to biopsies of both SCD and LD categories. The association of higher chronic histopathological Banff scores in biopsies from marginal donors with graft dysfunction or DGF has been reported by other studies (6, 27, 28). In our study, we did not find significantly higher interstitial fibrosis (ci) scores or higher expression of fibroblast-associated transcripts in early BL biopsies of ECD individuals compared to other donor categories. This corresponds to the results of a recent study where indication biopsies performed early after transplantation had higher expression of AKI-associated transcripts than fibroblast-associated transcripts (29).

The sample size for our analysis of graft function and survival ($n = 269$) among particular categories was satisfactorily large. Nevertheless, microarray transcriptome analysis was performed only in a small subgroup ($n = 21$) of patients, representing a possible limitation of our study. However, the main conclusion drawn from our transcriptome analysis is higher activation of immunity, inflammation, and extracellular matrix remodeling in biopsies from marginal donors seen even within 14 days post-transplant. Additionally, because of low number of differentially expressed genes after correction for multiple testing, the unadjusted p cutoff < 0.05 and fold change >2 were used instead in statistical analysis. Nevertheless, the high overlap of increased transcripts in marginal donors with the already described molecular AKI injury (12)–related transcript set supports our results of gene annotation analysis. The aim of our study was not to search for any biomarkers requiring larger sample size and validation, but to examine the main transcriptional pathways activated in marginal donors in the early posttransplant period.

In our study, the early borderline changes in ECD kidneys were characterized by the most increased regulation of inflammation, extracellular matrix remodeling, and AKI transcripts in comparison with SCD and LD grafts, respectively. It is likely that ECD-related transcripts were boosted by already present vascular changes in comparison with SCD kidneys and similarly in SCD kidneys by longer ischemia in comparison with LD kidneys. Therefore, chronic vascular changes and cold ischemia time aggravate inflammation and thus contribute to worse outcomes of these grafts. Our data are therefore in line

with current praxis where ECD kidney recipients often receive T cell–depletive induction therapy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Gene Expression Omnibus database under ID GSE134386.

ETHICS STATEMENT

The study was approved by Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital with Multi-Centre Competence under number G-16-06-09. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PH and OV designed and wrote the manuscript. PH, ZK, MD, JS, JM, and EH performed the research. VS, JK, and PH participated in the data analysis. OV supervised the research.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00423/full#supplementary-material

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The Differential Influence of Cold Ischemia Time on Outcome After Liver Transplantation for Different Indications—Who Is at Risk? A Collaborative Transplant Study Report

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Introduction: Despite increasing awareness of the negative impact of cold ischemia time (CIT) in liver transplantation, its precise influence in different subgroups of liver transplant recipients has not been analyzed in detail. This study aimed to identify liver transplant recipients with an unfavorable outcome due to prolonged cold ischemia.

Methods: 40,288 adult liver transplantations, performed between 1998 and 2017 and reported to the Collaborative Transplant Study were analyzed.

Results: Prolonged CIT significantly reduced graft and patient survival only during the first post-transplant year. On average, each hour added to the cold ischemia was associated with a 3.4% increase in the risk of graft loss (hazard ratio (HR) 1.034, $P < 0.001$). The impact of CIT was strongest in patients with hepatitis C-related (HCV) cirrhosis with a 24% higher risk of graft loss already at 8–9 h (HR 1.24, 95% CI 1.05–1.47, $P = 0.011$) and 64% higher risk at ≥14 h (HR 1.64, 95% CI 1.30–2.09, $P < 0.001$). In contrast, patients with hepatocellular cancer (HCC) and alcoholic cirrhosis tolerated longer ischemia times up to <10 and <12 h, respectively, without significant impact on graft survival ($P = 0.47$ and 0.42). In HCC patients with model of end-stage liver disease scores (MELD) <20, graft survival was not significantly impaired in the cases of CIT up to 13 h.

Conclusion: The negative influence of CIT on liver transplant outcome depends on the underlying disease, patients with HCV-related cirrhosis being at the highest risk of graft loss due to prolonged cold ischemia. Grafts with longer cold preservation times should preferentially be allocated to recipients with alcoholic cirrhosis and HCC patients with MELD <20, in whom the effect of cold ischemia is less pronounced.

Keywords: cold ischemia time, CIT, liver transplantation, extended donor criteria, EDC, collaborative transplant study, CTS, outcome

INTRODUCTION

Liver transplantation improves the underlying liver dysfunction, involves radical oncological resection, and is the only promising treatment for patients with end-stage liver disease and patients with hepatocellular carcinoma (HCC) (1–3). Because of the chronic organ shortage in most countries and in Eurotransplant, less than optimal, extended donor criteria (EDC) grafts are used to expand the organ pool (2, 4). Cold ischemia time (CIT) is a factor that occurs during the allocation and it is considered a major extended donor criterion (maEDC) that affects graft and patient survival along with macrovesicular steatosis and donor age (2, 5). Cold ischemia increases the risk of graft failure and early HCC recurrence, and graft outcome depends on its ability to recover from the ischemia injury (2, 6, 7). Therefore, organs with prolonged cold ischemia are often discarded as unsuitable for transplantation (2). To address this problem, we suggested an allocation algorithm that balances the maEDC with the recipient's health condition, and considers maEDC grafts an acceptable alternative for transplant candidates with lower laboratory Model of End-Stage Liver Disease (labMELD) scores who generally are in a better condition (2). Based on data from the Collaborative Transplant Study (CTS), we reported recently that donor age had a differential influence on graft survival depending on the indication for liver transplantation (8). Transplant recipients with HCC were less affected by advanced donor age whereas donor age influenced outcome strongly in patients with hepatitis C (HCV)-related cirrhosis. However, the impact of prolonged cold ischemia in patients with different underlying diseases was not investigated. Although the awareness of the negative impact of CIT has generally increased, the information on CIT's influence on outcome of maEDC grafts is scarce. Moreover, the accepted limits for CIT are subject to regional differences (5, 8–10). This study aimed to identify liver transplant recipients whose grafts are less affected from a prolonged cold ischemia, and to describe risk factors associated with an adverse outcome following transplantation of such organs.

METHODS

Study Population

All data were obtained from the CTS (www.ctstransplant.org). Since 1982, CTS collects data from solid-organ transplants worldwide on a voluntary base, continuously reports general information on transplantation outcomes and specific clinical issues, and takes into account the confidentiality of patients as well as transplant centers. The well-structured follow-up concept and the incorporation of available registry data guarantee a high level of data integrity (11).

We analyzed data from 40,288 deceased donor primary liver transplantations reported to CTS and performed from January 1st, 1998 to December 31st, 2017 in adult patients with alcoholic liver cirrhosis or cirrhosis due to HCV and HCC. Less frequent original diseases such as autoimmune disorders, cryptogenic cirrhosis, congenital diseases, hepatitis B, metabolic disorders, primary biliary cirrhosis, and primary sclerosing cholangitis were analyzed as a separate group. Patients with missing data on CIT, transplanted because of acute hepatic failure, recipients of organs from <18-year-old donors, split liver or multi-organ transplants were excluded. The MELD score was available to CTS after 2006.

Graft failure was defined as insufficient liver function to keep the patient alive, leading to death or re-transplantation, whereas patient survival was defined as the time between the primary transplantation and death or last known contact.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 25.0 (SPSS Inc., IBM Corporation, Somers, NY, USA). Survival rates were analyzed using the Kaplan-Meier method with the Mantel Cox log rank test of trend. To avoid possible influences from demographic differences, multivariable Cox regression analysis was used to calculate the hazard ratio (HR) and 95% confidence intervals (95% CI). The following confounders were considered: geographical region (country or region), year of transplantation, recipient age and race, donor age and race, cause of donor death, recipient and donor gender combinations, general evaluation of the patient, original disease, donation after cardiac death, donor history of hypertension, immunosuppressive regimen, induction therapy, urgency, and CIT. A two-sided P-value of <0.05 was considered statistically significant.

RESULTS

We analyzed 40,288 primary adult liver transplantations from 109 centers in 24 countries. 10,953 patients had HCC, 9,569 were transplanted because of HCV-related liver cirrhosis, 7,878 had alcoholic cirrhosis, and in 11,888 patients the underlying disease included autoimmune disorders, cryptogenic cirrhosis, congenital diseases, hepatitis B virus, metabolic disorders, primary biliary cirrhosis or primary sclerosing cholangitis. Confounders were unevenly distributed between the most common underlying diseases; e.g., patients with HCC received notably more grafts from ≥65-year-old donors and the lowest number of grafts with CIT ≥10 h. Demographics and confounders are shown in **Table 1**.

During 1998–2001, chronic HCV infection was the leading cause of liver cirrhosis (31%), however, the proportion of recipients with HCV-related liver cirrhosis declined continuously, especially after the introduction of the direct-acting antiviral agents (DAAs) in 2013. In contrast, alcoholic cirrhosis gained continuously on incidence and has become the second most common underlying disease that led to liver transplantation since 2014. The number of liver transplants for HCC also increased steadily from 13.3% during 1998–2001 to 28.8% during 2010–2013, but declined slightly to 26.6% after 2014 (**Figure 1A**). Recipient age and donor age increased significantly during 1998–2017. There were significantly more

Abbreviations: CI, confidence interval; CIT, Cold Ischemia Time; CTS, Collaborative Transplant Study; DAAs, Direct-acting Antiviral Agents; DRI, Donor Risk Index; EDC, Extended Donor Criteria; ET, Eurotransplant; ET-DRI, Eurotransplant Donor Risk Index; HCC, hepatocellular carcinoma; HCV, Hepatitis C Virus, HR, hazard ratio; IQR, interquartile range; labMELD, laboratory Model of End Stage Liver Disease; maEDC, major Extended Donor Criteria.

TABLE 1 | Demographics of study patients, *n* (%) or mean ± SD, $P < 0.001$ for all characteristics.

Characteristic	Unknown (%)	Underlying disease			
		HCC *n* = 10,953	HCV-cirrhosis *n* = 9,569	Alcoholic cirrhosis *n* = 7,878	Other *n* = 11,888
Geographical region	–				
Europe		10,110 (92%)	8,285 (87%)	7,264 (92%)	10,283 (86%)
Other		843 (8%)	1,284 (13%)	614 (8%)	1,605 (14%)
Transplant year	–				
1998–2007		4,766 (44%)	5,751 (60%)	3,619 (46%)	6,467 (54%)
2008–2017		6,187 (56%)	3,818 (40%)	4,259 (54%)	5,421 (46%)
Recipient sex	–				
Female		1,853 (17%)	2,461 (26%)	1,604 (20%)	5,271 (45%)
Male		9,028 (83%)	6,993 (74%)	6,244 (80%)	6,483 (55%)
Recipient age (years)	–				
18–64		9,420 (86%)	8,951 (94%)	7,286 (92%)	10,860 (91%)
≥65		1,533 (14%)	618 (6%)	592 (8%)	1,028 (9%)
Mean ± SD		56.2 ± 8.1	52.6 ± 8.4	54.0 ± 7.8	49.2 ± 12.3
Donor age (years)	–				
18–64		7,328 (67%)	7,552 (79%)	5,938 (75%)	9,579 (81%)
≥65		3,625 (33%)	2,017 (21%)	1,940 (25%)	2,309 (19%)
Mean ± SD		55.0 ± 16.9	50.2 ± 16.3	51.8 ± 16.4	49.2 ± 16.5
Cold ischemia time (h)	–				
≤5		1,576 (14%)	1,341 (14%)	1,038 (13%)	1,628 (14%)
6–9		6,405 (58%)	5,098 (53%)	4,183 (53%)	6,220 (52%)
10–13		2,659 (24%)	2,645 (28%)	2,276 (29%)	3,507 (30%)
≥14		323 (3%)	485 (5%)	381 (5%)	533 (4%)
Mean ± SD		8.1 ± 2.7	8.5 ± 3.3	8.5 ± 2.9	8.4 ± 2.9
Cause of donor death	5.4				
CVA		6,796 (65%)	5,512 (62%)	4,708 (63%)	6,982 (63%)
Trauma		2,212 (21%)	2,098 (24%)	1,508 (20%)	2,500 (22%)
Other		1,441 (14%)	1,273 (14%)	1,267 (17%)	1,643 (15%)
Calcineurin inhibitors	38.7				
Cyclosporine		2,021 (26%)	1,942 (34%)	1,074 (24%)	1,889 (28%)
Tacrolimus		5,182 (66%)	3,341 (58%)	3,005 (68%)	4,307 (64%)
None		613 (8%)	486 (8%)	346 (8%)	489 (7%)

HCC, hepatocellular carcinoma; HCV, hepatitis C virus; SD, standard deviation; CVA, cerebrovascular accident.

60–69-year-old recipients during 2014–2017 than during 1998–2001 (33.4 vs. 20.4%, $P < 0.001$), and the fraction of septuagenarian donors was with 25.2% highest during 2014–2017 (**Figures 1C,D**).

CIT and Outcome After Liver Transplantation

Over the study period, we observed a shift toward lower CIT. The fraction of transplant cases with ischemia time exceeding 12 h dropped dramatically from 21.5% during 1998–2001 to 6.5% during 2014–2017, and 6–9 h became the most prevalent CIT (**Figure 1B**). **Figure 2** illustrates the distribution of CIT in deceased donor liver transplantations in adult recipients that were performed during 1998–2017 and reported to the CTS. The arithmetic average of the CIT was 8.4 ± 3.0, the median 8, and the inter-quartile range 6–10 h.

As shown in **Table 2**, **Table S1**, CIT ≥8 h reduced graft as well as patient survival significantly during the first post-transplant year, but the impact of cold preservation on survival was uneven among liver transplant recipients with different underlying diseases. Overall, graft and patient survival rates declined in a linear fashion as CIT increased (all $P < 0.001$; **Figures 3A,C**). The multivariable Cox regression analysis indicated a linear influence of CIT, and with each hour added to cold ischemia, the risk of graft loss during the first post-transplant year increased by 3.4% (HR 1.034, 95% CI 1.027–1.041, $P < 0.001$). Remarkably, after the first post-transplant year, CIT did not show a significant effect in the univariate Kaplan-Meier analysis, neither on graft nor on patient survival ($P = 0.45$ and 0.94, respectively; **Figures 3B,D**).

The multivariable Cox regression analysis of the interactions of CIT with other confounders showed a significant interaction only with underlying disease. Prolonged cold ischemia exposed

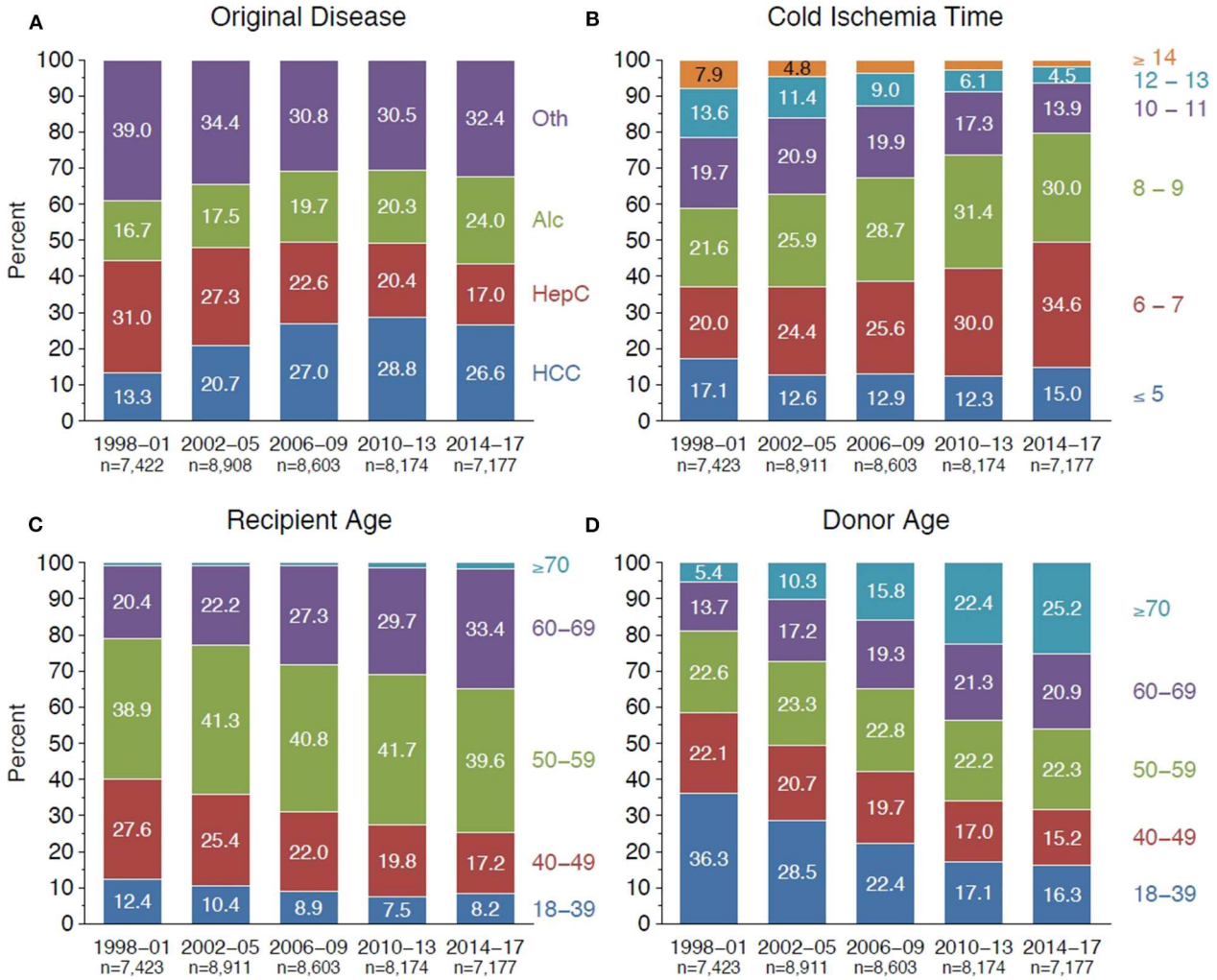

FIGURE 1 | Development of **(A)** original underlying disease, **(B)** cold ischemia time (h), **(C)** recipient age (years), and **(D)** donor age (years) for first deceased donor liver transplants of adult recipients ($P < 0.001$ for all parameters).

grafts at the highest risk of failure in patients with HCV-cirrhosis. Compared to the reference of <5 h, 8–9 h cold ischemia increased in HCV patients the risk of graft loss by 24% (HR 1.24, 95% CI 1.05–1.47, $P = 0.011$) and ≥14 h cold ischemia by as high as 64% (HR 1.64, 95% CI 1.30–2.09, $P < 0.001$; **Table 2**). In contrast, grafts transplanted into patients with HCC tolerated longer ischemia times and were at a significantly increased risk of graft loss only if the CIT was 10–11 h (HR 1.33, 95% CI 1.11–1.58, $P = 0.002$) or higher. Most resilient to the negative effect of CIT were grafts transplanted into recipients with cirrhosis due to chronic alcoholism and other underlying diseases (CIT 12–13 h, HR 1.45, 95% CI 1.13–1.84, $P = 0.003$; HR 1.36, 95% CI 1.11–1.65, $P = 0.003$, respectively; **Table 2**). Similar hazard ratios were obtained in the analysis of patient survival with the exception of HCC patients in whom the mortality risk was in all CIT categories constantly lower than the risk for graft loss (**Table S1**). Other than in recipients with HCV cirrhosis and other less frequent underlying diseases, 1-year graft survival decreased in a non-linear fashion in recipients with pre-transplant HCC and alcoholic cirrhosis (all $P < 0.001$; **Figure 4**).

Underlying Disease and Outcome After Transplantation

When the three most common underlying diseases were analyzed, grafts transplanted into patients with alcohol-induced liver cirrhosis showed the best (72.6%) and grafts transplanted into recipients with HCV-cirrhosis the worst 5-year survival (65.7%; $P < 0.001$; **Figure 5A**). Kaplan-Meier curves for patient survival had a similar trend ($P < 0.001$; **Figure 5B**). Patients with pretransplant HCC demonstrated the best 1-year graft and patient survival, but this worsened in time and declined at year 5 to a rate of 66.4 and 69.6%, respectively (**Figures 5A,B**).

We analyzed the HCC subpopulation separately for interactions between CIT and confounders. Despite the seemingly large differences in hazard risk ratios of the multivariable Cox regression analysis, no significant interactions

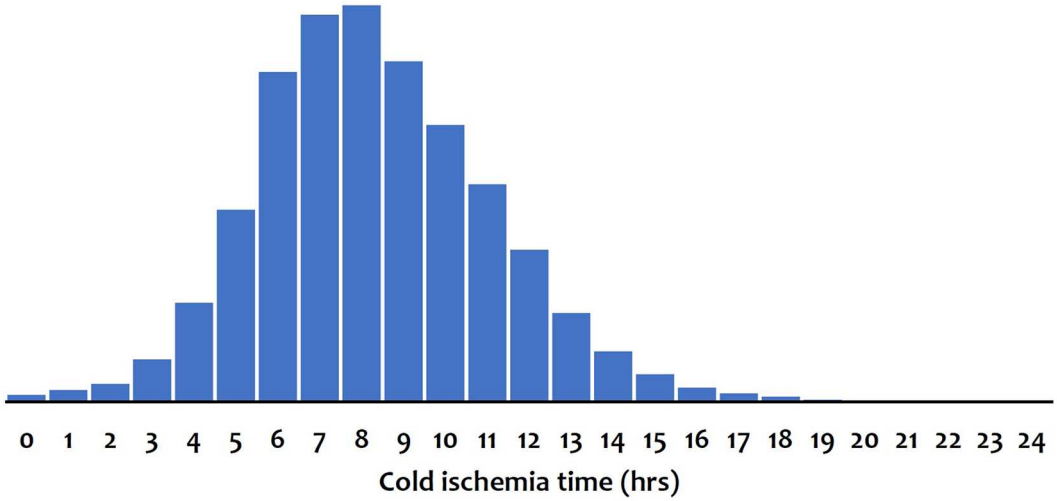

FIGURE 2 | Distribution of cold ischemia time in deceased donor liver transplantations that were performed during 1998–2017 in adult patients.

were observed, and transplant period, recipient sex, and recipient and donor age did not influence the effect of ≥10 h CIT on graft survival substantially (**Table 3**). Kaplan-Meier estimation of 1-year graft survival of HCC patients with respect to MELD score categories is shown in **Figure 6**. CIT ≥10 h worsened 1-year graft survival significantly in HCC recipients with a high MELD score of ≥20, whereas its influence on outcome was less pronounced in patients with a low MELD score of <20 ($P < 0.001$ and 0.035, respectively). In the multivariable Cox regression analysis, the risk of graft loss due to CIT ≥10 h was significantly increased in patients with a MELD score of ≥20 (HR=1.71, 95% CI 1.33–2.21, $P < 0.001$), whereas HCC patients with a MELD score of <20 showed a similar risk only after CIT ≥14 h (HR = 1.67, 95% CI 0.86–3.26, $P = 0.13$).

DISCUSSION

After prolonged cold ischemia, outcome of a graft depends on its ability to recover from the ischemia injury, which appears to be especially difficult in steatotic grafts or grafts from older donors (12–14). CIT influenced graft and patient survival in a linear fashion and only during the first year after transplantation. At later time points the effect of equidistant 1-h CIT intervals on graft and patient survival was no longer present, indicating that ischemia-reperfusion injury is relevant only during the early post-transplant phase and that if and once the liver has recovered from the influences of ischemia—its duration becomes irrelevant. This effect contrasts with the influence of donor age which has an impact on graft survival also at later time points (8).

CIT is a factor that occurs during allocation and can only be calculated retrospectively. Along with macrovesicular steatosis of >40% and donor age of >65 years, CIT >14 h is a maEDC (2). Prolonged cold ischemia increases the risk of graft failure and early HCC recurrence (2, 6, 7). In our study it affected graft survival in the most common indication groups and is therefore relevant for the organ allocation. However, its negative effects were unevenly distributed among recipients with different indications for liver transplantation. Increasing CIT had a dramatic impact on outcome in HCV recipients. Similar effect of donor age on outcome in HCV recipients has been reported (8, 15). Grafts transplanted into HCV patients appeared to have the lowest tolerance for cold ischemia and were already at a significantly increased risk of graft loss at CIT as low as 8 h. The mechanisms that determine the association between worse outcomes in HCV patients and longer CIT are multifactorial. Together with advanced donor age and macrovesicular steatosis, CIT, as the third maEDC, is an independent risk factor associated with preservation injury, delayed graft function, and biliary complications (2, 5). Preservation injury during cold storage affects post-transplant outcomes strongly, especially in HCV recipients because HCV patients with biopsy-proven preservation injury have been shown to have worse outcomes than HCV recipients without histologically proven injury (16, 17). The preservation injury that follows tissue inflammation, cellular edema, cholestasis, and progressive centrilobular necrosis increases the risk of rejection and biliary complications. After preservation injury and during the regenerative hepatocyte proliferation that follows cellular death, HCV could more effectively infiltrate into the proliferating cells, leading to early aggressive HCV recurrence (16–18). Moreover, preexisting illnesses, malnutrition, cytomegalovirus infection, and HCV-positive donors have been identified as factors that may also contribute to HCV recurrence after liver transplantation. However, HCV genotype 1, high viral load, induction immunosuppression before transplantation and overshooting immunosuppression during graft rejection episodes, alone or in combination with advanced donor age and biliary complications, are considered to be the most prominent causes responsible for the increased risk of aggressive HCV

TABLE 2 | Results of the multivariable Cox regression analysis for the influence of CIT on 1-year graft survival in liver transplant recipients with different underlying diseases.

Cold ischemia time (hours)	*n*	HR	95 % CI	*P*
All underlying diseases				
≤5	5,583	1 (ref)	–	–
6–7	10,800	1.04	0.95–1.13	0.40
8–9	11,106	1.14	1.05–1.25	0.003
10–11	7,448	1.22	1.11–1.34	<0.001
12–13	3,629	1.43	1.29–1.59	<0.001
≥14	1,722	1.67	1.47–1.89	<0.001
HCC				
≤5	1,576	1 (ref)	–	–
6–7	3,319	0.92	0.78–1.09	0.36
8–9	3,086	1.06	0.90–1.26	0.47
10–11	1,851	1.33	1.11–1.58	0.002
12–13	798	1.41	1.15–1.74	0.001
≥14	323	1.80	1.39–2.33	<0.001
HCV-induced liver cirrhosis				
≤5	1,341	1 (ref)	–	–
6–7	2,481	1.03	0.87–1.22	0.73
8–9	2,617	1.24	1.05–1.47	0.011
10–11	1,772	1.31	1.10–1.57	0.002
12–13	873	1.51	1.23–1.85	<0.001
≥14	485	1.64	1.30–2.09	<0.001
Alcoholic cirrhosis				
≤5	1,038	1 (ref)	–	–
6–7	1,985	1.14	0.93–1.40	0.21
8–9	2,198	1.07	0.87–1.32	0.50
10–11	1,497	1.09	0.88–1.36	0.42
12–13	779	1.45	1.13–1.84	0.003
≥14	381	1.73	1.31–2.28	<0.001
Other				
≤5	1,628	1 (ref)	–	–
6–7	3,015	1.12	0.94–1.32	0.20
8–9	3,205	1.18	1.00–1.39	0.055
10–11	2,328	1.15	0.96–1.37	0.12
12–13	1,179	1.36	1.11–1.65	0.003
≥14	533	1.59	1.25–2.01	<0.001

Hazard ratios (HR) with 95% confidence interval (CI) of categorized CIT are shown. HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ref, reference.

recurrence and subsequent graft injury or failure (18, 19). Aggressive immunosuppression regimens rather than the direct effect of a specific immunosuppressive agent might affect the outcome and reducing the intensity of immunosuppression in HCV patients to maintain adequate host immune responses could decrease the HCV recurrence and improve graft and patient survival (20). HCV-related cirrhosis is associated with high rate of recurrence and graft loss, but the introduction of DAAs in 2013 improved graft survival significantly in patients with HCV and modified the course of recurrent HCV-graft disease. HCV slowly but steadily disappears as an indication for liver transplantation, however, DAAs are expensive and not readily available worldwide and specific data on DAAs are not documented in the CTS (8, 16, 18–20). Therefore, it can be assumed that these agents were not comprehensively available for the entire study population.

Although cold ischemia dramatically increased the risk of failure in the HCV subgroup, merely allocating grafts with longer cold ischemia to non-HCV recipients would not sufficiently solve the problem of matching grafts and recipients adequately because grafts transplanted into recipients with alcoholic cirrhosis and patients with HCC were also affected by cold ischemia. Indeed, transplant outcome of patients with alcoholic cirrhosis was influenced by cold ischemia, but these patients were at increased risk of graft loss only after an ischemia time of 12 h. This observation is very interesting and may be attributed to fast recovery once the patient has ceased to consume alcohol. The influence of prolonged ischemia time was also less pronounced in HCC patients compared to the HCV subgroup. HCC patients may have a more suppressed immune state than HCV patients and generate less rigorous immune responses under CIT-mediated inflammation. A recent CTS report by Unterrainer et al. indicated that renal transplant recipients with different forms of pre-transplant cancer had a generally decreased risk of death-censored graft loss, which approximates the rate of immunological graft failures (21). This finding supported the assumption that the patients' deficient immunological surveillance against tumors was paralleled by a weakness in mounting rigorous immunological rejection against the transplant. This may also be true for patients with HCC in whom, due to a generally suppressed immune state, CIT-mediated ischemia-reperfusion injury results in a less rigorous inflammation and rejection. In contrast, CIT can cause a more rigorous inflammation and damage in HCV patients due to an immune environment that is strongly activated by HCV infection. HCC patients received most of the elderly grafts but with the shortest cold ischemia. Allocation of grafts from older donors to recipients with HCC can well be justified because they show the lowest rise in the donor age-dependent risk of graft loss (8). This may explain why HCC patients had the best 1-year graft and patient survival despite the negative influence of CIT with an obvious 10-h cutoff. Graft and patient survival of HCC patients worsened at later time points and were nearly similarly as low as in HCV-patients, but this may also be attributed to recurrence of HCC that led to death of the patient with functioning graft. This assumption could not be definitely verified, as death with functioning graft could not be reliably examined in this multicenter study. CIT had different effect on graft survival in patients with HCC and different MELD scores. While HCC patients with a MELD score of <20 tolerated cold ischemia of up to <14 h, more than 25% of the grafts with cold ischemia longer than 10 h succumbed to failure if the recipient had a MELD score of ≥20, which is an extremely poor outcome considering the current 1-year graft survival benchmarks in patients with HCC (9, 10). This clearly suggests that with the increase of the MELD score, the tolerance of prolonged cold ischemia decreases. Because allocating grafts with longer CIT to the aforementioned recipient category did not carry disproportionate risk, this type of matching (longer CIT

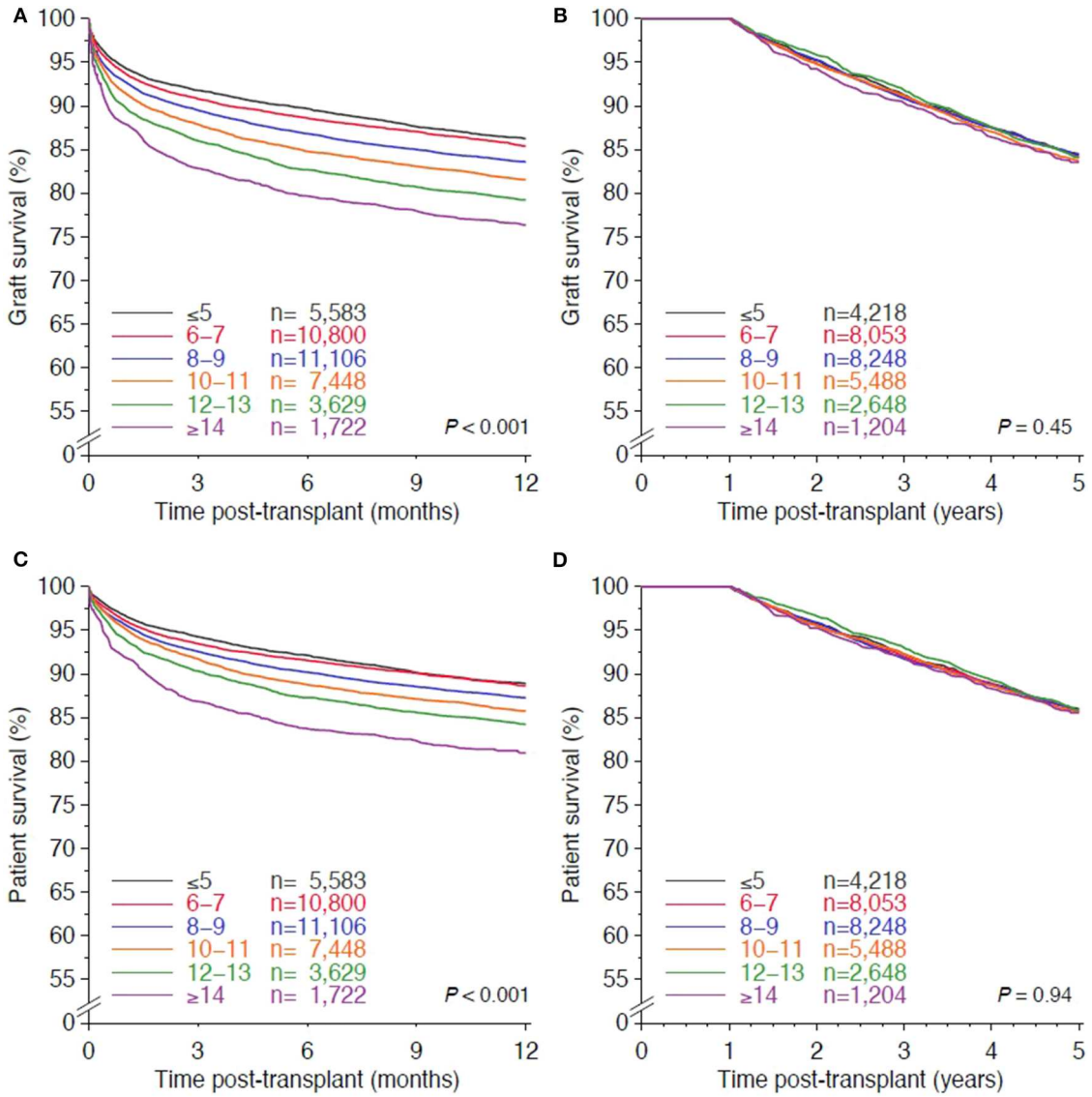

FIGURE 3 | Influence of cold ischemia time on overall graft survival **(A,B)** and patient survival **(C,D)** during first post-transplant year **(A,C)** and after first post-transplant year **(B,D)**. *P*-values of log rank test with linear trend are shown.

with HCC recipients and MELD < 20) is in line with previous findings and may be acceptable when facing organ shortage (2, 5). Patient survival constantly better than graft survival, also after longer CIT, was observed only in recipients with HCC. This may be attributed to the higher resilience of a re-transplant in patients with HCC. While Goldaracena et al. showed that patients with high labMELD scores benefit from transplantation as soon as possible and irrespective of the organ quality, our two recent studies pointed out that exact match between graft and recipient is important, and that grafts with maEDC could be allocated to low-risk patients with labMELD <20 e.g., patients with HCC (2, 5, 22). These findings were confirmed in a recent large cohort CTS study (8). Discrepant results may be attributed to the lack of uniform donor-recipient matching, but the aforementioned studies and the results of the current study indicate that matched allocation is plausible. However, the results of CIT with ≥14-h cutoff should be interpreted with great caution because cold ischemia exceeded 13 h only in 2.9% of HCC patients and in 4.3% of all recipients. Moreover, regarding the MELD score as a single surrogate parameter for the patient's condition bears a risk of bias. Also, for the purpose of this study, MELD score was only partially available since 2007 and has the known disadvantage of potentially inconsistent data entries due to the commingling of laboratory and exceptional MELD score values. Hence, the interaction of cold ischemia and MELD score demands further clarification. Nevertheless, to reduce the risk for individual patients, avoiding unfavorable constellations, e.g., HCV-patients and grafts with long cold preservation time, is prudent. The relevance of HCV-associated cirrhosis is decreasing owing to improved DAAs therapy and the new challenge is how to choose

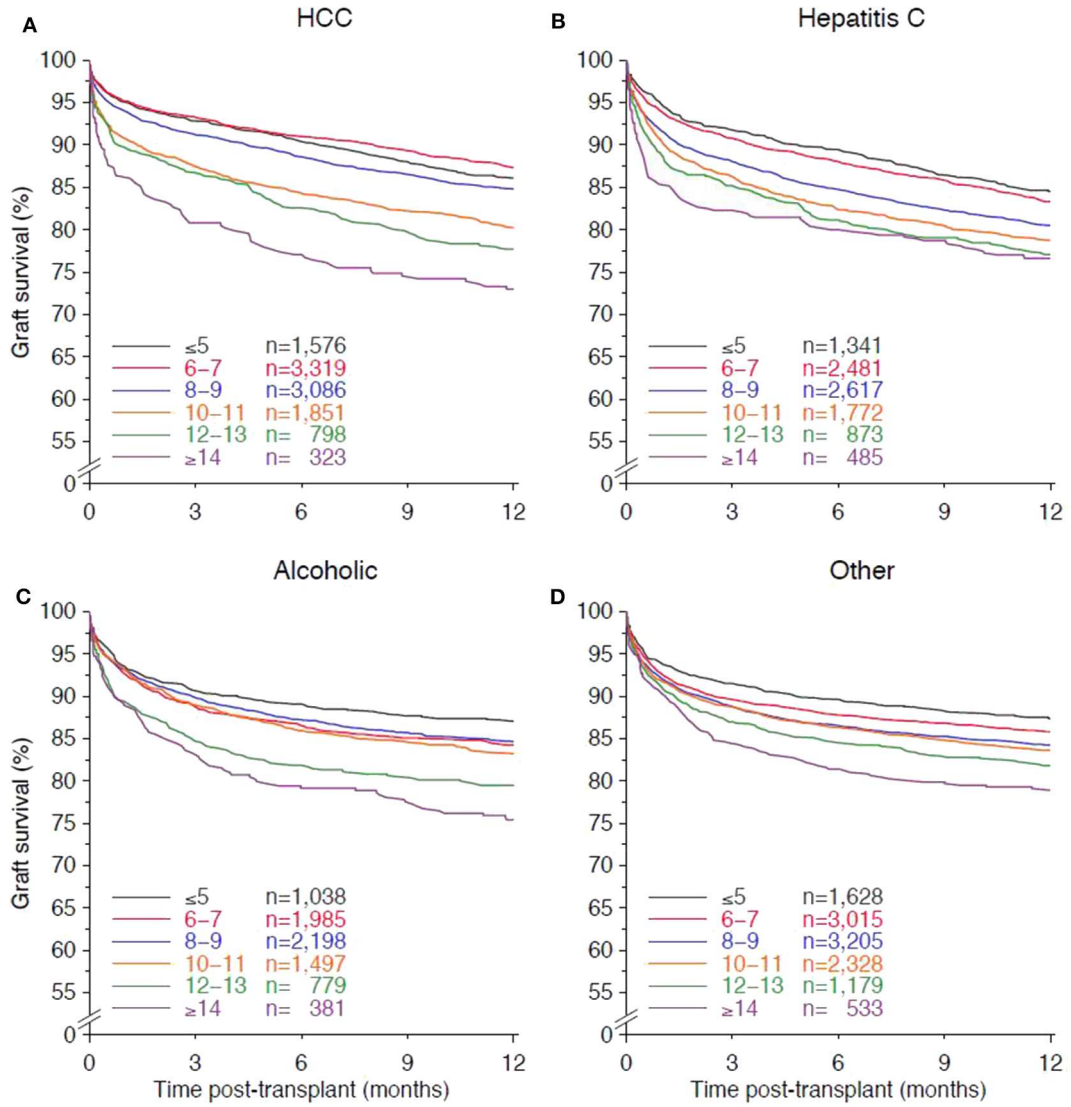

FIGURE 4 | Kaplan-Meier curves demonstrating the impact of cold ischemia time on 1-year graft survival for the main underlying disease categories **(A)** hepatocellular carcinoma (HCC), **(B)** hepatitis C, **(C)** alcoholic cirrhosis, and **(D)** the other less frequent original diseases (autoimmune disorders, cryptogenic cirrhosis, congenital diseases, hepatitis B, metabolic disorders, primary biliary cirrhosis, and primary sclerosing cholangitis). All log rank *P*-values with trend <0.001.

the most suitable candidate for grafts with longer cold ischemia out of recipients with HCC, alcoholic cirrhosis, and other diseases that gain on significance (23, 24). Organs with longer cold ischemia may be preferred for non-HCV recipients e.g., patients with HCC or alcoholic liver cirrhosis, but such ischemia time limits may only be useful in recipients with MELD scores below 20 as they do not impair outcome in this subgroup.

DRI and ET-DRI calculations include donor age, cause of death, donation after cardiac death, partial or split liver, location, and CIT (25, 26). With the exception of "location," all of the aforementioned risk factors were considered in our Cox regression model. Location was expected to play a less important role in our predominantly European cohort. We therefore took this parameter indirectly into account and used the confounder CIT instead. In line with the findings of Feng et al., we found the influence of CIT to be linear and similarly strong ($HR_{DRI} = e^{0.010} = 1.010$; $HR_{CTS} = e^{0.034} = 1.034$). However, our result showed that cold ischemia is important only during the first year following transplantation, and that its influence depends on the indication for transplantation. The studies of 20,023 recipients by Feng et al., and of 6,621 recipients by Braat et al. analyzed the effect on total available follow-up (DRI median 3 years; ET-DRI median 2.5 years), assumed constant linear influence of CIT, and did not consider indication for transplantation as confounder. Since the influence of ischemia time is clearly greatest at the beginning, the regression coefficient diminishes with the increase of the follow-up time, which is why we found it to be 3.4 times higher in our data than the

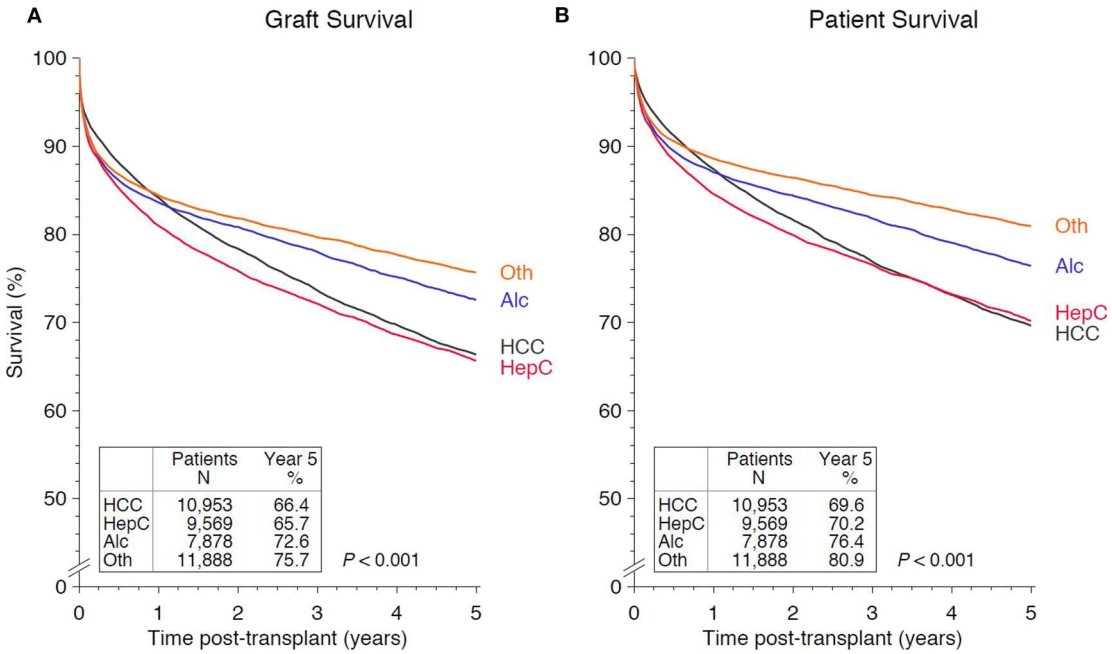

FIGURE 5 | Kaplan-Meier curves demonstrating the impact on **(A)** 5-year graft survival and **(B)** 5-year patient survival of the main original disease categories hepatocellular carcinoma (HCC), hepatitis C (HepC), alcoholic cirrhosis (Alc), and the other less frequent original diseases (autoimmune disorders, cryptogenic cirrhosis, congenital diseases, hepatitis B, metabolic disorders, primary biliary cirrhosis, and primary sclerosing cholangitis) (Oth).

TABLE 3 | Results of multivariable Cox regression analysis for the impact of CIT ≥10 h on 1-year graft survival in subpopulations of patients transplanted because of HCC.

Subpopulation	*n*	Regression coefficient	HR	95% CI	*P*
All patients with HCC	10,953	0.335	1.40	1.26–1.55	<0.001
Transplant year					
1998–2007	2,956	0.336	1.40	1.17–1.68	<0.001
2008–2017	7,997	0.336	1.40	1.23–1.59	<0.001
Recipient sex					
Female	1,853	0.139	1.15	0.89–1.49	0.29
Male	9,028	0.364	1.44	1.28–1.62	<0.001
Recipient age (years)					
<65	9,420	0.347	1.42	1.26–1.59	<0.001
≥65	1,533	0.296	1.34	1.04–1.74	0.026
Donor age (years)					
<65	7,328	0.322	1.38	1.21–1.57	<0.001
≥65	3,625	0.371	1.45	1.21–1.74	<0.001

Regression coefficients, hazard ratios (HR) with 95% confidence interval (CI) of CIT ≥10 h are shown. HCC, hepatocellular carcinoma.

coefficient used for the calculation of the DRI and ET-DRI (25, 26). According to the DRI- and ET-DRI calculations, CIT has an assumed linear influence on the outcome after transplantation (coefficient used for cold ischemia is 0.010) (25, 26). The linearity of cold ischemia can be assumed when the increase of ischemia time per hour would always affect the graft survival in a similar manner independent of the range of the ischemia time. By assuming the linear influence and by setting a fixed coefficient as in the aforementioned formulas, the categorical effect of CIT cannot be observed, especially when there is evidence of the opposite, non-linear influence. Not being able to retrieve DRI from the CTS database limits our study. However, the influence of cold ischemia is clustered in a non-linear fashion in recipients with HCC and alcoholic liver cirrhosis, and several cutoffs stand out. In HCC recipients, CIT only makes a difference when the comparison is made between ischemia time <10 h and ≥10 h, whereas in patients with alcoholic cirrhosis the two cutoffs are at 10 and 12 h. Therefore, similar to donor age, a categorical model that also considers the underlying disease should be preferred to a linear one in the case of CIT (8). The awareness of the importance of cold ischemia has increased significantly over the years, and the formulas for the calculation of DRI and ET-DRI are based on data from 2002 to 2007, respectively. Therefore, entering the indication for transplantation and CIT as categorical variable for HCC and for alcoholic cirrhosis with 3 different categories (HCC: <10 h, 10–13 h, and ≥14 h; alcoholic cirrhosis: <12 h, ≥12 h), and their respective coefficients may be worth considering as it might increase the specificity of the DRIs.

The allocation process is complex, but CIT can be managed by improved internal organization and regional allocation if estimated cold ischemia exceeds certain limits (2, 27). Our study of more than 40,000 patients revealed a strong negative linear impact of CIT on 1-year graft and patient survival. Remarkably, the negative influence of different CIT vanished after the first year

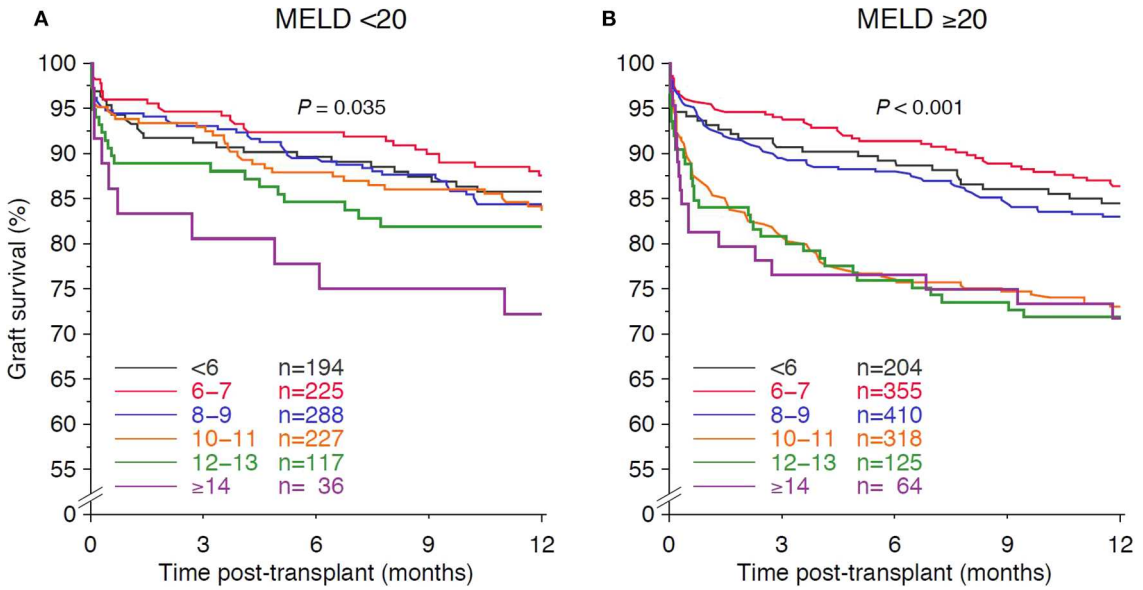

FIGURE 6 | Influence of cold ischemia time on 1-year graft survival in subpopulations of patients transplanted because of hepatocellular carcinoma with **(A)** low and **(B)** high Model of End Stage Liver Disease (MELD) score. Log rank *P*-values with trend are shown.

suggesting that other factors come into play. We narrowed the parameters that did not contribute substantially to the negative effect of longer cold ischemia to recipient gender and age ≥65 years, and HCC patients with a MELD score of <20. The negative cold ischemia effect depends strongly on the underlying disease. While HCC patients and recipients with alcoholic cirrhosis are able to compensate better for the effect of longer CIT, the impact of CIT is most severe in patients with HCV-related cirrhosis and should not exceed 8 h. Optimal donor-recipient matching is crucial in achieving reasonable outcomes after transplantation, and taking underlying disease into consideration is important especially in allocation of maEDC organs.

DATA AVAILABILITY STATEMENT

The raw data are available to the Collaborative Transplant Study in accordance with the consents of the patients, the participating transplant centers and registries.

ETHICS STATEMENT

The work of the CTS is approved by the Ethics Committee of the Medical Faculty of Heidelberg University (No. 083/2005) and performed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles in the currently valid version.

AUTHOR CONTRIBUTIONS

VL designed the study, analyzed data, and wrote the manuscript. BD participated in writing of the manuscript, performance of research, and data analysis. KW participated in data analysis. AM designed the study and analyzed data. CS designed the study, analyzed data, and participated in 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/fimmu.2020.00892/full#supplementary-material

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frontiers in Immunology

MINI REVIEW
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Assessment of Organ Quality in Kidney Transplantation by Molecular Analysis and Why It May Not Have Been Achieved, Yet

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Donor organ shortage, growing waiting lists and substantial organ discard rates are key problems in transplantation. The critical importance of organ quality in determining long-term function is becoming increasingly clear. However, organ quality is difficult to predict. The lack of good measures of organ quality is a serious challenge in terms of acceptance and allocation of an organ. The underlying review summarizes currently available methods used to assess donor organ quality such as histopathology, clinical scores and machine perfusion characteristics with special focus on molecular analyses of kidney quality. The majority of studies testing molecular markers of organ quality focused on identifying organs at risk for delayed graft function, yet without prediction of long-term graft outcome. Recently, interest has emerged in looking for molecular markers associated with biological age to predict organ quality. However, molecular gene sets have not entered the clinical routine or impacted discard rates so far. The current review critically discusses the potential reasons why clinically applicable molecular quality assessment using early kidney biopsies might not have been achieved yet. Besides a critical analysis of the inherent limitations of surrogate markers used for organ quality, i.e., delayed graft function, the intrinsic methodological limitations of studies assessing organ quality will be discussed. These comprise the multitude of unpredictable hits as well as lack of markers of nephron mass, functional reserve and regenerative capacity.

Keywords: marginal organs, molecular diagnostics, implant biopsies, organ quality, surrogate marker

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Abbreviations: AKI, acute kidney injury; CIT, cold ischemia time; D, donor; DBD, donation after brain death; DCD, donation after cardiac death; DD, deceased donor; DGF, delayed graft function; ECD, extended criteria donor; EVKP, score *ex vivo* kidney perfusion score; GFR, glomerular filtration rate; HCV, hepatitis C; I/RI, ischemia reperfusion injury; IRRATs, injury and repair response associated transcripts; KDPI, kidney donor percentile index; KDRI, kidney donor risk index; LD, living donor; MAPI, Maryland aggregate pathology index; PBTs, pathogenesis based transcript sets; PRA, panel reactive antigen; R, recipient; SCD, standard criteria donor; TPL, transplantation.

BACKGROUND

Good *organ quality* is the basis for successful long-term transplant outcome. The ability to withstand and repair immune and non-immune mediated injury and the number of nephrons to match the increased and persistent metabolic demand to a single kidney characterize optimal kidney organ quality with the potential to best long-term function. Hence, a robust assessment of kidney quality at time of transplantation is needed, in particular in donors with suboptimal conditions, i.e., marginal donors with old age, uncertain medical history, long ischemia time or pre-donation renal failure. In case of doubt clinicians will err on the side of caution and decide on discarding the organ, despite organ shortage and growing waiting lists. This is reflected in the high kidney discard rates in the US despite significant efforts to expand the donor pool. Nearly 20% of kidneys recovered are discarded, mainly based on procurement biopsies as method to assess organ quality (1–4). In Europe, where procurement biopsies are rarely performed, kidney discard rates are significantly lower and this is associated with saved patient life years (4, 5). This difference between US and European allocation practice underscores the need for more reliable and objective methods for organ quality assessment, especially in marginal donors, to decrease the number of discarded organs. So far, no evaluation process has sufficient discriminatory potential to guide the clinician and implanting surgeon team whether to accept or discard an organ. Currently available methods for assessment of organ quality are summarized in **Table 1** and discussed in the following paragraphs.

CURRENTLY AVAILABLE METHODS FOR ASSESSMENT OF ORGAN QUALITY

Histopathology

In 1995, the seminal paper on procurement biopsies by Gaber et al. presented a significantly increased rate of delayed graft function (DGF) and graft loss with glomerulosclerosis of >20% (6). However, accumulating data in the last 25 years questions the utility of procurement biopsies for evaluating donor kidneys (3, 7, 8). A systematic review by Wang et al. reported that all 47 published studies on kidney biopsies were retrospective, poor in design, and the results were heterogeneous. The percent glomerulosclerosis was most often examined and failed to predict graft failure in 7 out of 14 studies (7).

Analyzing biopsy findings it is necessary to distinguish between pre-implantation biopsies, performed immediately before implantation, and procurement biopsies, taken at time of donor kidney retrieval (9). Histology, in contrast to molecular changes, is expected to be similar in pre-implantation and procurement biopsies. For allocation purposes, focus lies on the procurement biopsy. As time is an important factor in the allocation process, these biopsies are evaluated on frozen sections stained with hematoxylin-eosin and not in paraffin-embedded tissues stained with periodic-acid-schiff, masson trichrome, and methenamine silver. Also, evaluation is done by on-call pathologists often not by an experienced renal pathologist. Furthermore, no consensus exists regarding use of wedge biopsies or core needle biopsies. All these factors pose problems. Hence, classification of histological lesions might differ when evaluated on frozen *vs.* paraffin embedded sections and interpretation might vary between on-call pathologists vs. experienced nephro pathologists contributing to the poor quality with missing information, lack of concordance and reproducibility (8–12). Even the agreement between expert renal transplant pathologists were only moderate to poor at Banff Histopathological Consensus meeting for preimplantation kidney biopsies with most interclass correlations less than 0.5 (12). In addition, intrinsic differences between wedge biopsies, that preferentially evaluate the subcapsular zone overestimating glomerulosclerosis, and core needle biopsies, that preferentially represent the cortex, further impact comparisons between various practices of procurement biopsies (9).

Finally, no consensus exists regarding the grading system to be used for interpretation of procurement biopsies. Besides the Banff grading system scoring individual lesions (12), several composite histological scoring systems have been described (7, 9). Yet, most histological composite scores lack validation in independent cohorts as well as testing of their predictive power in multivariate analyses including donor age and organ function and hence they might erroneously appear as independent predictors of graft failure. These facts underline the difficulty to predict long-term graft outcome based on histological evaluation of procurement biopsies (13). All these limitations translate into high discordance between two biopsies obtained of the same kidney (8) and also contribute to the high discrepancy in discard rates between centers (2, 3).

Graft survival rates of unilaterally discarded kidneys might indeed still be acceptable for some patients (2, 8). One-year death censored graft survival rate of recipients from unilaterally discarded kidneys due to donor factors (in particular biopsy findings) has been reported to be over 90% and five-year death censored graft survival was >85% (2). This underscores the fact that the currently available scores for organ assessment using histology inaccurately capture organ quality and the gain of life years for the individual patient.

Clinical Scores

The first clinical parameter found to be negatively associated with graft survival was age (13, 14). Besides age, established cardiovascular co-morbidities also associate with graft survival (15). Hence, common variables used in all scoring systems include donor age, history of hypertension and serum creatinine, altogether being surrogate markers of reduced nephron mass and extent of established injury and repair capacity, key donor factors contributing to long-term graft outcome (13, 16). However, these clinical markers lack robustness and standardization as organ quality metrics.

The recently introduced KDRI score (resp. kidney donor percentile index KDPI) reflects the rate of graft failure relative to a healthy 40-year old donor. This score was originally based on 14

TABLE 1 | Comparison of different assessment tools to evaluate organ quality in kidney transplantation.

Method		Scores	Strength	General intrinsic limitations
Organ inspection by surgeon			Identification of renal tumors and vascular and anatomical variations and quality of perfusion after retrieval	Interobserver variability, unclear predictive value
Kidney biopsy		Different scores evaluating either individual lesions or composite scores e.g., Banff Score Pirani-Remuzzi Score Maryland Aggregate Pathology Index (MAPI)	Offers the potential to detect preexisting lesions associated with donor medical diseases, e.g., hypertension, diabetes mellitus	Interobserver variability Interpretation on frozen sections differ from paraffin embedded formalin fixed samples. This however is time consuming (up to 5 hours), hence increasing cold ischemia time Sampling error (wedge versus needle biopsy different results) Low predictive value on outcome
Clinical classification models		Classification in SCD/ECD (introduced in 2002)	Practical for clinical routine application (easy, quick, information available at time of decision making)	Categorical classification underestimating variability Original model defining ECD did not include validation cohort
		Donor risk scores Kidney Donor Risk Index (KDRI) (including 14 variables) Donor-only KDRI (including 10 donor characteristics) (most recently introduced)	Assessment of graft quality as a spectrum Can easily be calculated based on donor factors	Does not account for injury during procurement Does not account for anatomical abnormalities Does not account for any recipient parameter (including immunological risks) Overall c statistic low with 0.62; c statistic for upper and lower quartile of KDRI 0.78 Falsely elevated in HCV + organs and increased creatinine due to acute kidney injury Not intended to be used as discriminatory tool to determine discard/acceptance but to better characterize organs
Machine perfusion characteristics	Hypothermic machine perfusion	Renovascular resistance index		Overall c statistic low with 0.58 for prediction of DGF, association with graft survival unclear
		Biomarkers within perfusate		Biomarkers (e.g., NAG, H-FABP, miR21), predictors of DGF but not graft survival
	Normothermic machine perfusion	Assessment of functional parameters		EVKP score (macroscopic appearance, blood flow, urine output), urine biomarkers (Endothelin 1, NGAL, KIM 1 and others)

donor characteristics (donor age, race, history of hypertension, history of diabetes, serum creatinine, cerebrovascular cause of death, height, weight, donation after cardiac death, hepatitis C virus status, HLA-B and -DR mismatch, cold ischemia time, *en-bloc* kidney transplant, dual kidney transplant) (17–19) and later reduced to 10 variables, as some information may be missing at time of transplantation. This is a far more granular tool for physicians to evaluate the offer and assess generic donor quality and outcome than the previously used dichotomous extended criteria donor (ECD) vs. non-ECD classification. Yet, despite introduction of this more detailed risk index, discard rates in the US remained unchanged at roughly 18–20% (20).

The differences in discard policies and application of the KDRI scores are highlighted in a recently published analysis by Aubert et al. (4). The probability of organ discard for the same KDRI is significantly higher in the United States compared to France and the interpolation of a similar organ use strategy in the United States would generate additional 132'445 allograft life-years over a ten-year observation period with greatest gain of life years through reduced discard of the organs with highest KDRI. These differences in applying the KDRI for accepting organ offers also reflect its limited predictive power. A recent study showed no significant difference in 5-year death-censored graft survival between DCD KDPI 61–81 and DCD KDPI $\geq$ 85 when used for donation after cardiac death (DCD) kidneys (18). In line with the limited discriminative power regarding graft failure very high KDPI kidneys may reveal acceptable outcomes (21–24). Another group showed 5-year graft survival of 91% using kidneys with KDPI score of 97% as dual transplants, highlighting that besides KDRI, nephron mass plays a major role with respect to graft survival (14, 25). A further a critical issue when using KDRI/KDPI is Hepatitis C virus (HCV) status having the largest contribution to KDPI (KDRI b coefficient 1/4.24; "Xb" component 1/4.24). However, HCV + kidneys are mostly young donors and at current era with available excellent antiviral treatment for HCV, clinical outcomes are excellent in HCV negative patients receiving HCV + deceased-donors (26). The other important critical

component of KDPI is the pre-donation serum creatinine level, which might be "falsely" high due to acute kidney injury from acute tubular necrosis. In a multicenter deceased donor study of 2,430 kidneys transplanted from 1,298 deceased donors 585 (24%) were from donors with AKI. The analysis did not show any significant difference in graft survival at 4 years by donor AKI stage (27).

All these articles question the utility of KDRI/KDPI as single decision tool with respect to kidney discard policies. Even though KDRI/KDPI has repeatedly been shown to associate with graft failure, a high KDRI/KDPI is not synonymous with graft failure and underscores its limited discriminative power as single decision tool.

Machine Perfusion

Research and applications of machine-based organ preservation have experienced a significant revival with the goals to reduce peri-transplant ischemia reperfusion injury, to facilitate assessment of organ quality and directed organ therapies, and to decrease the number of marginal organs to be discarded. First described as early as 1935 by Carrel and Lindbergh (28), interest in organ perfusion has re-emerged with the landmark trial published by Moers et al. (29). Machine perfusion for organ preservation was associated with a reduction of DGF compared to cold storage and its application has led to reduced discard rates of organs (30). However, these positive effects on short-term function did so far not translate into a marked improvement in long-term outcomes (31, 32). However, more sophisticated perfusion methods and cell-based therapies are investigated. Currently hypothermic machine perfusion is the most widely used technique, in recent years normothermic machine perfusion is gaining interest (33).

In addition to positively impacting reperfusion injury and organ preservation, machine perfusion also offers the opportunity for organ quality assessment based on perfusate analysis or measurement of perfusion dynamics such as intravascular renal resistance. The largest randomized controlled trial prospectively assessing renal intravascular resistive indexes on hypothermic machine perfusion and its association with graft outcome by Jochmans et al. showed that renal resistance at the end of hypothermic machine perfusion is an independent risk factor for both DGF and 1-year graft failure, yet the predictive power was low with a c-statistic of only 0.58 (34). Similar findings are reported by de Vries et al. and Parikh et al., showing only modest correlation with early graft function (35, 36). Likewise, perfusate analyses indicated that biomarkers, such as NAG or H-FABP, are associated with DGF, but again with low predictive value in differentiating functioning versus non-functioning grafts (37). Another group described levels of microRNA-21 (miR-21) to correlate with early graft function, but no data on association with long-term graft function is available (38). Hence, so far, neither dynamic machine perfusion characteristics such as renal resistance nor machine perfusate biomarkers can be used as stand-alone criteria for organ quality assessment with sufficient precision (36, 39).

Yet, novel techniques using normothermic perfusion allow for further assessments of functional parameter in addition to the above described flow/resistance markers. Hosgood et al. (40). described an *ex vivo* kidney perfusion quality assessment score (EVKP score) based on macroscopic appearance, renal blood flow and urine output after *ex vivo* normothermic kidney perfusion correlating with DGF but not long-term outcome. The same group correlated urine biomarkers of injury with this score. They measured a significant correlation between levels of urinary endothelin-1 and NGAL and perfusate parameters as well as between the EVKP score and donor creatinine at organ retrieval, while no correlation was found for KIM-1 (41). Similar results, reporting a lack of correlation of KIM-1 with donor AKI, have also been reported by other groups, likely due to the fact that KIM-1, in contrast to NGAL, is a rather late marker of kidney injury. However, the predictive power of these urinary biomarkers, despite being sensitive for structural kidney damage, is still unclear. Of note, a large multicenter deceased donor study of 2,430 kidney transplant recipients from 1298 donors did not find an association of the donor urine injury biomarkers microalbumin, NGAL, KIM-1, IL-18, and L-FABP with graft failure at a median follow-up of 4 years, questioning the predictive utility of urinary biomarker measurements during normothermic *ex vivo* perfusion (42).

Future evaluations will show whether novel techniques, such as normothermic machine perfusion, may allow better assessment of organ quality and function under near-physiological conditions (43). A key advantage of machine perfusion might well be the additional time gained for organ evaluation and the clinical decision to use or not use the organ.

MOLECULAR DIAGNOSTICS USING KIDNEY IMPLANTATION BIOPSIES

As outlined above, evaluation of organ quality by clinical scores, histopathology or perfusion characteristics lacks discriminatory power to guide clinicians to accept or discard an organ, in particular in the situation of marginal donors.

Over the recent years molecular analysis of biopsy samples has become a reliable, technically robust, not too expensive methodology including transcriptome, proteome and metabolome technologies. The unbiased, quantitative "omics' approaches have become standard of care in oncology, classifying tumors and individualizing therapy. Hence, great expectations have been based on molecular diagnostics as they potentially offer an alternative, more objective and quantitative method for organ evaluation. Molecular profiling indeed demonstrated to go beyond histopathologic evaluation being able to detect changes not captured by histopathology. In a previous review we have summarized molecular studies of 0-hour biopsies (both pre-implantation and post-reperfusion) published till 2010 (44). It could be shown that transcriptome profiles provide a quantitative measurement of inflammatory burden, detect coordinated activation of pathways of immune activation, defense response, oxidative stress and a parallel inhibition of metabolism and transport or ion binding. In particular, transcriptome patterns identified changes in kidneys such as susceptibility to DGF, which was not reflected using clinical

and histopathological scores (45). However, despite the number of promising findings, no robust set of predictive molecular markers for organ quality measurement had been identified in these early studies.

Since then a number of new studies have been conducted to further assess the potential to evaluate organ quality and transplant outcomes. **Table 2** summarizes studies on molecular analyses of peri-transplant biopsies assessing organ quality that have been published since 2011 and are listed in PubMed.

A majority of studies focused on DGF as a surrogate marker for organ quality and early outcome (46–52). They largely confirm the earlier findings that DGF is usually better predicted with molecular changes than histology or clinical scores at time of transplantation (46, 49–52). They identified molecular changes associated with kidney injury (such as NGAL, syn. LCN2, KIM1, syn. HAVCR1, NTN1) (46, 49) and aging (CDKN2A) (50–52) as DGF biomarkers. However, these peri-transplant changes associated with DGF did not allow a robust prediction of medium- to long-term functional outcomes.

Mas and her group showed that transcript changes associated with early kidney function but not with DGF *per se* correlate with outcome. Kidneys with DGF and also a low GFR at 1-month post-transplant showed inferior medium- to long-term outcomes. The pre-implantation biopsies of these kidneys showed an increased expression of pathways associated with immune activation and inflammation. Gene transcripts of CCL5, CXCR4, and ITGB2 discriminated best between low *vs.* high GFR. This difference in kidney function remained throughout the period of observation of 2 years (48, 53). Findings of the Halloran group confirm the lack of predictive power of gene changes associated with DGF (54, 55). They identified gene transcript changes associated with AKI in transplant biopsies. These so-called injury and repair associated transcripts (IRRATs) correlate with degree of injury, repair capacity and functional outcome but not with DGF (54, 55). However, with a sufficient long-term follow up of more than 2 years, peri-transplant molecular phenotypes at time of, or early after transplantation seem not to correlate with medium- to long-term transplant function. Molecular changes in 6-week protocol biopsies correlated with atrophy and scarring at 6 months but not with future functional decline (47), implant biopsies did not predict late function (54, 55). In contrast, long-term function correlated with histopathology changes associated with aging or clinical scores, in particular donor age (53, 55).

Consequently a number of studies focused on molecular markers for biological age as parameters for organ quality (50–52). In particular increased expression of CDKN2A associated with graft function, probably better reflecting the allostatic load of "wear and tear" of an organ and its resilience to cope with the peri- and post-transplant stressors (50–52). However, the clear added value of markers of biological age like CDKN2A or others like telomere length, microRNAs or epigenetic changes to the simple measurement of chronological age is not clear. In addition, the age allocation bias, i.e., old kidneys are predominantly given to old recipients, and hence likely poorer quality organs are transplanted into recipients with more comorbidities and inferior outcomes, makes it difficult to identify and validate robust quality markers in old kidneys (55).

An interesting, recent study analyzed gene expression in cell infiltrates at time of transplantation and 4 months post-transplant (56). This study indicated gene expression of inflammatory and fibrotic markers at 4 months, and differences between 4 months and baseline, correlated negatively with renal function up to 5 years. Another small, exploratory but cutting-edge methodology study by Kaisar et al. (57). suggests that proteomics analyses are able to discriminate different outcomes that were not predicted by common evaluation methods such as clinical (KDPI), histology or AKI scores (57). These promising studies need further validation and larger numbers.

In general none of the molecular analyses outlined here have entered the clinical routine diagnostics and organ quality is still evaluated exclusively by clinical and histopathology-based scores.

The question is why these molecular analyses have not yet identified robust quality markers and hence successfully translated into clinical useful tests? This might be due to intrinsic limitations of molecular studies, selection of insufficient surrogate markers and end points for outcome studies, or the principal unpredictability of long-term outcomes with donor organ characteristics given heterogeneity and multitude of hits during the post-transplant life of the donor kidney.

Molecular analyses of donor kidney biopsies might not depict structural changes or reflect nephron mass. They measure tissue cell mixtures depending on the location of the biopsy site, cannot predict the multitude of additional immune and non-immune hits and recipient factors that occur in the long run. They are drowned by the tidal wave in expression changes due to brain death and the associated SIRS-like syndrome.

The surrogate markers for kidney quality used for the identification of molecular changes is another likely reason for the lack of established kidney quality profiles. Delayed graft function, chronological rather than biological age, incomplete disease phenotyping, weak markers of kidney function (such as creatinine), short follow-up periods, small samples sizes or lack of validation studies all contribute to the still unfulfilled promise of molecular diagnostics for organ quality assessment.

ORGAN QUALITY ASSESSMENT OF NON-KIDNEY TRANSPLANTS

Pre-transplantation assessments of organ quality in non-kidney solid organs primarily rely on clinical scores and markers assessed during *ex vivo* machine perfusion. Comparable to kidney transplantation there is no established molecular assessment of biopsy samples and few examples are given below. In-depth analysis of organ quality assessment measures for other organs than the kidney is out of the scope of this review.

In liver transplantation, organ quality has been correlated with cumulative bile acid production and coagulation parameters (58). Also metabolomic signatures associated with early graft function comprising key pathways involved in lipid homeostasis and histidine pathway have been described (59). With respect to analysis of molecular markers, investigation of microRNA profiles in graft preservation solutions has been

TABLE 2 | Molecular diagnostics of early kidney transplant biopsies [summarizing studies published since 2011, studies published before were previously summarized (44)].

References	Time of biopsy	Patient/biopsy numbers	Follow up	Test and outcome markers	Findings	Strengths and limitations with focus on quality assessment
			DGF as surrogate for organ quality and early outcome			
(46)	Implantation[1]	34 DD + 9 controls (tumor-nephrectomies)	Early post-TPL	4 candidate AKI genes studied in micro-dissected tubulointerstitial vs. glomerular segments: KIM1 (i.e., HAVCR1), NGAL (i.e., LCN2), CYR61, NTN1 *Tested outcome:* *DGF*	• Upregulation of NGAL and KIM1 in DGF • In multivariate model only D age significantly associated with DGF	**Strengths:** • Analysis of micro-dissected samples **Limitations:** • No validation set • Low numbers • No long-term outcome
(47)	6 weeks post-TPL	107 in total: 14 LD + 93 DD Indication biopsies	≥ 2 years	Pathogenesis based transcript sets (PBTs) for inflammation and injury *Tested outcome:* *DGF, GFR*	• The molecular phenotype correlates with previous DGF • No difference in PBTs between LD and DD • Molecular phenotype correlates with 6 month atrophy and scarring • Molecular phenotype did not predict future functional decline or allograft loss	**Strengths:** • long-term analysis incl. assessment of graft loss **Limitations:** • no validation set • No peri-TPL biopsies
(48)	Pre-implantation (back bench)	92 DD (91 analyzed) cold perfusion and pump perfusion	≥ 1 year	Microarrays (validation in independent sample set) *Tested outcome:* *DGF and 1 month GFR (≤ / > 45 ml/min/ 1.73 m^2)*	• Clinical variables pre-transplant did not identify kidneys with better or poorer function during first year • 1 month function predictive of 1 year function • Low *vs.* high GFR within DGF group differ in inflammation and immune activation transcripts pre-TPL, at month 1 and throughout first year • DGF not associated with 3 month and 1 year function • Molecular phenotype does not separate DGF vs. no DGF	**Strengths:** • validation set • Unbiased gene selection approach • GFR at 1 month and 1 year as outcome marker and not only DGF **Limitations:** • no analysis of longer-term graft function (incl. graft loss)
(53)	Pre-implantation (back bench)	112 biopsies in 100 DD	29 months (median)	Unbiased microarray gene expression approach: four differentially expressed genes selected (validated in an independent set) *Tested outcome:* *1 month GFR (≤ / > 45 ml/min/1.73 m^2)*	• Groups with high *vs.* low 1 month GFR stay different at 24 months post-TPL • D age only clinical marker different in high *vs.* low 1 month GFR groups, not race, gender, CIT, PRA and cause of death • CCL5, CXCR4 and ITGB2 expression in pre-implantation biopsies discriminate GFR high *vs.* low group at 1 month	**Strengths:** • validation set [overlap with (50)] • Unbiased gene expression approach • GFR up to 24 months as outcome marker **Limitations:** • no analysis of longer-term graft function (incl. graft loss)

(Continued)

TABLE 2 | Continued

References	Time of biopsy	Patient/biopsy numbers	Follow up	Test and outcome markers	Findings	Strengths and limitations with focus on quality assessment
(54)	Early post-TPL AKI	28 biopsies (26 patients with AKI)	3.9 years	Unbiased microarray gene expression approach (validation set of 27 kidneys) 11 protocol biopsies *Tested outcome: GFR, graft loss, dialysis*	• No difference in kidney outcome with or without AKI • Histology did not correlate with DGF, GFR, recovery of function or IRRAT score • 30 injury repair response transcripts (IRRATs) in AKI correlate with function, future recovery, need for dialysis but not future graft loss • IRRAT transcripts differ to DGF transcripts	**Strengths:** • validation set • Unbiased gene expression approach • Analysis of AKI in allografts • Analysis of longer-term function, incl. graft loss **Limitations:** • small number • No peri-TPL biopsies
(55)	Implantation[1]	70 biopsies from 53 DD 8 control nephrectomies	4.2 years	AKI associated. transcripts (IRRATs), see Famulski et al. (54) *Tested outcome:* *early and 1 and 3 year GFR, histology, clinical scores, graft loss*	• D and R age, not histology correlate with early dysfunction • CAVE: age bias, old kidneys are often allocated to old Rs • D age predicts late function • D age, D age-dependent models such as KDRI and Irish and histology correlate weakly with late function • IRRATs predict early but not late function in SCD	**Strengths:** • independently validated gene set • Analysis of clinical, morphological and molecular markers as predictors of early and late function **Limitations:** • no validation set • No longer-term follow-up (incl. graft loss)
(49)	Sequential biopsies: Procurement and Pre-implantation and Implantation[1]	105 biopsies in 38 DD	1 year	92 pre-selected genes associated with I/RI injury *Tested outcome:* *DGF*	Gene expression heterogeneity increases from procurement to pre-implantation to implantation biopsies suggesting different organ vulnerability • Cold storage not associated with significant transcript changes • Reperfusion associated with activation of innate and adaptive immune response and apoptosis • Low netrin-1 (NTN1) and higher tubular atrophy on histology predictive of DGF	**Strengths:** • investigation of sequential biopsies from the same graft **Limitations:** • no validation set • No long-term follow-up
Molecular markers for biological age as markers for organ quality						
(50)	Pre-implantation	120 DD	1 year	Comparison of predictive capacity of biomarkers of aging (CDKN2A expression and telomere length) *Tested outcome:* *6 and 12 month function, DGF*	• CDKN2A, stronger than telomere length, predict DGF and 6 and 12 month graft function • Pre-TPL D risk classification based on CDKN2A and ECD criteria possible	**Strengths:** • assessment of markers of biological age (yet additive value to chronological not clear) **Limitations:** • no validation cohort • Short follow-up • No assessment of long-term graft outcome

(Continued)

TABLE 2 | Continued

References	Time of biopsy	Patient/biopsy numbers	Follow up	Test and outcome markers	Findings	Strengths and limitations with focus on quality assessment
(51)	Pre-implantation	94 DD	1 year	Pre-selected microRNAs, CDKN2A expression (validation cohort) *Tested outcome:* *t1/2 creatinine fall, DGF, 3, 6 and 12 month function*	• A score using senescence associated miRNAs (hsa-miR-217, hsa-miR-125b; regulators of CDKN2A) combined with D age and organ type predicts occurrence of DGF (Sens > 90%, Spec > 60%) • CDKN2A expression and hsa-miR-217 correlate positively with 12 month function	**Strengths:** • Investigation of markers of biological age • Concept of allostatic load **Limitations:** • Questionable clear added value of miRNAs and CDKN2A to chronological age alone • No assessment of long-term graft function or loss
(52)	Pre-implantation and Implantation[1] (paired biopsies)	55 DD	1 year	Unbiased, RNASeq, genome, transcriptome and epigenetics analysis *Tested outcome:* *DGF, creatinine fall 1st week, 3, 6 and 12 month function*	• Transcriptional response to reperfusion injury similar for allografts irrespective of post-TPL outcome, but magnitude is greater for those exhibiting DGF • DGF specific transcripts reveal differential promotor methylation status • Pre-implantation TP53, CDKN1A/p21, CDKN1B/p27 ("BioAge") associated with 3 and 6 month function • Molecular signature for allostatic load (burden of "wear and tear") reflects age-related physiological capability and resilience • DGF is a manifestation of its allostatic load	**Strengths:** • Unbiased analysis investigating genome, transcriptome and epigenetics • Concept of allostatic load **Limitations:** • No validation set • Questionable clear added value of biological age markers to chronological age alone • Short term follow-up • No assessment of long-term graft function or loss
				Analysis of cell infiltrates and proteomics		
(56)	Pre-implantation and 4 months post-TPL	94 biopsies (60 DD and 34 LD)	≤5 years	Pre-selected genes, macrophage infiltration *Tested outcome:* *graft survival, 4, 24 and 60 month function*	• Baseline expression of selected genes did not correlate with GFR at any time point • Higher pre-implantation levels of inflammation, monocyte recruitment, and M1/M2 macrophage transcripts in DD compared to LD • 4 month fibrosis transcript levels correlate with long-term function in DD • Expression of inflammation and fibrosis markers at 4 months and difference between 4 months and baseline correlate negatively with medium- and long-term renal function in DD • TGFb1 best predictor of long-term renal function in DD	**Strengths:** • Broad gene set • Analysis of graft survival and outcome up to 5 years **Limitations:** • No validation set • LD *vs.* DD impacted by multitude of factors (D/R age, CIT, dialysis time) not *per se* reflecting tissue quality

(Continued)

TABLE 2 | Continued

References	Time of biopsy	Patient/biopsy numbers	Follow up	Test and outcome markers	Findings	Strengths and limitations with focus on quality assessment
(57)	Pre-implantation	38 donors (proteomic analysis done in 10 donors) always one mate kidney when both had same 3-months function to select for donor factors	12 months *Tested outcome: 3 and 12 month GFR*	Pilot study, unsupervised proteomics analysis	• Common evaluation methods not predictive of outcome (KDPI, histology, AKI score) • Pathways of cellular response to stress, cell surface receptor signaling and enrichment of reactive oxygen species detoxification differ between optimal and suboptimal donors • Proteomics analysis differentiates between different outcomes	**Strengths:** • Novel "mate kidney approach" analyzing proteomics • Unbiased proteomics analysis **Limitations:** • No validation set • Small number • Short follow-up • Highly selected groups

[1]*Taken post-reperfusion, i.e., within one hour after opening of the anastomosis.*

shown to be predictive of ischemic-type biliary lesions after liver transplantation, which are the second most common cause of graft failure after liver transplantation (60). The ratio of hepatocyte to cholangiocyte-derived miRNAs (with special focus of miR 122 and miR 222) was predictive of graft viability (60–62). In pancreas transplantation, assessment of organ quality is performed during machine perfusion measuring insulin secretion, acid-base balance and perfusion characteristics (63). Likewise, in lung transplantation organ quality assessment is reported through ventilation parameters, analysis of arterial blood gases on perfusate samples with recent focus on metabolic components of glucose consumption and lactate production (64). Other groups indicated that levels of inflammatory cytokines (65, 66), endothelin-1 (67), adhesion molecules (68) or neutrophil extracellular traps (69) in lung perfusate are associated with post-transplant primary graft function. Similarly, assessment of donor heart quality prior to transplantation is attempted by analyzing perfusate during machine perfusion (70).

CRITICAL DISCUSSION OF SURROGATE MARKERS USED FOR KIDNEY QUALITY ASSESSMENT

The majority of published studies on molecular assessment of organ quality used *DGF*, i.e., transient renal failure immediately post-transplantation, as surrogate marker for graft quality and outcome. This is based on the association of reduced graft survival of DGF kidneys in standard brain death donors (DBD) shown in some, but not in all studies. The limitations of DGF as a surrogate outcome marker for poorer organ quality is highlighted by the excellent quality and long-term outcomes of DCD organs. Despite the high percentage of DGF cases these positively selected cases with usually young age and lack of comorbidities show good long-term outcomes. Similar lack of correlation with longer-term outcomes and DGF is seen analyzing mate kidneys. Donor characteristics rather than ischemia times or DGF rates determine the long-term performance (71–74).

Moreover, definition of DGF is not uniform (71). More than 10 different definitions are used and most importantly none of them was associated with poorer graft survival in DCD kidneys (71). The limitations of DGF as a quality marker is further underlined as so far no treatment of DGF translated into significant improvement in long-term outcome (75).

Patho-physiologically the higher risk of DGF in DCD donors compared to DBD donors can be explained by the unavoidable extended warm ischemia time and associated increased ischemia-reperfusion injury. However, full recovery and excellent long-term graft outcome underline repair capacity and nephron mass as organ quality determinants.

Hence, not DGF *per se* but rather ability to recover from DGF as indicated, e.g., by GFR at 1 month might be a more reliable marker of long-term graft outcome and quality, as recently reported by Lee et al. (76). Donor age, donor final creatinine and cold ischemia time were significantly associated with DGF recovery status (76). DGF is a syndrome and duration of DGF and

degree of acute kidney injury is associated with renal outcome, in transplant and non-transplant settings (77, 78). Extent of recovery presumably reflects the intrinsic repair capacity of the donor organ. Age strongly defines repair capacity and this might explain donor age as the most widely used criterion in all clinical scores assessing organ quality pre-transplantation (73, 79).

In summary, the post-transplant course is determined by donor factors, acute peri-transplantation injury as well as recipient factors. DGF *per se* is a poor, but most frequently used, surrogate marker for organ quality (see **Table 2**). Hence, the focus on identifying DGF-associated molecular patterns might be one reason that so far molecular diagnostics of organ quality has not translated into clinical decision making. In addition, molecular assessment of repair capacity and biological tissue aging is still ill defined. Ongoing work on robust molecular markers of biological age is promising (see **Table 2**) but again has not yet translated into clinical utility.

Successful organ transplantation is largely defined by a good and long-term functioning kidney graft. This requires a sufficient nephron mass to meet the increased, long-term metabolic demand and stresses of a single kidney in a transplant recipient. In the unstable setting of brain death and organ donation donor serum creatinine or estimated GFR are unreliable markers of nephron mass or reserve capacity. The same applies to histopathology and clinical scores. The identification of molecular markers for nephron mass in addition to repair capacity would be most valuable but yet, has not been achieved. This might be due in part to the lack of long-term studies. As shown in **Table 2** most studies focus on short-term function. The identification of molecular changes in peri-transplant biopsies that correlate with long-term function is needed.

PROPOSED APPROACHES TO OPTIMIZE MOLECULAR DIAGNOSTICS FOR CLINICAL ROUTINE APPLICATION

Assessment of Nephron Mass by Molecular Methods: Paired Kidney Transplantation Study

- Comparison of molecular profiles at implantation biopsy between kidney pairs from the same donor both with high eGFR at 1 year post transplantation (i.e., eGFR $>$ 60 ml/min/1.73 m^2) and kidney pairs from the same donor with both low eGFR at 1 year post transplantation (i.e., eGFR $<$ 30 ml/min/1.73 m^2). This should primarily reflect intrinsic donor factors rather than post-transplant hits and recipient factors. Note: Ratio for taking follow up of 1 year only: if taking too long follow up (longer than 1 year) recipient factors might become additionally relevant.

Assessment of Kidney Regeneration Capacity: Recovery From AKI (i.e., DGF)

- Comparison of molecular profiles at implantation biopsy between kidney with low delta of expected and observed creatinine at 1 year post transplantation (e.g., delta 25%; i.e., kidney with good regeneration capacity) and kidneys with high delta of expected and observed creatinine at 1 year (delta $>$ 25%; i.e., kidney with impaired regeneration capacity). Note: make sure taking only kidney with good match of recipient/donor weight (i.e., R/D weigh ratio of 0.8–1.2 allowed) (80).

Assessment of Effect of Pumping on Recovery Form AKI in High Risk Patients (i.e., Patients With Low Regeneration Capacity)

- Once molecular profiles of kidney with low regeneration capacity is characterized: comparison of delta expected-observed creatinine at 1 year in high risk kidneys preserved with pumping as compared to delta expected-observed creatinine in high risk kidneys preserved with cold storage.

AUTHOR CONTRIBUTIONS

SM and TM designed the first draft of the manuscript. EA and VM revised the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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Transplanting Marginal Organs in the Era of Modern Machine Perfusion and Advanced Organ Monitoring

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Organ transplantation is undergoing profound changes. Contraindications for donation have been revised in order to better meet the organ demand. The use of lower-quality organs and organs with greater preoperative damage, including those from donation after cardiac death (DCD), has become an established routine but increases the risk of graft malfunction. This risk is further aggravated by ischemia and reperfusion injury (IRI) in the process of transplantation. These circumstances demand a preservation technology that ameliorates IRI and allows for assessment of viability and function prior to transplantation. Oxygenated hypothermic and normothermic machine perfusion (MP) have emerged as valid novel modalities for advanced organ preservation and conditioning. *Ex vivo* prolonged lung preservation has resulted in successful transplantation of high-risk donor lungs. Normothermic MP of hearts and livers has displayed safe (heart) and superior (liver) preservation in randomized controlled trials (RCT). Normothermic kidney preservation for 24 h was recently established. Early clinical outcomes beyond the market entry trials indicate bioenergetics reconditioning, improved preservation of structures subject to IRI, and significant prolongation of the preservation time. The monitoring of perfusion parameters, the biochemical investigation of preservation fluids, and the assessment of tissue viability and bioenergetics function now offer a comprehensive assessment of organ quality and function *ex situ*. Gene and protein expression profiling, investigation of passenger leukocytes, and advanced imaging may further enhance the understanding of the condition of an organ during MP. In addition, MP offers a platform for organ reconditioning and regeneration and hence catalyzes the clinical realization of tissue engineering. Organ modification may include immunological modification and the generation of chimeric organs. While these ideas are not conceptually new, MP now offers a platform for clinical realization. Defatting of steatotic livers, modulation of inflammation during preservation in lungs, vasodilatation of livers, and hepatitis C elimination have been successfully demonstrated in experimental and clinical trials. Targeted treatment of lesions and surgical treatment or graft modification have been attempted. In this review, we address the current state of MP and advanced organ monitoring and speculate about logical future steps and how this evolution of a novel technology can result in a medial revolution.

Keywords: transplantation, machine perfusion, marginal, graft, immunogenecity, immunomodulation, reconditioning, expanded criteria donor

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INTRODUCTION

The velocity and significance of progress in transplantation started to decrease around the turn of the century. Up until that point, the number of transplantations performed was ever-increasing, and the outcomes continued to improve. The clinical benefit to the patient from the immediate and effective treatment of organ failure was defined by improved patient survival. Patient survival as an endpoint followed by graft survival as a surrogate and indicator of judicious organ attribution were the framework of significant momentum that succeeded in making transplantation a standard of care. This success was fueled, however, not only by the healthcare quality gain but also by a flourishing industry producing high-quality and expensive immunosuppressive medication—to be taken for life after transplantation (1–3).

The honeymoon period came to an end when both donor rates and short-term results stagnated. The established endpoints for clinical trials are difficult to improve in the short run after transplantation, and the field was slow in anticipating and responding to the changing circumstances—specifically the shortage of standard criteria donors (SCD). The field of transplantation now finds itself in very difficult circumstances: 90%+ patient and graft survival rates leave little room for improvement but also little room for error. At the same time, the conditions and circumstances in organ donation are radically changing, with increasing donor age and comorbidities and donation from non-heart-beating donors. In countries spearheading the evolution, donation after cardiac death (DCD) and extended criteria donor (ECD) organs make up more than 50% of the organ pool. With the pressure to maintain and further improve the excellent short- and long-term results, but working with very different resources, the most prominent immediate challenge is to better define and preserve, if not improve, organ quality in transplantation.

Abbreviations: A2AR, Adenosine A2A receptor; AST, Aspartate aminotransferase; ATP, Adenosine triphosphate; CIT, Cold ischemia time; CMV, Cytomegalovirus; CVA, Cerebrovascular accident; DAMP, Danger-associated molecular pattern; DBD, Donation after brain death; DC, Dendritic cell; DCD, Donation after cardiac death; DGF, Delayed graft function; D-HOPE, Dual hypothermic oxygenated perfusion; DRI, Donor Risk Index; EAD, Early allograft dysfunction; ECD, Expanded criteria donor; ECMO, Extracorporal membrane oxygenation; EMS, Exsanguinous metabolic support; ESP, Eurotransplant senior program; ET-1, Endothelin-1; EVLP, *Ex vivo* lung perfusion; FABP, Fatty Acid- Binding Protein 1; GFP, Green fluorescent protein; GST, Glutathione S-Tranferases; H-FABP, Heart-type fatty acid-binding protein; HOPE, Hypothermic oxygenated perfusion; HMP, Hypothermic machine perfusion; HMPO, Oxygenated hypothermic machine perfusion; IFNγ, Interferon gamma; IL-10, Interleukin-10; IRI, Ischemia-reperfusion injury; KDRI, Kidney Donor Risk Index; LDH, Lactate dehydrogenase; MP, Machine perfusion; MSC, Mesenchymal stromal cells; NAG, N-acetyl-beta-D-glucosaminidase; NMP, Normothermic machine perfusion; NK, Natural killer cell; NKT, Natural killer T cell; NRP, Normothermic regional perfusion; OCS, Transmedic Organ Care System; PDR, Pancreas Donor Risk Index; PGD3, Prostaglandin D3; PNF, Primary non-function; PV, Pressure volume; RCT, Randomized controlled trial; ROS, Reactive oxygen species; SCD, Standard criteria donor; SCS, Static cold storage; SOFT, Survival Outcomes Following Liver Transplantation score; SOP, Standard operating procedure; SOT, Solid organ transplantation; TNF-α, Tumor necrosis factor α; UNOS, United Network for Organ Sharing; VEGF, Vascular endothelial growth factor; WIT, Warm ischemia time.

With this as a driving force, modalities to prolong and improve preservation together with the tools to assess organ quality and function are emerging as the new frontier. Beyond serving an immediate medical need, the technologies evolving herald a more fundamental potential for change in healthcare: with the extracorporeal long-term preservation and assessment of organs, the capability for human organ treatment and repair arises. Hence, 20 years after transplantation advanced to become a standard of care, the field has the potential to play a key role and add a chapter to healthcare once again (4–10).

In this review, we aim to address the current state of the field. A clarification of the terminology and the definitions of marginal organs, including an overview and comparison between the definitions for each organ, shall help to provide a better picture of the actual status. Considering the changing circumstances, the actual impacts of a condition on organ quality and function need to be addressed not only from the viewpoint of an empirical correlation. The individual parameters need to be further addressed for their reversibility and treatability. Building on this concept, the various modalities of MP in their different stages of development and actual benefit require a critical reflection. While the assessment of organ quality and function during extracorporeal perfusion is in its infancy, the growing body of data may help to eventually develop a comprehensive picture of the quality of the components of organs as relevant to transplantation and beyond. An important focus in this context is the assessment, definition, and relevance of the immune system of organs if isolated from the human body. Immunomodulation and immunomasking seem more realistic if attempted under the conditions of an isolated and perfused organ. The vision of a realization and refinement of the repair, treatment, and modification of organs is starting to trigger a boost in interest both in academia and in industry.

DEFINING "MARGINAL" ORGANS

In the light of organ shortage, critical reflection on the expansion of the donor pool is essential. The definition of organs that are marginal for transplantation is often considered a well-defined entity. In reality, however, the definition is very different for each organ, and there is incongruence between the various definitions. It is clear and well-justifiable that different organs are considered to be extended criteria organs going by different criteria, since the relevance of one single factor may be very diverse. The assessment of the outcome after transplantation of extended criteria organs, however, needs to be seen and interpreted with reference to the diverse definitions used throughout the years.

ECD Kidneys

The determination of ECD evolved from the term "marginal donors," and much of the literature focuses on criteria for living donors. Since this is not the focus of this article, we will restrict the research to deceased donors and deceased donor organs.

It is well-known that a number of factors may impact the eventual outcome after transplantation. The definition of cadaveric kidney organs marginal for transplantation emerged from prioritization of and preference for factors that may outrank

TABLE 1 | Commonly used "ECD" criteria for the kidney.

Donor condition	Donor age category (years)				
	<10	10–39	40–49	50–59	≥60
CVA+ HTN+ creatinine >1.5 md/dL				x	x
CVA+ HTN				x	x
CVA+ creatinine >1.5 md/dL				x	x
CVA+ creatinine >1.5 md/dL				x	x
CVA+ creatinine >1.5 md/dL					x
CVA					x
HTN					x
Creatinine >1.5 mg/dL					x

CVA, cerebrovascular accident was the cause of death; HTN, history of hypertension.

other contributors in magnitude and simplicity of assessment (11). In the deceased donor situation, the prior medical history is not completely known, and the exact determination of hypertension, for example, often remains incomplete regarding details such as manifestation, duration, or response to treatment. Some of the understanding of donor risk factors also stems from living donors (12), where early definitions of marginal donors relate to the risks for the recipient rather than the risk for the donor, which was only assessed and understood much later. Hence, the definition of marginal or ECD kidneys needs to be used with caution since the definitions used are building on a set of parameters that have not been formally established, validated, and re-validated.

Still, compared to other organ systems in SOT, a comparatively good definition of marginal or ECD has been established. Noteworthily, the term "marginal" should be avoided in favor of ECD, as "marginal" may be considered pejorative by the patients who receive them and also by the programs that transplant them (13).

The ECD criteria most widely used in kidney transplantation are the OPTN-approved criteria, as described by Metzger et al. (13). They are shown in **Table 1**.

A helpful tool that combines such parameters is the Kidney Donor Risk Index (KDRI), which was generated by weighting the following factors into a single number in order to predict the risk of post-transplant graft failure: age, weight, height, race, history of hypertension, history of diabetes, cause of death, hepatitis C status, serum creatinine, and DCD. The association of KDRI with the outcome in the Eurotransplant senior program (ESP) was evaluated by Schamberger et al. (14). Kidney transplantation from Maastricht category-III donors after circulatory death (DCD), which introduces a higher risk of primary non-function and delayed graft function (DGF), has been added to ECD criteria (15). Furthermore, kidneys from a donor with an acute kidney injury before organ procurement correspond to marginal quality (16). Importantly, kidneys from donors with a history of extracorporal membrane oxygenation (ECMO) have not been addressed specifically but can be classified as marginal organs since deteriorated microperfusion can be assumed (17–19).

TABLE 2 | Common criteria defining "marginal" or "expanded criteria donor (ECD)" livers, as well as "ECD DCD" livers.

DBD	
Cardiac arrest (min)	>15
Prolonged hypotensive periods	<60 mmHg for >1 hr
Age (yrs)	>55
BMI (kg/m^2)	>30
HBV	Positive
HBC	Positive
BMI (kg/m^2)	79 (100.0)
Macrosteatosis (%)	>30
Hypernatriemia (mEq/L)	>155
ICU stay (days)	≥ 5 (mechanical ventilation)
Nosocomial infection	Positive blood cultures or pneumonia
Split liver	yes
AST (U/L)	>170
ALT (U/L)	>140
CIT (hrs)	>12
Vasopressor drug requirement	Dopamine dose >10 μg/kg/min or any doses of other amines)
Non-heart-beating	Yes
DCD	
Age (yrs)	>50
BMI (kg/m^2)	>35
Functional WIT (min)	>30
Macrosteatosis (%)	>30

DCD, donation after cardiac death; DBD, donation after brain death; min, minutes; yrs, years; hrs, hours; CIT, cold ischemia time; HBV, hepatitis B virus; HCV, hepatitis C virus; BMI, body mass index; ICU, intensive care unit; AST, aspartate aminotransferase; ALT, Alanin-aminotransferase.

ECD Livers

In liver transplantation, the term "marginal" does not refer to a number of distinct internationally accepted criteria that could characterize a graft, and nor does "ECD." Instead, both terms encompass a variety of factors that have been observed to limit graft quality, but the impact of each of these factors remains to be defined. Therefore, several models for the assessment of the sum of such risk factors have been introduced (20). In this regard, valuable predictive quality of patient and graft survival at 3 months was achieved using the Survival Outcomes Following Liver Transplantation (SOFT) score (21). Death censored graft survival at 60 months follow-up was well-predicted by the Donor Risk Index (DRI; c-index 0.59) and Eurotransplant-DRI (c-index 0.58) (20).

Table 2 lists those criteria that overlap in the mentioned scoring systems and that are widely applied when defining "marginal" or "ECD" liver grafts (22, 23). Apart from clinical and metric parameters, these include histological criteria, since liver biopsies are considered helpful in order to evaluate the organ quality. Whereas, liver fibrosis has been reported to predict a high incidence of early graft failure (24), Liu et al. (25) suggested a macrovesicular steatosis of 30 percent as a cutoff value for marginal grafts.

TABLE 3 | Common criteria defining a "marginal" or "ECD" pancreas.

Age (yrs)	<10/>45 (>50)
BMI (kg/m^2)	>30
Trauma	Yes
Pancreatitis	Yes
Alcohol intake	Yes
DCD	Yes

yrs, years; BMI, body mass index; DCD, donation after cardiac death; ECD, expanded criteria donor.

TABLE 4 | Common criteria defining "marginal" or "ECD" hearts.

Age (yrs)	>40 (32)/>55 (33)
BMI mismatch donor/recipient (%)	>20
HCV	Positive
BMI (kg/m^2)	79 (100.0)
LV hypertrophy (mm)	>14
Ejection fraction (%)	<45
High-dose catecholamine administration	Yes
Tobacco or illicit drug use (cocaine)	Yes
History of diabetes	
Prolonged cardiopulmonary resuscitation	Yes
Transient reversible hypotension or cardiac arrest	Yes

LV, left ventricular; BMI, body mass index; HCV, hepatitis C virus.

Noteworthily, while livers from non-heart-beating donors are *per se* considered as ECD, Mihylov et al. suggested criteria for "ECD DCD" livers in order to subclassify DCD organs (26) (**Table 2**).

ECD Pancreas

In analogy to other organs in SOT, no clear definition exists for a "marginal" or ECD pancreas graft. **Table 3** summarizes the criteria that have been commonly used by various authors (27–30). In an attempt to bring such criteria into a clinically useful context, Axelrod et al. (31) presented a Pancreas Donor Risk Index (PDR). The score builds a prediction model of graft survival on donor factors together with ischemia time and type of transplantation (31).

ECD Hearts

Due to the imminent gap between the supply of donor hearts and the demand for transplantable organs, strategies have emerged to overcome this clinical dilemma. The numbers of left ventricular assist devices as bridge-to-transplant therapy have significantly increased over the last decade. Simultaneously with this trend, marginal or "extended criteria" hearts are routinely evaluated in order to increase the donor pool. While standardized criteria have not been published by societies so far, there is a general consensus when it comes to the definition of marginal donor hearts. The literature on this topic is primarily driven by US data. A Meeting Report by the American Society on Transplantation on adult cardiac transplantation is still lacking a unified formal definition.

TABLE 5 | Common criteria defining standard criteria (SCD), therefore not ECD lung.

Age (yrs)	<55
BMI mismatch donor/recipient (%)	>20
Clear chest X-ray	Yes
PaO_2 (mm Hg)	>300 (FIO2 1.0, PEEP 5 mm Hg)
History of smoking (pack yrs)	<20
Absence of chest trauma	Yes
Absence of microbiologic organisms endobronchial	Yes
Absence of malignancy	Yes
Absence of purulent secretions or signs of aspiration endobronchial	Yes
Negative virology	Yes

yrs, years; BMI, body mass index; FIO2, fraction of inspired oxygen; PaO2, partial pressure of oxygen; PEEP, positive end-expiratory pressure.

Based on the current literature, the factors that should be considered as extended criteria in heart donation are given in **Table 4**.

In order to facilitate a better risk-assessment of allografts, donor profiles have been implemented in Risk Scores. Smits et al. have identified 12 variables associated with donor non-use in the Eurotransplant region and have generated the Eurotransplant Heart Donor Score (32). Based on the UNOS database, a risk score model (UNOS Donor Risk Score) has been developed by Weiss et al. (33).

ECD Lungs

Based on the standard criteria donor lung definitions published in 2003 by Orens et al. (34), one can define an ECD lung if the donor does not fulfill at least one criterion of the SCD criteria suggested by the International Society for Heart and Lung Transplantation (**Table 5**).

MACHINE PERFUSION

The concept of MP was part of organ preservation long before solid organ transplantation (SOT) started to become a clinical reality and routine. With the publication by Collins et al., in which the authors described a method for the transportation of kidneys on ice using a preservation solution with the result that the organs could be shipped in a small box and showed almost no damage after 30 h, the era of static cold storage (SCS) had begun (35). The implementation of SCS led to satisfactory results within the entire field of SOT. However, with the increasing use of organs procured from ECD and DCD, SCS alone is not able to deliver the post-transplant results we aim to achieve for our patients. The lack of oxygen, the continuation of anaerobic metabolism leading to organ-damage and recipient-harming IRI after reperfusion, is significantly pronounced and more detrimental in these marginal donor organs (36).

Figure 1 illustrates the different possibilities for combining all preservation techniques available today clinically for almost all organs: static cold storage (as the golden standard,

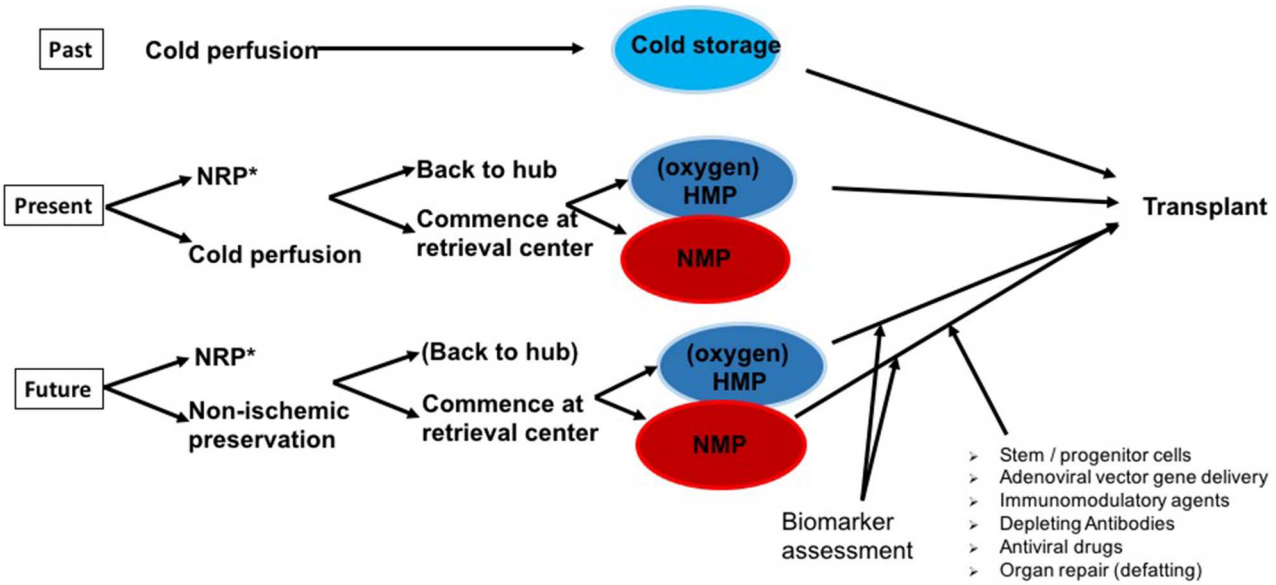

FIGURE 1 | The past, present, and potential (near) future of organ preservation. Whereas, static cold storage (SCS) has been successfully applied over decades with good outcomes in standard criteria donor (SCD) organs, at present, marginal or expanded criteria donor (ECD) organs in particular are increasingly preserved by hypothermic (HMP) or normothermic machine perfusion (NMP) technologies. After initial cold perfusion [*and optimally following normothermic regional perfusion (NRP) in Donation after cardiac death (DCD) organs], machine perfusion is commenced either at the retrieval center or at the transplant center in a "back to hub" approach. The next step is to effectively use the prolonged preservation times of ECD grafts achieved by optimized machine perfusion protocols to characterize ECD organ quality and transplantability via the assessment of biomarkers. To improve graft quality, NMP provides an ideal platform for future immunomodulatory modifications and organ repair.

so far), hypothermic preservation with/without oxygen, and normothermic perfusion. Normothermic regional perfusion (NRP) is mentioned in **Figure 1**, as it has become an important tool for use before the procurement process of DCD starts. The following sections will not present NRP data in particular, as the review is about machine preservation in marginal donor organs. However, good and satisfying results after kidney, pancreas, heart, and liver transplantation were reported by several groups in the United Kingdom, Spain, and France and have been summarized by previous reviews (37–42).

Recently, Ruiz et al. presented a series of 46 livers transplanted after NRP and concluded that their results, including 23% early allograft dysfunction, are superior to standard DCD organ procurements and comparable to donation after brain death (DBD) results (43). The Cambridge group (United Kingdom), clear NRP proponents, commented on this publication in a way similar to our suggestions illustrated in **Figure 1**. The publication by Ruiz et al. (43), like many others before it, did not compare the results to an adequate prospectively organized control group.

It is noteworthy that, only recently, a protocol for NRP was established even for DCD heart transplantation. Due to a restoration of function of the arrested heart, organ assessment via echocardiography, pressure-volume loops, and cardiac-output measurements could be implemented (44).

Accepted criteria for subsequent heart transplantation after weaning from mechanical support are defined as: cardiac index >2.5 L/min/m^2; central venous pressure < 12 mm Hg; pulmonary capillary wedge pressure < 12 mm Hg; left ventricular ejection fraction > 50% in transoesophageal echocardiography.

NRP leads to a meaningful increment of survival benefits and helps to enlarge the available donor pool by utilizing marginal donor organs in a safe way (45, 46). Future trials are needed to compare DCD organ transplantation +/– NRP followed by +/– a combination of preservation techniques (42, 45).

Since 2009, dynamic cold storage—hypothermic machine perfusion (HMP)—has progressed to become clinical routine in several fields of SOT—above all, in kidney preservation. Moers et al. published their landmark paper in the *New England Journal of Medicine* and demonstrated a significantly lowered DGF rate in recipients receiving a hypothermically perfused kidney compared to a renal graft stored on ice in the common way (47). Applying the same technology (HMP without supplemental oxygenation), numerous publications on DCD and ECD kidneys have followed over the past 10 years, showing similar results: ECD and DCD kidneys and their recipients clearly benefit from MP as long as the duration of cold ischemia time (CIT) is reasonable (48–52). During the second half of the recent decade, oxygen as a supplement has been focused on for kidney HMP. In 2016, Jochmans et al. published an overview of ongoing MP trials in kidney and liver transplantation, including those assessing oxygenated HMP (53). One of these trials run by the COPE® consortium (*http://cope-eu.com/work%20programme/trials.html)* has finished recruiting, and 1-year-follow-up results were presented during the American Transplant Congress 2019. This international RCT on oxygenated HMP of DCD kidneys included 197 kidney pairs, of which 106 were successfully hypothermically perfused and transplanted. Approximately 80% of the transplanted kidney pairs were eligible for the primary

analysis and resulted in a similar eGFR at 1 year after transplant for oxygenated (HMPO) and standard HMP. Analysis of all-cause graft failure, however, showed a higher eGFR in HMPO than in HMP. Overall graft loss was significantly lower in HMPO, leading the authors to suggest that HMPO improves 1-year kidney graft function when accounting for the beneficial effect on allograft survival.

In contrast, in the field of pancreas transplantation, dynamic preservation technologies have not yet experienced a breakthrough. Neither hypothermic nor NMP technologies are well-established, and SCS remains the standard procedure. A recent review outlined nine studies on HMP and 10 studies on NMP. All of them were experimental; none of the human pancreatic grafts were transplanted. However, the common conclusion of all of the published articles considered was that IRI, thrombosis, and morbidity after whole organ pancreas transplantation might be reduced by both technologies (54–56).

Liver transplantation can be regarded as the field in SOT that primarily focuses on the development of preservation techniques, currently. The most commonly used perfusion types in the daily routine are HOPE (hypothermic oxygenated perfusion), D-HOPE (d for dual oxygenation via hepatic artery and portal vein), and normothermic liver preservation. HOPE and D-HOPE studies have produced promising results, showing higher adenosine triphosphate (ATP) levels during preservation, less IRI, excellent graft survival after DCD liver transplantation, and a significantly reduced rate of bile duct injuries (57–60). The Birmingham group was the first to publish its experiences on livers undergoing HOPE and NMP consecutively (61). Although such livers were not transplanted, HOPE/NMP livers developed less oxidative injury and inflammation and achieved enhanced metabolic recovery compared to livers undergoing NMP only (61). Liver NMP had its great appearance when the first prospective international multicenter RCT was published in April 2018 (62). The trial on DBD and DCD livers with NMP durations up to 24 h could show a significantly lower level of graft injury, measured by reduced peak aspartate aminotransferase (AST), longer preservation times, and a 50% lower discard rate of organs compared to SCS. Bile duct complications and graft and patient survival were comparable in both groups (62).

NMP has been shown to be beneficial in terms of immediate graft function in all organ types in which it has been explored clinically: this includes the kidney (63–65), liver (62, 66), and the lung (67, 68). The mechanism by which this occurs has not yet been conclusively proven, but the concept is that reperfusion (rewarming and oxygen delivery) in the artificial context of the perfusion circuit is beneficial by allowing recovery of cellular energetics in the absence of the effector mechanisms needed for an acute inflammatory response (as in ischemia-reperfusion).

ORGAN PRESERVATION VS. ORGAN CONDITIONING VS. ORGAN REPAIR

With a new technology, the accompanying terminology is often somewhat unspecific and unprecise in the beginning. This also applies to MP. It is paramount, however, to eventually clarify the terminology and define a uniformity for its correct use. Simply put, no other terminology than organ preservation would be justified at this point in the clinical application, since no targeted means for organ reconditioning (let alone repair) have been established. While this remains a glorious goal for the future, it also represents a significant legal and ethical challenge, since the framework for the treatment of human organs remains to be established. It remains entirely unclear whether extracorporal organ reconditioning and/or repair can be achieved. The hope and the hype are building on a growing body of experimental data that indicates the feasibility of success or organ-specific treatment. While this remains a most significant opportunity for the field, we herein aim to adequately define organ preservation, organ (re-)conditioning, and organ treatment.

Organ Preservation—A Definition

It is interesting to note that no entry exists on organ preservation in Wikipedia (search, 21.08.2019), while a PubMed search yields 17189 entries (*https://www.ncbi.nlm.nih.gov/pubmed/?term=organ+preservation).* It seems to be a term where the definition appears so clear and easy that it does not need to be written down. Yet again, a uniform description of organ preservation is not straightforward since it is not necessarily defined by organ retrieval, temperature, metabolic state, or any other sole condition. The one possible common ground for a definition is the purpose. Putting an organ into a state that allows later reactivation and restitution of its original function with the aim of minimizing damage during the period of mal- and non-perfusion.

One essential limiting factor in the determination of the quality of the preservation method is the poor definition of endpoint for a clinical readout. Primary non-function (PNF) seems relatively well-defined at first glance, but the actual definitions leave room for interpretation. PNF does not indicate no organ function at all but rather a function insufficient to prevent/avoid longer-term organ replacement therapy.

For example, in liver transplantation, according to Ploeg et al., PNF is defined by poor initial function, requiring re-transplantation or leading to death within 7 days after the primary procedure without any identifiable cause of graft failure (69). Such parameters inevitably introduce non-specificity into the definition. The call for the need for re-transplantation and the risk for death are clinician's calls built on medical facts, the interpretation of the physician, and a projection based on the parameters and the experience of the doctor. Since alternatives are insufficiently well-established, studies relating preservation quality to PNF need to be read in the light of this limitation. While PNF is seen as a suitable measure for organ preservation, other factors impacting on the PNF rate need to be considered. The fact that re-transplantation is a risk factor for PNF indicates that the role of recipient factors may be underestimated (70). Further to this, no clinical preservation trial can seriously build on PNF as an endpoint, since the PNF rates reported more recently are in the range of 2–5%. Primary poor function is even more vague in its sufficiency as a definition and clinical endpoint. Very recently, Dutkowski et al. (71) critically reflected on the current parameters used to determine the success of preservation

in liver transplantation trials. While the current practice might be reasonable in the sense that it is building on the combination of parameters currently best established, alternatives are needed but need to be more formally established prior to considering them new clinical standards.

A Brief History

The original vision of organ preservation as formulated in 1813 was built on the continuation of circulation and blood supply (72). The substitution of blood with perfusion solutions led to prolonged successful preservation of tissues, as described by Carrell (73). While prolonged successful preservation of tissues seems plausible according to these early findings, the actual suitability for transplantation remained to be established (72). Successful kidney preservation for 3 to 5 days was achieved by the use of continuous perfusion with cooled, oxygenated blood or plasma (74). Since continuous warm perfusion, oxygenation, and nutrition of an organ during transportation was technically very demanding, the alternative concept eventually surpassed MP in the 1960s. The chemical composition of advanced solutions for hypothermic organ storage resulted in excellent short-term preservation with good outcomes after transplantation. As with any achievement, the good results are a blessing and a curse. With patient and graft survival as the only hard endpoints for clinical trials, the room for improvement became very small—at least with standard criteria organs. The search for superior technologies found a platform for clinical realization only when the results after transplantation became less convincing, with a greater effect on bile ducts as a target for early damage in liver transplantation (72, 75–77). The organ shortage, together with the push to use lower-quality organs and organs with additional damage as a result of DCD, triggered the clinical realization of MP in liver transplantation.

Current Situation

The current status of organ preservation remains largely defined by SCS. The well-established and standardized procedures of organ storage in three sterile plastic bags filled with preservation solution (bag 1) or saline/ringer lactate/other (bags 2/3) is well-established, with a relatively good understanding of the risk accumulating with the duration of preservation. The major advantage of SCS is the safety of the procedure. Pretty much nothing can happen to an organ in plastic bags, on ice, in a plastic container. The major limitations of this technique relate to the fact that metabolism continues—even though at a very low rate—eventually resulting in the accumulation of succinate and reactive oxygen species (ROS) (78–81). The immediate exposure to normothermia and oxygen results in an inflammatory response and organ-specific damage early after transplantation. The mechanisms responsible for the eventual organ damage not only in organ transplantation but also in other diseases such as cardiac infarction and stroke are well-established but not are the focus of this review. The key question in the consideration of NMP as an alternative to SCS is how effectively the mechanisms leading to an IRI are interrupted through either immediate NMP or NMP following SCS. While preclinical evidence (81) and the phase II/III trial by Nasralla et al. (62) have delivered evidence suggesting that NMP is superior to SCS when applied immediately, the actual net benefit of NMP following SCS remains to be more clearly established. While a key elements in such assessments are the proper definition of and agreement in the community on suitable endpoints regarding the preservation quality, the accompanying benefits and alternative endpoints should not be overlooked. The major benefit of NMP vs. SCS is the time gained with (as it appears) a relatively safe prolongation of preservation times under close to physiological conditions. The ability to monitor the viability and function of the organ adds important parameters for decision making and significantly ameliorates the logistical challenges in transplantation. The benefit of transforming transplantations into scheduled procedures has the potential to significantly enhance safety but also training and teaching in transplantation.

Organ Reconditioning

The term organ reconditioning is used in a rather unspecific manner in our field. According to Wikipedia, reconditioning means "to restore to a functional state, or to a condition resembling the original" (*search "reconditioning," September 2019*). The way this can be read in reference to human organs is that no intervention aiming beyond the restoration of the condition of an organ prior to retrieval (a) or beyond the optimal (naïve) condition of a healthy human organ (b)—depending on how the term "original" is interpreted in this context—shall be included. These two possible views already point toward very different states of an organ. The way the term was used in the recent scientific transplant literature is in agreement with repair of the immediate injury prior to and during organ retrieval and storage. The key element of reconditioning/repair as clinically applied at this point relates to the prevention of an eventual ROS burst by mitochondria and subsequent disruption of ATP production, mitochondrial permeability transition pores, and danger-associated molecular pattern (DAMP) release upon reperfusion. An electron shift toward succinate and subsequent reverse electron transfer upon reperfusion is discussed as a mechanism of damage and target of therapy in liver HMP (82). NMP is suggested to mitigate post-reperfusion hyperfibrinolysis (83) and inflammation (84). While these may be indirect effects of NMP, the actual immediate therapeutic mechanisms (if any) may be glycogen repletion and ATP regeneration (85). Whether these mechanisms are a simple effect of the restoration of close to physiological conditions or specific for NMP remains to be established. It is likely, however, that the benefit of NMP is mostly defined by the replacement or shortening of SCS and the indirect effects on logistics and preparation, organ monitoring, and decision making. Further to this, NMP may emerge as a platform enabling organ treatment, while it may not have a therapeutic/reconditioning effect *per se*.

Organ Repair

Human organ repair, apparently, is treated as an equivalent to organ regeneration (*Google search "human organ repair," September 2019*). While the common understanding of organ repair might be more mechanistic and that of organ regeneration more biological, the distinction between the terms seems vague.

Albeit experimental, therapeutic interventions in lungs seem most closely approaching possible clinical implementation (86).

Bronchoalveolar lavage, surfactant replacement, and alveolar recruitment maneuvers seem to positively affect organ tissue morphology and organ function. A hurdle to clinical application may be the legislation. Directive 2004/23/EC of the European Parliament and of the Council "on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells" largely focusses on tissues and not organs but represents an important reference. Since preservation times can be prolonged with the technologies being developed and urgency may no longer serve as justification for immediate processing (transplantation) without quality assurance, the legal framework for tissues and cells could be applied to organs. The second element of the legal consideration relates to the element of organ therapy. Inevitably, the issues of responsibilities and possession require clarification for attempts to introduce *ex vivo* human organ treatment into clinical application. As long as an organ is labeled as discarded because it seems unsuitable for immediate transplantation, the organ will not be allocated to an individual, and hence considerations of possession and responsibilities become less demanding. Once organs possibly suitable for immediate transplantation have been treated for reasons such as quality improvement or possibly organ reconditioning, the allocation process may be completed and the future recipient indicated. If and how an individual can consent to interventions and/or a trial involving organs will eventually require clarification.

Directive 2010/45/EU of the European Parliament and of the Council of 7 July 2010 on standards of quality and safety of human organs intended for transplantation (*https://eur-lex.europa.eu/eli/dir/2010/53/oj)* does not provide much guidance regarding this issue.

MACHINE PERFUSION IN CLINICAL PRACTICE

SCS is a simple, safe, and cheap method to preserve and transport organs, and it has been successfully applied over decades with good outcomes in SCD organs. However, to date, the large discrepancy between patients on the waiting lists and available donor organs has forced the transplant community to cope with the demand by pushing the limits and expanding the donor pool by accepting increasing numbers of marginal organs from ECD and reestablish DCD programs (87). Importantly, ECD and DCD organs have been identified to be particularly prone to IRI. Although SCS can decelerate degradation of a preserved organ, especially in high risk or marginal organs, limited applicability is the consequence (36, 78). Attempting to overcome this issue, several MP technologies have been developed and now find their way into clinical practice in SOT.

HMP of the Liver

Liver HMP has been translated from the experimental state to clinical reality over the last decade. In 2010, the first phase I prospective cohort study was published, reporting on 20 patients who underwent liver transplantation of grafts preserved by non-oxygenated HMP. Outcomes were compared to a matched SCS control group. HMP time ranged from 2 to 7 h, with a total CIT below 12 h. Except for a significantly lower peak serum AST in the HMP group, no further significant differences were observed in regard to PNF, early allograft dysfunction, or graft and patient survival. However, clinical feasibility, as well as non-inferiority to SCS, was proven, making way for further studies (88). Five years thereafter, the same group showed promising results by transplanting 31 ECD livers declined by the United Network for Organ Sharing (UNOS) region and preserved under hypothermic dynamic conditions at 4–8°C. Compared to well-matched SCS controls, significantly fewer biliary complications and shorter hospital stays were observed. This study represents a landmark, since the effectiveness of MP was shown within the most susceptible livers in the donor pool (89). The impact of HMP on marginal organs was further investigated by Dutkowski et al. In an international matched-case analysis, 25 DCD livers were subjected to HOPE and compared to matched SCS DCD livers. HOPE resulted in a significant reduction of ischemic cholangiopathy (HOPE: 20% vs. SCS: 46%) as well as a significant improvement of 1-year graft survival rates (90 vs. 69%) (58). The beneficial effect of HMP on DCD grafts was confirmed by a prospective case-control liver transplantation study, comparing 10 grafts with at least 2 h of DHOPE to 20 grafts without. An 11-fold increase in cellular ATP during oxygenation was documented. In addition to good early graft function, both 6-month and 1-year graft survival were 100%, while 6-month graft survival and 1-year graft and patient survival in the control group were 80, 67, and 85%, respectively (57). Moreover, the same group could demonstrate an attenuation of IRI-associated bile duct damage after transplantation of DCD end-ischemic DHOPE liver grafts (90).

HOPE in particular has evolved to become a widely used technology, showing excellent longer-term results. Schlegel et al. provided data on 5-year outcomes of patients receiving DCD livers preserved with 1–2 h of end-ischemic HOPE ($n = 50$) and compared them to the equal numbers of DCD liver transplants without HOPE or DBD liver transplantations. Five-year outcomes of HOPE-treated DCD liver transplants were similar to those of DBD primary transplants and superior to those of untreated DCD liver transplants (HOPE DCD 94% vs. SCS DCD 78%) (91).

NMP of the Liver

Preserving an organ under physiological conditions is the ideal condition to prevent deleterious effects of ischemia as well as IRI following SOT. Similar to HMP, NMP has recently been implemented in clinical routine at a variety of centers. The transfer from the experimental stage to clinical application was first described by Ravikumar et al. in 2016. In this phase 1 (first-in-man), non-randomized prospective trial, short-term outcomes of 20 recipients of NMP-perfused donor livers were matched in a ratio of 1:2 to cold-stored livers. In the study group, livers were preserved under normothermic conditions over the entire preservation period (ranging from 3.5 to 18.5 h). Except for a significantly reduced peak serum AST in NMP-liver recipients,

no differences regarding short-term recipient survival, PNF rate, or early allograft dysfunction were observed. Therefore, the safety and feasibility of this dynamic preservation method could be confirmed, opening the field for further clinical investigations with major implications on logistics (66).

Feasibility and reproducibility following transplantation were further underlined in a U.S. phase I non-randomized pilot study (92). However, this trial also pointed out that NMP of an organ is not a trivial procedure and runs the risk of graft loss if human/technical errors occur. Out of 10 procured livers, one organ had to be promptly discarded due to an unrecognized portal vein twist. The remaining nine organs (four DCD Maastricht class III, 5 DBD) were successfully transplanted, and outcomes were comparable to matched control liver transplant recipients of SCS grafts. The proof of concept and feasibility gave way for larger-scale trials.

Nasralla et al. were first to perform RCT to test the efficacy of NMP vs. SCS. After informed consent had been obtained from the recipient, the allocated liver was either randomized to SCS or NMP. NMP was performed over the entire preservation period. Outcomes of 121 NMP-graft recipients were compared to outcomes from 101 well-matched SCS-graft recipients. The key findings of this study were a significantly reduced peak serum AST, a significant reduction of post-reperfusion syndrome, and a 72% lower adjusted odds rate of developing early allograft dysfunction (EAD) in the NMP liver recipients compared to controls. Furthermore, NMP of the liver graft resulted in increased organ utilization with a 50% lower discard rate in this group (62).

The additional positive effect of NMP is the "almost" physiological aspect of this method, which opens up new possibilities by means of organ assessment and organ selection. This fact was a matter of investigation in an observational study, where livers considered unsuitable for transplantation as well as highest risk liver were included. In total, 47 livers were biochemically assessed during the preservation, resulting in 39 liver transplants. Remarkably, two out of 19 livers deemed unsuitable were transplanted (93).

In summary, both HMP/HOPE and NMP are accepted and clinically established preservation methods, particularly interesting in the preservation and preconditioning of high-risk organs. Especially in this organ category, including ECD and DCD livers, they have both been shown to be superior to SCS. Whether HMP/HOPE or HMP is superior to the other is still a matter of debate since the two methods have not been correlated with each other in clinical settings, and further RCTs are needed.

HMP of the Kidney

The amount of clinical evidence for dynamic preservation of kidney grafts is rapidly increasing. Moers et al. were first to perform an international RCT on hypothermically perfused kidneys for transplantation. One kidney from 336 deceased donors was randomly assigned to HMP, and the other to SCS. A reduced risk of DGF and improved graft survival in favor of MP-preserved organs was shown (47). These promising results were confirmed in a subsequent RCT from the Eurotransplant region. Jochmans et al. investigated the efficacy of HMP in preserving DCD kidneys. Similar to Moers et al., DCD kidneys were assigned in a 1:1 match to HMP or SCS, resulting in a total of 164 kidney transplants (82 HMP vs. 82 SCS). HMP resulted in a significantly reduced DGF rate as well as better early graft function after 1 month compared to SCS. However, 1-year graft survival was comparable in both groups (49).

Furthermore, in another RCT, kidneys were either preserved by HMP or SCS. In accordance with Moers as well as Jochmans, the primary end-point was the occurrence of DGF. Secondary endpoints were primary non-function as well as graft survival. Both the risk of DGF and the incidence of non-function were significantly reduced by HMP in their cohort. One-year graft survival and function could be improved by dynamic cold graft preservation (52). Despite these findings, the importance of different factors such as the impact of CIT and the combination of CIT with HMP have to be mentioned. CIT is a known independent risk factor for DGF. In order to investigate which kidney graft may further benefit from HMP prior to transplantation, Kox et al. analyzed prospectively collected data from the "Machine Perfusion Trial." A total of 752 renal transplants were included with 376 dynamically preserved kidneys and 376 statically preserved grafts. They identified CIT as an independent risk factor for DGF. Furthermore, HMP did not prevent DGF if CIT exceeded 10 h (51).

The optimal timepoint for, as well as the optimal duration of, HMP is still a matter of debate. HMP can be used either as a primary preservation method or in the end-ischemic phase as a reconditioning tool. Matos et al. were able to show a faster recovery of renal function if grafts were subjected to HMP prior to transplantation after a mean SCS period of 22 h. MP time was at least 6 h. However, this beneficial effect was limited to donors under the age of 50, who represent a rather selective group (94). Further studies followed, indicating a beneficial effect of MP in the end-ischemic phase by displaying a reduction of DGF, as well as an improved organ acceptance. This last positive trend is attributed mainly to the possibility of organ monitoring (95, 96).

NMP of the Kidney

NMP of the kidney may eventually resolve many issues related to damage induced by ischemic phases. The idea of preserving kidney grafts under almost physiological conditions with the possibility of real-life assessment of the graft prior to transplantation sounds intriguing. However, while the number of preclinical studies using animal models as well as discarded human kidneys is steadily increasing, evidence for NMP of the kidney in a clinical setting is rather poor. Hosgood et al. were first to transform preclinical experience into clinical reality by transplanting a 62-year-old ECD kidney into a 55-year old recipient. After 11 h of SCS, the graft was perfused with a plasma-free red cell-based solution at 33.9°C for 35 min prior to transplantation. Although a slow graft function was observed, the patient remained dialysis free with a serum creatinine level of 132 μmol/L at 3 months post-transplantation. In contrast, the recipient of the opposite kidney developed DGF (64). Based on this first success, the Leicester group translated NMP into clinical reality on a larger scale. Eighteen ECD kidneys were subjected to NMP reconditioning prior to transplantation. The average NMP

time was prolonged to 63 min. Outcomes were compared to 47 matched ECD kidneys preserved by SCS. In this study, NMP resulted in a significantly reduced DGF rate compared to controls (NMP: 5.6% vs. SCS: 36.2%). However, no differences could be observed in regard to 1-year graft or patient survival (97).

Taken together, HMP of the kidney has been shown to have its place, especially in the preservation of marginal grafts, including grafts from DCD and ECD, with superior outcomes in the early phase compared to SCS. However, CIT is still the limiting factor influencing short- as well as long-term outcomes. In contrast, although the amount of preclinical data is rapidly increasing, NMP is still in its early phase. However, feasibility and non-inferiority to SCS have been described at this early stage, and multicenter RCTs are planned.

Machine Preservation of the Pancreas

In contrast to the dynamic preservation of liver and kidney, pancreatic MP is challenging. This is attributed to the physiologic as well as the anatomical characteristics of the pancreatic graft (98). MP of pancreatic grafts poses major challenges due to the susceptibility of the organ to IRI-associated alterations, including acinar necrosis, edema formation, and endothelial disruption. These factors in particular are recognized risk factors for early graft pancreatitis and thrombosis, eventually resulting in graft loss (98, 99).

In contrast to the liver as well as the kidney, the pancreas is characterized by a low-flow and low-pressure environment, which impedes the direct translation of the existing perfusion machines for use in dynamic pancreas preservation (100). However, the feasibility of pancreatic HMP has been demonstrated in several experimental settings involving the preservation of porcine, dog, and discarded human pancreas grafts (55, 101, 102). Although results are promising in this experimental setting, clinical translation is still pending.

Therefore, assessment and evaluation of a pancreatic graft prior to transplantation would be of the utmost importance, since changes in donor demographics result in an increased need for the acceptance of higher-risk grafts at high-volume centers. However, MP of pancreatic grafts must still be considered experimental at this stage.

Machine Preservation of the Lung

Due to pre- and peri-retrieval management and direct examination of the donor organ by the explant surgeon, some extended criteria organs can be directly used with acceptable risk for the recipient, but a notable number are rejected during the retrieval process. There is still a lack of clear decision criteria for lung suitability for transplantation or attempts at lung reconditioning using an *ex vivo* lung perfusion (EVLP) (103). Needless to say, MP could improve organ availability, which is critically needed in the face of a waitlist mortality that is reported to be up to 30% depending on the allocation system (104).

Importantly, *ex vivo* prolonged lung preservation resulted in successful transplantation of high-risk donor lungs (105), and MP is now indispensable for evaluating lung graft quality from an uncontrolled DCD (106). Very promising data have come from experimental EVLP studies, which are outlined later in this article.

Machine Preservation of the Heart

At present, SCS of donor hearts remains the standard practice in most transplant units, whereas MP is limited to a few centers in the US and Europe. To date, only one device is available for clinical use—the Transmedic Organ Care System (OCS) Heart. At mild hypothermia (34°C), the system uses a combination of donor blood and a proprietary solution as perfusate for the heart. In 2014, heart NMP has led to the first distant procurement DCD heart transplantation. To date, over 100 DCD heart transplants have been performed using a cardiac perfusion system (107), and MP of hearts exhibited safe preservation in RCTs (108).

EX VIVO MONITORING OF ORGAN FUNCTION AND QUALITY

Currently, the decision as to whether organs are suitable for transplantation is determined on the basis of more or less subjective empirically established clinical parameters that have been shown to be associated with an increased rate of early allograft dysfunction or graft failure. Parameters such as donor past medical history, last known laboratory values, findings during procurement, and other procurement variables such as expected ischemia times primarily determine the acceptability of a graft. These parameters include prolonged warm ischemia time (WIT) >30 min during DCD, prolonged CIT, and parenchymal alterations within the graft (e.g., steatosis, fibrosis, arteriosclerosis). Viability testing and functional assessment prior to transplantation are likely to extend the utilization of suboptimal or marginal organs (109–111).

HMP is an alternative method to standard SCS for the preservation of organs. For different types of kidney grafts, HMP offers superior preservation compared with SCS. In terms of graft quality assessment during HMP, some biochemical parameters of the released perfusate and hydrodynamic parameters are found to independently correlate with the outcome—a finding that may help clinicians in their decision making.

Increased Glutathione S-Tranferase (GST), N-acetyl-beta-D-glucosaminidase (NAG), heart-type fatty acid-binding protein (H-FABP), or lactate dehydrogenase (LDH) concentrations during MP may serve as indicators for suboptimal graft quality. Furthermore, levels of redox-active iron measured in the perfusate have been correlated with DGF rates (112–114).

Moreover, the measurement of vascular resistance during MP represents an additional and objective source of information that can assist clinicians in their decision-making process. High vascular resistance as a hydrodynamic parameter has been shown to be associated with an increased risk of DGF development and predictive for 1-year graft failure (115–117). Taken together, to date, increased vascular resistance and high injury marker concentrations in the perfusate are risk factors for DGF and helpful parameters, but they are not accurate enough to justify a decision for discard based on their interpretation alone (112–114, 117).

Concerning kidney NMP, a scoring system has been developed by Hosgood and Nicholson. The decision on whether to transplant an NMP-kidney was based on macroscopic appearance, renal blood flow, and total urine output during NMP. The authors conclude that, currently, a high percentage of retrieved kidneys are being unnecessarily discarded (80, 118).

Compared to the kidney, data on liver monitoring during HMP remain scarce, although recent publications suggest promising parameters. A correlation between the AST levels in the perfusate during HMP and the peak of AST after liver transplantation has been described. Furthermore, during HMP of porcine livers, a cumulative release of Fatty Acid- Binding Protein 1 (FABP) or AST may be represented by a linear or logarithmic equation, respectively. Each equation is characterized by a b-coefficient that is able to discriminate between livers likely to fail. Similarly, during HMP of human livers discarded for transplantation, AST release in the perfusate could discriminate between livers suitable for transplantation and unsuitable ones. More sophisticated methods to determine graft viability include evaluation of ATP content assessed by magnetic resonance imaging and spectroscopy (89, 91, 119–121). In this regard, van Rijn et al. used cellular ATP content during oxygenated dual (arterial and portal perfusion) HMP as a viability marker for liver grafts. Cellular ATP content correlated with biochemical function early after transplantation (57).

Furthermore, there is evidence that a decline in arterial flow in a pressure-controlled system of MP can be used as a marker for decreasing graft viability. However, in general, it cannot be advised to use flow values as an indicator of liver damage and viability during human liver MP (61, 91, 122).

Concerning NMP of liver grafts, this technology has been applied with promising results for utilization and outcomes (62, 66, 92, 123–126), and the option for the assessment of liver viability during NMP has been highlighted. Imber et al. (127) suggested that bile production is directly associated with liver viability. Matton et al. (128) were able to show that a biliary bicarbonate concentration greater than 18 mmol/L has a high negative predictive value in terms of histological bile duct injury. Moreover, biliary pH greater than 7.48, biliary glucose concentration less than 16 mmol/L, bile/perfusate glucose concentration ratio less than 0.67, and biliary LDH concentration less than 3,689 U/L may serve as indicators for high biliary viability. Watson et al. (93) published their experience of transplanting declined livers following NMP and graft viability assessment. They observed that NMP resulted in a reduced incidence of post-reperfusion syndrome and described biliary pH as a predictive marker for post-transplant cholangiopathy. Perfusate lactate and transaminases and bile production during NMP were suggested by Op den Dries et al. (129) to serve as viability markers. Recently, Mergental et al. (130) published viability criteria (applicable within 3 h of perfusion) for livers considered suitable for transplantation. These include lactate clearance, bile production, PH- homeostasis, and stable pressure and flow dynamics of the graft.

There is growing evidence that hepatic ATP content may serve as a viability marker. In clinical studies, hepatic ATP levels prior to organ retrieval or during preservation correlated with primary liver function after transplantation. Bruisma et al. showed that differences in mitochondrial respiration and restoration of cellular ATP contents are possible viability markers in the setting of NMP. Moreover, NMP offers the option to perform dynamic metabolomic analyses in both bile samples and sequential liver biopsies (111, 131).

However, although these results seem impressive, none of the above-mentioned viability markers have been validated in larger studies, and the small numbers of patients included in the studies make it difficult to draw robust conclusions.

Regarding a potential viability assessment of human pancreas grafts during HMP, flow indices and histological assessment of duodenal and pancreas-parenchyma biopsies throughout the perfusion duration have been proposed. Branchereau et al. described an absence of edema, a decrease in resistance indices, and normal staining for insulin, glucagon, and somatostatin as markers for good graft quality (56, 101). Barlow et al. suggested the assessment of amylase levels in the perfusate, fat infiltration (detected using standard histology), and exocrine pancreas function as criteria to assess graft viability during NMP (55).

Concerning heart transplantation, organ assessment during NMP mainly relies on metabolic parameters and measurements of lactate levels in the aortic root and pulmonary artery. In the PROCEED II trial, three hearts were discarded due to rising total perfusate lactate concentrations. Histopathological examination showed myocardial injury (necrosis, hemorrhage, scarring) or left ventricular hypertrophy in those organs (108). Calculations of pressure volume loops in the left and right ventricle have been described by switching the heart into a "working mode" during NMP. Based on pressure-volume (PV) loop data, end-systolic elastance could be calculated as a parameter for ventricular contractility (44, 132). Due to complicated clinical interpretation and difficulties in reproducibility, "working mode" has been removed from the OCS perfusion module. Contrast echocardiography or intravascular ultrasound of coronary arteries might emerge as tools for anatomical and mechanical assessment during NMP but have not been implemented in clinical routine so far.

In EVLP, assessment of the organ relies primarily on functional and macroscopic parameters, as has been reviewed recently (103). Adequate gas exchange, stable hemodynamic and ventilatory parameters, macroscopic evaluation (absence of oedema), bronchoscopy (absence of purulent secretion and erythema of the bronchus), and deflation after endotracheal tube disconnection (collapse test) during the evaluation period are the commonly used decision criteria for acceptance of the lung. Yet these criteria are not standardized and differ between transplant centers (103). As physiological parameters are in the lead for decision making in EVLP, clinical single-center studies assessed different biomarkers and metabolic parameters of the perfusate or the bronchioalveolar lavage to predict organ acceptance and occurrence of early prostaglandin D3 (PGD3). Machuca et al. (133) showed that high levels of endothelin-1 (ET-1) and Big ET-1 measured in perfusate samples have prognostic value for decline in the lung because of subsequent development of poor physiological performance. Subsequently, the same group suggested IL-8 as a powerful predictor for early graft dysfunction

(134). Furthermore, IL-1β and IL-8 levels measured in the perfusate during EVLP were reported to inversely correlate with the recipient oxygenation 24 h post-transplantation (135).

Interestingly, given the observation that perfusate IL-1β and TNF-α at 30 min during EVLP were effective markers for differentiating between in-hospital survival and non-survival post-transplant, blocking the IL-1β pathway during EVLP might reduce endothelial activation and subsequent neutrophil adhesion on reperfusion (136).

IMMUNOLOGICAL ASPECTS OF (MARGINAL) ORGANS DURING MACHINE PERFUSION

The definition of marginal organs *per se* is a rather arbitrary one. The most commonly used is the dichotomy between standard criteria (SCD) and extended criteria donors (ECD), which is simply discriminated by age and three donor criteria for the DBD (and, increasingly, DCD) kidney cohort (13, 137, 138). However, in clinical routine, it is not always straightforward to decide if an organ is within "standard criteria," as there are plethora of factors interfering with organ quality, i.e., estimated duration of CIT, (functional) WIT, or the grade of steatosis in liver and pancreas, just to mention a few.

When we focus on the donor age only, a vast amount of literature exists. There is evidence that the immunogenicity of elderly organs is increased, as it is believed that aging in combination with injuries (i.e., brain death) induces a pro-inflammatory environment, which could lead to an activation of innate and adaptive immune responses (139–141). Aging influences cellular repair mechanisms, and it is known that the number of MHC molecules present on the cellular surface is increased by aged parenchymal cells (139). In experimental studies, transplantation of elderly organs was associated with a more powerful early immune response compared to transplantation of younger ones. Recipients of such old grafts presented with a higher concentration of effector/memory T-cells with an increased alloreactivity, leading to acute rejection, which results in a particular high burden of damage for the older donor organ (140). Elderly organs do not have the same repair mechanism capabilities, so the clinical damage of acute rejection leaves a more severe defect, explaining the increased rates of DGF and/or graft loss in this donor cohort (140, 142).

An alteration of immunogenicity in aging organs could be one reason to focus on preservation techniques (143) such as HMP and NMP to learn more about the process or even to (re-)condition an organ for reperfusion in the recipient. Due to the fact that the reconstitution of a near-physiological environment might work best to study immune cells, the following paragraphs will bring NMP into focus.

Human organs are equipped with a sophisticated resident immune system. The kidney, for example, not only hosts tissue-resident macrophages (144); the organ also harbors lymphocytes, innate lymphoid cells, natural killer (NK) cells, natural killer T (NKT) cells, and γδ T cells (145). The residential immune cells of human organs remain in a balanced homeostatic state unless they are stimulated—by the insult of brain death in an organ donor, for example—when an immunological activation might be induced. Such a response to a significant injury like brain death in an organ donor includes both local and systemic inflammation, comprising cellular infiltrates and a cytokine storm (146). With the current gold-standard preservation technologies such as SCS, the organs will be transplanted unchanged, in an immunologically activated status, into recipients in whom alloantigens will trigger another immune response immediately after reperfusion, presented by donor-derived antigen presenting cells to recipient T cells (147, 148). How important donor-derived leukocytes are in the allorecognition and rejection processes was demonstrated by Lechler and Batchelor years ago. They impressively showed that placing a rat kidney into an intermediate recipient before implanting it into the final recipient could significantly prolong allograft survival. These experiments demonstrated that "clearing" the organ of dendritic cells (DC), the "passenger leukocytes" responsible for activating recipient T cells, by using the intermediate rat led to the desired success (149, 150). "Parking" the organ in an interim host is not a feasible strategy for implementation in the clinical routine. However, NMP offers the possibility to represent the intermediate recipient as a conditioning tool to achieve the same end result, as previously published by Lechler et al. (150). An *ex vivo* setting might provide not only a more physiological preservation method but also the ability to assess the organ prior to transplantation and to investigate its immunological characteristics in isolation (42).

One of the leading groups analyzing marginal and SCD organs after *ex vivo* perfusion is the Manchester group around Fildes and Stone. Already in 2015, they compared the clinical outcome of patients transplanted with marginal donor lungs after undergoing EVLP compared to standard transplantation of acceptable lungs (151). Despite having transplanted marginal lungs, there was no significant difference in the clinical outcome up to 12 months after transplantation concerning the overall incidence of acute rejection and the number of treated infection episodes (151). This work was followed by a study on passenger leukocyte migration from donor lungs into the recipients and the effects of donor leukocyte depletion prior to transplantation using EVLP (152). In this experimental work using a porcine model, Stone et al. illustrated that donor leukocyte transfer into the recipient was reduced by EVLP and therefore reduces direct allorecognition and T-cell priming (152). The same group transferred their experience to an *ex vivo* NMP model to analyze donor-derived leukocytes in a porcine renal transplantation model. Stone et al. were able to demonstrate that an inflammatory cytokine storm and the release of mitochondrial and genomic DNA were initiated in the NMP circuit prior to transplantation. However, the renal function was not impacted at all after transplanting those organs. They suggest that NMP could be used to immunodeplete and to saturate the capacity of inflammation of donor kidneys before transplanting them (152). Amin et al. (153) evaluated the impact of a post-SCS-preservation flush on the inflammatory burden of a limb allograft (a porcine model for vascularized composite allotransplantation). The venous effluent

after flushing the limbs following either 2 or 6 h of SCS comprised a large population of viable leukocytes, significant concentrations of pro-inflammatory cytokines and mitochondrial DNA. These results support the hypothesis that flushing or dynamically preserving the organs prior to transplantation impacts the inflammatory burden otherwise transferred to the recipient upon reperfusion unchanged (153).

Liver NMP has recently been implemented as a standard procedure in the United Kingdom and been shown to improve organ utilization and post-transplant outcomes following phase I and phase III prospective randomized trials (62, 66). Jassem et al. (84) transplanted, compared, and analyzed 12 NMP-preserved livers against 27 SCS-preserved livers to assess the impact of NMP on IRI, necrosis, platelet deposition, neutrophil infiltration, and the degree of steatosis after NMP or SCS pre- and post-perfusion. Their results showed that, with NMP, there were altered gene-expression profiles of liver tissue from pro-inflammation to regeneration, reduced numbers of interferon gamma (IFNγ)- and interleukin-17-producing T cells, and an enlarged pool of regulatory T cells. In addition, NMP liver tissue was less necrotic and apoptotic compared to SCS-preserved livers, with less neutrophils infiltrating the periportal area (84).

Overall, with the clinical and experimental evidence gained so far for the field of SOT, dynamic preservation methods - and NMP in particular - provide the means to introduce organ conditioning so as to lower immunogenicity as well as pro-inflammatory markers while ensuring the promotion of graft regeneration.

IMMUNOMODULATION DURING MP

Machine perfusion (MP) offers a previously non-existent link between basic and clinical science. The opportunity to treat an organ during preservation creates a new field of translational research. In this context, one of the main targets that can now be addressed is the modulation of the immunogenicity of a specific graft. In theory, such immunomodulation could allow the obviation of lifelong immunosuppression or even forge a path to the holy grail in transplantation—the achievement of tolerance. Importantly, MP offers a platform to apply immunomodulatory strategies that have been elucidated in *in vitro* or preclinical models in the past.

In the existing literature, three major such strategies have been investigated, which are discussed herein. One of the most promising is the application of stem or progenitor cells, with the potential to suppress immunogenicity and help repair injured tissue. Another strategy is the administration of anti-inflammatory/immunomodulatory drugs and agents, and, finally, gene transduction by adenoviral vector gene delivery.

The majority of studies have been carried out under normothermic conditions. However, oxygenated perfusion of a graft *per se* seems not only to protect against preservation injury but also to downregulate the immune system and blunt the alloimmune response, as was shown in a rodent model of hypothermic oxygenated perfusion (HOPE) (154). In particular, the constant flow of fluids in the vessels during MP is regarded to promote the expression of vasoprotective endothelial genes, alleviating the microcirculatory failure associated with ischemia-reperfusion injury (IRI) (155–157). On the other hand, when it comes to additional modification, it is perfusion under normothermic conditions that seems to represent the ideal platform, since NMP grants a physiological metabolic state (81, 158–160).

Many of the ongoing studies addressing the field of immunomodulation during MP are carried out in a porcine setting, as pigs have appropriate size and anatomy as well as immunologic characteristics (161). Whereas, much of our knowledge on organ preservation is derived from various animal studies (162), the porcine model above all has now evolved as an ideal model, making *ex vivo* porcine organ perfusion models a suitable platform for translational transplant research. According to a recent review, in 2017, 22 articles discussed *ex vivo* porcine organ perfusion within the context of transplant preservation surgery (162), but the number of articles has steadily increased since then. However, also in this setting, it is important to highlight potential limitations when translating experiences between species (158).

Stem and Progenitor Cells

Several candidate cells have been investigated, including stromal mesenchymal cells (MSCs), induced adult pluripotent stem cells, fetal stem cells from placenta, membranes, amniotic fluid, and umbilical cord, and hematopoietic cells (163). Among these, MSCs have been reported to represent the most promising cell subset. MSCs are multipotent cells that are found in adult tissues, including adipose tissue and bone marrow, where they support function and repair. Importantly, they have been shown to abate immune and inflammatory responses via the release of paracrine effectors (164, 165).

This circumstance has led to a broad application of MSCs in a variety of pathologies. Therefore, a prerequisite for research on MSCs is the agreement on criteria that define these cells and allow comparability between studies. The International Society for Cellular Therapy has established the minimum criteria that a cell must meet to be considered an MSC: first, an MSC must be plastic-adherent when maintained in standard culture conditions; second, an MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules; third, an MSC must differentiate into cell types of mesodermal origin *in vitro* (166, 167).

The fact that MSCs are known for their potent anti-inflammatory and regenerative capacities, combined with the finding that a therapeutic effect can be achieved with either autologous or allogeneic MSCs (168), has led to the conduction of several studies investigating whether their application is feasible in the context of MP. However, the detailed mechanisms by which MSCs exert their anti-inflammatory and regenerative potential have not yet been depicted. The main mechanism of action is supposed to rely on secreted mediators. Therefore, an appropriate timeframe is likely to be required for these cells in order to mediate beneficial effects (169). This hypothesis is underlined by the findings of a recent study in a porcine *ex vivo* lung perfusion (EVLP) model. Twelve-hour NMP of DCD lungs with intravascular delivery of MSCs (150×10^6) resulted in reduced levels of interleukin-8 (IL-8), a pro-inflammatory

cytokine associated with reperfusion injury, along with increased levels of vascular endothelial growth factor (VEGF) (170). The fact that IL-8 was suppressed is of particular note, since MSCs are known to produce the anti-inflammatory cytokine IL-10, which in turn has been reported to significantly suppress the production of IL-8 in a dose-dependent manner (169).

In human kidneys, Brasile et al. demonstrated actual renal regeneration mediated by MSCs under 24 h of *ex vivo* normothermic perfusion. The authors used an exsanguinous metabolic support (EMS) tissue-engineering platform to study five pairs of kidney allografts from DCD donors. Whereas, one kidney of each pair solely underwent perfusion, the partner kidney was EMS perfused with MSC (1×10). Indeed, a reduced inflammatory response was observed along with increased synthesis of adenosine triphosphate and growth factors and a normalization of the cytoskeleton and mitosis (171).

Even though these findings are promising, more insight needs to be gained regarding the effects of the NMP milieu on MSC themselves and the comparability between human and porcine data. These aspects were highlighted in a kidney NMP model, showing that while the suspension conditions reduced the viability of porcine MSC by 40% in both perfusion fluid and culture medium, the viability of human MSC was reduced by suspension conditions by 15% in perfusion fluid, and no differences were found in survival in culture medium. Furthermore, it was shown that a freeze-thawing process impaired survival, metabolism, and the ability to adhere to endothelial cells. The authors concluded that NMP conditions affect MSC but show sufficient support of their function and survival that MSC administration through NMP should be considered, and secondly that slight differences in the behavior of porcine and human MSC need to be taken into account (158).

Another recent analysis focused on the characteristics of culture-expanded MSC, investigating heir viability and homin during NMP. Kidneys were perfused for 7 h in the presence of 10^5, 10^6, or 10^7 human adipose tissue-derived MSC. Intact MSCs were detected in the lumen of glomerular capillaries, but only in the 10^7 MSC group. After a rapid decline of cell numbers during NMP, only a small portion of the MSCs were intact, and these were clustered in a minority of glomeruli. Apart from outlining the complexity of MSC therapy during MP, the authors concluded that "*an exciting new window of opportunity might emerge to actively pre-condition isolated organs in a fully controlled setting and in the absence of an immune response, before they are transplanted*" (172).

It is noteworthy that such promising findings have recently led to the creation of the international "MePEP consortium" (173) in order to study this novel modality of treatment in preparation for human trials. Therefore, more findings on MSC and immunomodulation can be expected in the near future.

Anti-inflammatory Agents

Alternatively, pharmacologic interventions to decrease immunogenicity or prevent recurrent disease can be applied during MP. The seemingly most obvious strategy to influence the inflammatory profile is the delivery of anti-inflammatory agents directly into the machine perfusion circuit, treating the liver *ex vivo* during the preservation period to obviate the need for lifelong immunosuppression or to improve long-term outcomes separate from the physiological quality of the organ at the time of transplantation (174).

Several targets for potential agents have been identified. Amongst these is TNF-alpha, one of the most potent proinflammatory cytokines, which is released in response to and has been implicated in the pathogenesis of IRI (175, 176). Therefore, in a recent clinical HMP study, it was hypothesized that the administration of the TNF-α inhibitor etanercept could improve outcomes following kidney transplantation. However, no significant differences were found concerning kidney machine perfusion parameters, including average flow and vascular resistance, nor did the authors observe significant changes regarding DGF, rejection episodes, or allograft survival (177). However, it has to be taken in mind that this study was carried out under hypothermic conditions. Although possible in other machine-perfusion techniques, NMP seems to be ideal, as active metabolism permits graft intervention and modification during preservation, circumstances that are not present in HMP (178).

More promising data come from a porcine study in which a variety of agents were added to act at different levels. Prostaglandin E1, a prostacyclin analog with vasodilator, antiplatelet, fibrinolytic, and several other anti-inflammatory properties, was continuously administered. In addition, n-acetylcysteine was added due to its free radical scavenging properties. Sevoflurane was administered due to its protective properties on endothelial cells, and carbon monoxide was added with the objective of improving vasodilatation and reducing inflammation. Indeed, during a 3-day follow-up after transplantation, this treatment resulted in lower AST levels as well as lower levels of IL-6, tumor necrosis factor α (TNF- α), and galactosidase and increased IL-10 levels (179).

Other candidate markers for modulation during MP are anti-inflammatory receptors. In this context, the adenosine A2A receptor downregulates inflammation, including the suppression of $CD4^+$ and $CD8^+$ T cells, and increases endothelial cell nitric oxide (174). Since experiences in rabbit experiments showed that adenosine A2A receptor activation can diminish IRI (180), a subsequent study evaluated whether treatment with an adenosine A2A receptor agonist could be beneficial during normothermic *ex vivo* lung perfusion (EVLP). Indeed, EVLP with targeted A2AR agonist treatment could attenuate IRI after transplantation of DCD donor lungs subjected to prolonged 12-h cold preservation in a preclinical porcine model (181).

Adenoviral Vector Gene Delivery

As cellular metabolism is preserved during normothermic perfusion, it represents a potential platform for effective gene transduction in a specific graft (182). To test this hypothesis in donor lungs, Yeung et al. used a porcine model of EVLP and treated them with an E1-, E3-deleted adenoviral vector encoding either green fluorescent protein (GFP) or the immunosuppressive interleukin-10 (IL-10). They observed a decreased expression of inflammatory cytokines such as IL-1,

TNF-α, and IL-6, as well as attenuation of the alloimmune response following transplantation (174, 182).

This finding is also of particular interest, since the delivery of adenoviral vectors could result in a prompt innate immune response by macrophages, recruiting circulating neutrophils, which in turn propagate the inflammatory response. Moreover, in the transplant setting, this preexisting inflammation could potentiate subsequent IRI (182, 183). Therefore, the authors compared *in vivo* and *ex vivo* administration and showed that donor lung is superior to *in vivo* delivery since it leads to less vector-associated inflammation (182).

Concerning the kidney, already in 2002, proof of principle of a similar technique had been reported by Brasile et al., using a recombinant adenovirus, Ad5, CMV5 GFP encoded with green fluorescence protein. They achieved effective transfection and synthesis during 24 h of *ex vivo* normothermic perfusion (184).

Only recently has a normothermic *ex vivo* organ perfusion delivery method for cardiac transplantation gene therapy been reported. Adenoviral vector transduction was utilized to deliver particles of an Adenoviral firefly luciferase vector with a cytomegalovirus (CMV) promotor to porcine donor hearts during NMP and prior to heterotopic implantation. Along with a high copy number of vector genomic DNA in transplanted hearts, there was no evidence of vector DNA in either the recipient's native heart or liver, substantiating the applicability and safety of the protocol.

These findings make it likely that this technology could be feasible for other organ systems as well. A wide variety of interesting genes could be targeted. As has been reviewed lately (159), promising possibilities arise when translating the findings of rodent models using adenoviruses expressing CTLA4Ig (185) to the setting of NMP; also, NMP could be used to deliver gene therapies that induce cytoprotection against IRI, such as myr-Akt (159, 186).

Outstandingly, Figueiredo et al. recently showed that antigen silencing is feasible during NMP. In a porcine model of lung NMP, short hairpin RNAs were delivered by lentiviral vectors, successfully reducing the immunogenicity of the lung by silencing MHC expression on the endothelium. The authors concluded that the decrease in immunogenicity carries the potential to generate immunologically invisible organs to counteract the burden of rejection and immunosuppression (187).

Organ Reconditioning and Repair

Recent experimental data primarily focusing on marginal livers furthermore suggested that NMP offers a platform for organ reconditioning and repair.

Impressive data from Birmingham, UK, showed that livers discarded due to pronounced steatosis could be effectively treated with defatting agents during 6 h of NMP. Tissue triglycerides were lowered by 38% and macrovesicular steatosis by 40%, which was associated with a down-regulation of inflammatory marker expression relevant for oxidative injury and activation of immune cells (CD14; CD11b), combined with reduced inflammatory cytokines in the perfusate (TNFα, IL-1β) (188). In addition, arterial vasospasm can be modulated by the application of ET-1 antagonists, prostacyclin analog, or calcium channel antagonists during NMP (189). Furthermore, NMP can be used for the direct and efficient application of antiviral agents, as shown in a porcine NMP model, in which Miravirsen, an inhibitor of hepatitis C virus (HCV) replication, produced improved uptake compared to SCS control livers. The authors concluded that this approach might offer a future strategy to prevent HCV reinfection after liver transplantation (190). Likewise, HCV-infected human donor lungs could effectively be treated during short-time EVLP via physical viral clearance combined with germicidal light-based therapies (191).

In summary, immunomodulation in the context of MP seems to offer a plethora of novel strategies. A series of porcine and human studies distinctively underline its effectiveness and feasibility. However, as formulated in a recent review (191, 192), the studies conducted so far just scratch the surface of conceivable interventions. In particular, NMP provides an ideal platform for immunomodulatory modifications. The next step will be the translation of findings from preclinical and rodent models as well as HMP trials into the setting of NMP.

THE IMMEDIATE FUTURE IN THE TRANSPLANTATION OF MARGINAL ORGANS

Much speculation about the changes in transplantation is currently energizing the field. The conceptual approach of extracorporal organ preservation and monitoring begs for speculation about organ reconditioning, repair, and treatment. While these are all valid considerations and perfectly reasonable hopes, the implementation of machine perfusion is the first step. Immediate and widespread adoption of HMP and NMP is unlikely since this technology does not immediately improve the outcome with regard to the primary endpoints in transplantation: patient and graft survival. Authority approval and market entry are largely based on secondary endpoints such as delayed graft function (DGF, kidney) or primary poor function (PPF, liver), which are considered to serve as surrogates for improved long-term results. The conceptual limitation with surrogates is, however, that the predictive value is not uniformly robust or formally established and immediately reproducible. The DGF rate, for example, in kidneys from DCD donors is high, but the predictive value of DFG in these cases is low (193). The definition of PPF is mostly based on high AST/ALT values early after transplantation. While these parameters indicate hepatocyte damage, correlation with the eventual outcome is very limited (194). Hence, the adoption of the technology is possibly built on the wish for innovation and the belief in the value of the time gain with NMP. Preliminary data on HOPE in recent and ongoing trials indicate a possible benefit in organ survival. If such an outcome is eventually formally achieved, the arguments for implementation will be more substantial.

For clinical implementation, several regional and center factors play an important role. First, the retrieval team may be different from the team eventually performing the transplantation. Even if the retrieval team is familiar with the technology, the preparation of the graft prior to NMP (more than HMP) is more substantial and more definitive for the

fate of the graft. Hence, in a system with longer-distance organ exchange, additional steps and a new routine would need to be established. The technology and the shifting of the work of the backtable procedure to the retrieving team need to be considered. The alternative approach where the organ is transferred to the recipient center and is then machine perfused is currently being pursued, but data indicating the actual advantage of this approach are lacking (195). For now, NMP serves as a technology that allows one important asset in surgery to be gained: time. The value of time and flexibility is dependent on the regional circumstances but may hold great potential for easing the surgery logistics, working hours, and surgical training but also gives rise to higher risks of nighttime procedures. The actual value of time in this context, however, requires further attention and needs to be better defined.

The consideration of longer-term organ preservation under normothermic conditions further carries the challenge of defining the responsibilities for managing and monitoring the organs. Eventually, the working place description of health care workers involved in this will require adoption and formal training. Standard operating procedures (SOP) and safety parameters need to be established for a wider spread use of the technology. It is likely that the advanced technological requirements and the need for 24-h availability of knowhow and personnel would favor the establishment of regional hubs for MP.

Despite the hype in the field, the clinical adoption of MP is slow. In addition to the above-mentioned factors, the lack of reimbursement is a stringent limitation in many regions. For the technology to be reimbursed, the authorities will require hard facts. Hence, the path to more widespread clinical use might be long, and the focus should remain on the demonstration of superiority regarding the most relevant clinical endpoints.

One major important step toward collective advancement in this field would be the definition of data points during and after MP and the establishment of registries for coordinated data collection. Such data points should include the definition and terminology of the various time points and actions such as cold flush and storage prior to MP, temperature and flow during MP, parameters indicating metabolic function and bile/urine production during MP, second flush, second cold ischemia time, and others. One of the hurdles in improving NMP is the large number of variables added to preservation. Identifying the relevance of individual parameters will require attention to the details of the procedure and adequate data collection and handling. To the knowledge of the authors, no routine data collection and no consensus toward data points have been established at present.

The decision-making process in MP is relatively arbitrary since the data collected during MP are suggestive but not formally established as quality-defining parameters. Since the decision to transplant an organ or not is extremely meaningful, great care needs to be applied in the process, and detailed documentation of the reasoning for decision making should be carried out. A greater effort toward orchestrated data collection could help to streamline and eventually enhance the robustness of the decision-making process.

The preference for MP to be used for the preservation of marginal organs results from the greater need for organ assessment and the greater potential benefit of better preservation. This might be particularly true and relevant for organs from uncontrolled DCDs, where the circumstances and the accumulated damage to the organs might be less clear. The value of an additional assessment under these conditions is not only meaningful with respect to the number of additional organs for transplantation but also for preventing the transplantation of organs that are severely damaged. While the assessment of this subject would be highly valuable and is much needed, the behavior of the investigators in the NMP trial by Nasralla et al. indicates the limitations and conflicts observed with this approach (62). Since the trial cannot be fully blinded, the bias generated by the fact that a technology is used that is deemed superior and the data generated during MP define a deviation in the behavior of the decision makers.

THE FAR FUTURE OF (MARGINAL) ORGAN TRANSPLANTATION

The fantasies building on the realization of extracorporal organ preservation under physiological conditions are currently fuelling hopes that this technology could facilitate tissue regeneration, organ repair, immunomodulation, xenograft humanification, and many other things. MP as a platform may impact medicine far beyond transplantation and delivers a unique chance to alter the treatment of organ failure and organ disease. Between the imagination and the realization of medical advancement stands a mountain of work and an as yet unknown but probably large number of technical and methodological challenges. An important initial goal is the expansion of the duration of MP and the establishment of an equilibrium of the condition of the organ. Preservation of organs for several days will likely require additional modifications from the currently existing technologies. Preservation of the acid-base equilibrium, nutrition organ weight-induced pressure, electrolyte shift, hemolysis, and many other challenges may require closer attention. A tissue- and/or cell-specific and targeted treatment is a realistic consideration, and proof of concept trials indicate the feasibility of this approach (196, 197). The treatment and replacement of damaged or displaced elements of organs may evolve as a new discipline and help the field to make the leap to serve one of the greatest unmet needs: The effective treatment of damaged organs.

AUTHOR CONTRIBUTIONS

TR and SS conceived and designed the study and wrote the manuscript. BC, RO, AW, JD, CK, and CB wrote sections of the manuscript. DO and MG helped to revise the manuscript, tables and figures. All the authors contributed to manuscript revision, read, and approved the submitted version.

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Impact of Combinations of Donor and Recipient Ages and Other Factors on Kidney Graft Outcomes

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As the availability of kidneys for transplantation continues to be outpaced by its growing demand, there has been an increasing utilization of older deceased donors in the last decades. Considering that definition of factors that influence deceased donor kidney transplant outcomes is important for allocation policies, as well as for individualization of post-transplant care, the purpose of this study was determine the risks for death censored graft survival and for patient survival conferred by older age of the donor in the context of the age of the recipient and of risk factors for graft and/or patient survival. The investigation was conducted in a single-center cohort of 5,359 consecutive first kidney transplants with adult deceased donors performed on non-prioritized adult recipients from January 1, 2002, to December 31, 2017. Death censored graft survival and patient survival were lower in older donors, whereas graft survival was higher and patient survival was lower in old recipients. The analyses of combinations of donor and recipient ages showed that death censored graft survival was lower in younger recipients in transplants from 18 to 59-year old donors, with standard or extended criteria, but no difference in graft survival was observed between younger and older recipients when the donor was ≥ 60-year old. Patient survival was higher in younger recipients in transplants with younger or older donors. Two to six HLA-A,B,DR mismatches, when compared to 0-1 MM, conferred risk for death-censored graft survival only in transplants from younger donors to younger recipients. Pre-transplant diabetes conferred risk for patient survival only in 50–59-year old recipients, irrespectively, of the age of the donor. Time on dialysis ≥ 10 years was a risk factor for patient survival in transplants with all donor-recipient age combinations, except in recipients with ≥ 60 years that received a kidney from an 18–49-year old donor. In conclusion, the results obtained in this study underline the importance of analyzing the impact of the age of the donor taking into consideration different scenarios.

Keywords: kidney transplantation, donor age, recipient age, death censored graft survival, patient survival

INTRODUCTION

Transplantation is considered the preferred treatment option for patients with end stage renal disease offering survival advantage over long-term dialysis, independently of patient age. As the availability of kidneys for transplantation continues to be outpaced by its growing demand, there has been an increasing utilization of older deceased donors in the last decades (1–6). The proportion of elderly individuals is also increasing among patients on the waitlist (2, 7–9).

With the aim of reducing waiting time for older patients, the Eurotransplant Senior Program or "old for old" was implemented within the Eurotransplant kidney allocation algorithm. This program is based on regional allocation of kidneys from ≥ 65-year old deceased donors to ≥ 65-year old recipients and has been very successful in increasing the number of transplants in elderly recipients (2, 7, 10–12).

The negative impact on kidney graft outcomes of older age of donors and of recipients has been repeatedly reported in the literature (4, 8, 13–15), but there are fewer studies on the impact on graft outcomes of combination of these two variables (16, 17).

Considering that definition of factors that influence deceased donor kidney transplant outcomes is important for allocation policies, as well as for individualization of post-transplant care, the purpose of this study is to investigate the risk for death censored graft survival and patient survival conferred by the combination of the age of the donor and the age of the recipient, along with other factors that may interfere with graft and/or patients survival, such as recipient sex, donor-recipient sex mismatch, pre-transplant diabetes, time on dialysis, cold ischemia time and HLA mismatches (6, 13, 14, 18–38).

MATERIALS AND METHODS

Study Population and Data Source

This is a retrospective single center study on data from 5,359 consecutive first kidney transplants with adult deceased donors performed in non-prioritized adult recipients, from January 1, 2002, to December 31, 2017.

The kidney allocation was performed following the Brazilian national criteria, which is based on HLA-A, B, DR, with emphasis on HLA-DR, compatibility. Kidneys from donors under 18 years of age (not part of this study) are allocated to < 18 year-old recipients. In addition, < 18 year-old recipients also compete for adult donor kidneys (39). Patients in high risk of losing their last vascular access to dialysis are prioritized on the waitlist and were not included in this study. All the data concerning recipients, donors, and transplant follow-up were obtained from the database of the São Paulo State Registry of Transplants. This registry requests post-transplant follow-up to centers at 3, 6, and 12 months, and yearly thereafter. Failure to comply within 90 days of a request causes a center to have its right to register new patients for transplantation to be suspended until all requested data is provided.

Among the donors, there were 3,066 (57.2%) males and 2,293 (42.8%) females. Four donor age groups were considered: (1) 18–49 years ($N = 2,783$), (2) 50–59 years with standard criteria (SCD) ($N = 567$), (3) 50–59 years with extended criteria (ECD), ($N = 980$), and (4) with 60 or more years ($N = 1,027$). ECD was defined according to the United Network for Organ Sharing, i.e., donors with 60 or more years or with 50–59 years with at least two of these three criteria: history of hypertension, serum creatinine ≥1.5 mg/dL, or death by cerebrovascular accident. For two donors with 50–59 years it was not possible to determine whether they belonged to standard or extended criteria categories and they were excluded from any analysis concerning donor age.

Among the recipients, there were 3,298 (61.5%) males and 2,061 (38.5%) females, 932 (17.4%) had pre-transplant diabetes, and 3,027 (57.1%) were on dialysis for ≥ 10 years. Three age categories were considered: 18–49 years ($N = 2,730$), 50–59 years ($N = 1,562$) and ≥ 60 years ($N = 1,067$).

Cold ischemia time above 24 h occurred in 2,412 (45%) transplants. Concerning HLA compatibility, 1,226 (22.9%) transplants were performed with 0-1 HLA-A,B,DR mismatches.

Statistical Analysis

The endpoints analyzed were death censored graft survival and patient survival, during the first 5 post-transplant years. Analyses were performed with the GraphPad Prism® 5.0 (GraphPad Software, Inc, La Jolla, CA) and SPSS (Statistical Package for the Social Sciences) (SPSS Inc, Chicago, IL). Graft and patient survival curves were constructed with the Kaplan-Meier method and compared with log rank test or Cox regression analysis. In the Cox regression analyses were included variables with P-value < 0.10 in the log rank test. Cases with any missing value were excluded. A two-sided P-value ≤ 0.05 was considered statistically significant.

RESULTS

Univariate Analyses

The univariate analyses results are presented in **Table 1**. Donor's older age negatively impacted both death-censored graft ($p < 0.001$) and patient ($p < 0.001$) survival, whereas no impact was observed regarding donor sex. Recipient's older age positively impacted death-censored graft survival ($p < 0.001$) and negatively impacted patient survival ($p < 0.001$). No significant differences were observed regarding recipient sex, although a tendency ($p = 0.062$) was observed toward a higher patient survival in female recipients. Donor-recipient sex mismatch had no influence on death-censored graft or patient survival. Cold ischemia time > 24 h and 2–6 HLA-A,B,DR mismatches impacted negatively on death-censored graft survival ($p = 0.009$ and 0.004, respectively) whereas pre-transplant diabetes and time on dialysis ≥ 10 years had a negative impact on patient survival ($p < 0.001$ for both variables).

Multivariate Analysis

The multivariate analysis included all variables with $p < 0.10$ in the univariate analyses and the results are presented in **Table 2**. Concerning death censored graft survival, all the variables, except cold ischemia time, remained significantly associated. Regarding patient survival, all the variables with a $p < 0.05$ in the univariate analysis remained significant, whereas sex of the recipient and

TABLE 1 | Univariate analysis (log-rank) of the influence of donor, recipient and transplant characteristics on death censored graft survival and patient survival during the first 5 post-transplant years.

Characteristic	Number (%)	Missing values, *n*	5 year death censored graft survival		5 year patient survival	
			Survival (%)	*p*	Survival (%)	*p*
Donor age (years)		2				
18–49	2,783 (52.0)		88.8	<0.001	89.6	<0.001
50–59 SCD	567 (10.6)		88.0		86.0	
50–59 ECD	980 (18.3)		83.7		85.5	
≥ 60	1,027 (19.2)		77.4		84.4	
Donor sex		0				
Female	2,293 (42.8)		85.4	0.88	87.2	0.83
Male	3,066 (57.2)		85.9		87.7	
Recipient age (years)		0				
18–49	2,730 (50.9)		83.3	<0.001	92.7	<0.001
50–59	1,562 (29.1)		87.3		85.9	
≥ 60	1,067 (19.9)		90.2		76.3	
Recipient sex		0				
Female	2,061 (38.5)		86.3	0.53	88.6	0.062
Male	3,298 (61.5)		85.3		86.8	
Donor-Recipient sex mismatch		0				
Female-Female	879 (16.4)		85.6	0.64	89.4	0.41
Male-Female	1,182 (22.1)		86.8		88.1	
Male-Male	1,884 (35.2)		85.3	0.86	87.4	0.38
Female-Male	1,414 (26.4)		85.3		85.8	
Pre-transplant diabetes		0				
Yes	932 (17.4)		87.3	0.17	80.3	<0.001
No	4,427 (82.6)		85.4		89.0	
Time on dialysis (years)		58				
1–9	2,274 (42.9)		83.7	0.28	90.2	<0.001
≥ 10	3,027 (57.1)		86.3		85.5	
Cold ischemia time (hours)		2				
0–24	2,945 (55.0)		86.7	0.009	88.1	0.23
> 24	2,412 (45.0)		84.4		86.5	
HLA-A, -B, -DR mismatches		0				
0–1 MM	1,226 (22.9)		88.6	0.004	88.7	0.087
2–6 MM	4,133 (77.1)		84.8		87.1	

HLA-A,B,DR mismatches that presented borderline ($0.05 > p < 0.10$) significance in the univariate analysis were not significant in the multivariate analysis.

Impact of Donor Age on Death-Censored Graft Survival and on Patient Survival

Death-censored graft survival did not differ between 18–49 and 50–59-year old SCD (88.8 vs. 88.0 %, $p = 0.78$). Considering transplants from 18–49-year old donors as reference, graft survival was lower in transplants from 50–59-year old ECD (hazard ratio (HR) 1.51, 95% confidence interval (CI) 1.23–1.86, $p < 0.001$) and from ≥ 60-year old donors (HR 2.11, 95% CI 1.75–2.55, $p < 0.001$) (**Figure 1A**). The difference in graft survival between 50–59-year old ECD and ≥ 60-year old donors was statistically significant ($p = 0.002$). Considering these results, three age groups of donors (18–59-year old SCD, 50–59-year old ECD and ≥ 60-year old donors) were considered in the remaining analyses.

Patient survival was significantly lower in transplants from donors of any age group > 50 years, as compared to transplants from 18–49-year-old donors (**Figure 1B**). The patient survival did not differ among transplants from 50–59-year old SCD, 50–59-year old ECD and ≥ 60-year old donors and these three age categories were combined for the remaining analyses.

Impact of Recipient Age on Death-Censored Graft Survival and on Patient Survival

Death-censored graft survival was higher in recipient aged 50–59 years (HR 0.72; 95% CI 0.60–0.86; $p < 0.001$) and ≥ 60 years (HR 0.56; 95% CI 0.44–0.71; $p < 0.001$), in comparison with recipients aged 18–49 years (**Figure 2A**).

TABLE 2 | Multivariable Cox regression analyses for death censored graft survival and patient survival during the first 5 post-transplant years.

Variables	5 year death censored graft survival			5 year patient survival		
	p	HR	95% CI	*p*	HR	95% CI
Donor 18–49 years	Reference			Reference		
Donor 50–59 years SCD	0.78	–	–	0.045	1.32	1.01–1.72
Donor 50–59 years ECD	<0.001	1.51	1.23–1.86	0.011	1.32	1.07–1.65
Donor ≥60 years	<0.001	2.11	1.75–2.55	<0.001	1.53	1.25–1.89
Recipient 18–49 years	Reference			Reference		
Recipient 50–59 years	<0.001	0.72	0.60–0.86	<0.001	1.85	1.50–2.27
Recipient ≥60 years	<0.001	0.56	0.44–0.71	<0.001	3.10	2.52–3.82
Recipient sex: male	–	–	–	0.53	–	–
Pre-transplant diabetes	–	–	–	<0.001	1.48	1.22–1.79
Time on dialysis: ≥10 years	–	–	–	<0.001	1.84	1.53–2.21
Cold ischemia time: >24 h	0.082	–	–	–	–	–
HLA-A, -B, -DR: 2–6 mismatches	0.013	1.29	1.06–1.57	0.22	–	–

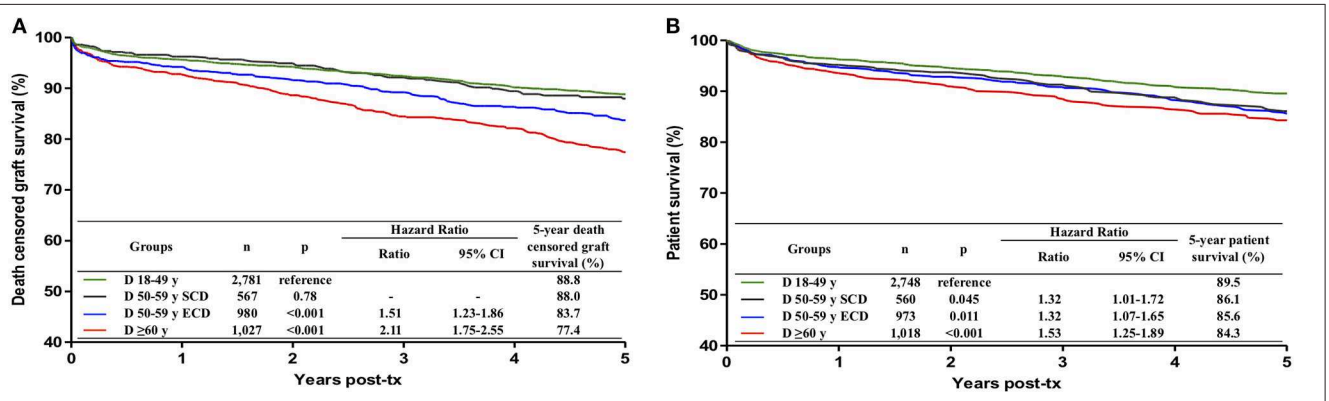

FIGURE 1 | Influence of donor age on death censored graft survival **(A)** and patient survival **(B)** during the first 5 post-transplant years. Donors were divided into four groups, 18–49 years, 50–59 years with standard criteria (SCD), 50–59 years with extended criteria (ECD) and with 60 or more years. ECD were defined according to the United Network for Organ Sharing definition. Kaplan-Meier curves were compared with multivariate Cox regression analysis.

As the groups with 50–59 years and ≥ 60 years were not significantly different ($p = 0.093$), they were combined for the remaining analyses.

Patient survival was significantly lower in recipients with 50–59 years (HR 1.85; 95% CI 1.50–2.27, $p < 0.001$) and ≥ 60 years (HR 3.10; 95% CI 2.52–3.82, $p < 0.001$), in comparison with recipients aged 18–49 years (**Figure 2B**). As the groups with 50–59 years and ≥ 60 years were significantly different ($p < 0.001$), the three groups were maintained separately for further analyses.

Impact of Different Combinations of Donor and Recipient Ages on Death-Censored Graft Survival

The results are presented in **Figure 3A**. Graft survival was lower in 18–49-year old recipients than in ≥50-year old recipients in transplants with 18–59-year old SCD (HR 1.80; 95% CI 1.44–2.24; $p < 0.001$) and with 50–59-year old ECD (HR 1.66; 95% CI 1.18–2.33; $p = 0.004$). There was no difference, however, in the graft survival in younger and older recipients (76.6 vs. 78.2%, $p = 0.80$) when the donor was ≥ 60-year old.

Impact of Different Combinations of Donor and Recipient Ages on Patient Survival

The results are presented in **Figure 3B**. In any donor age category, in reference to 18–49-year-old recipients, recipient age of 50–59 conferred a risk for lower patient survival and this risk was even higher in recipients with ≥ 60 years of age. The survival of recipients aged ≥ 60 years did not differ in transplants with 18–49-year old and ≥ 50-year old donors (77.7 vs. 74.8%, $p = 0.40$).

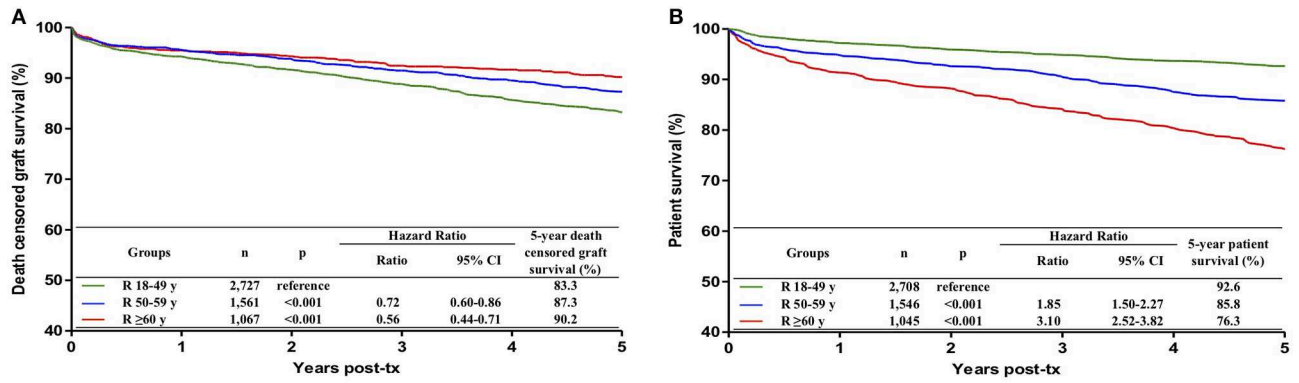

FIGURE 2 | Influence of recipient age on death censored graft survival **(A)** and patient survival **(B)** during the first 5 post-transplant years. Recipients were divided into three groups, 18–49 years, 50–59 years and with 60 or more years. Kaplan-Meier curves were compared with multivariate Cox regression analysis.

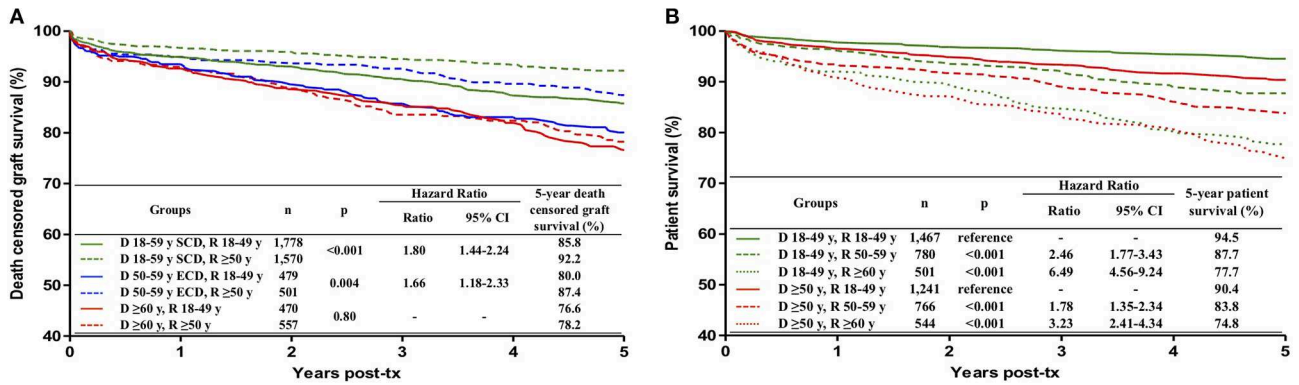

FIGURE 3 | Influence of the combination of donor and recipient ages on death censored graft survival **(A)** and patient survival **(B)** during the first 5 post-transplant years. **(A)** Based on previous results, in death censored graft survival analysis, donors were divided into three groups: 18–59 years with standard criteria (SCD), 50–59 years with extended criteria (ECD) and ≥ 60 years; recipients were divided in two groups: 18–49 years and ≥ 50 years. In patient survival analyses **(B)**, donors were divided into two groups, 18–49 years and ≥ 50 years, and recipients in three groups, 18–49 years, 50–59 years and ≥ 60 years. ECD were defined according to the United Network for Organ Sharing definition. Kaplan-Meier curves were compared with the log rank test.

Impact of HLA Mismatches on Death-Censored Graft Survival in Different Donor-Recipient Ages Combinations

Two to six HLA-A,B,DR mismatches, when compared to 0-1 MM, conferred a significant risk for death-censored graft survival only in transplants from 18–59-year old SCD in 18–49-year-old recipients (84.3 vs. 90.2%, HR 1.58; 95% CI 1.17–2.13; $p = 0.003$) (**Figure 4**).

Impact of Pre-transplant Diabetes on Patient Survival in Different Donor-Recipient Ages Combinations

Pre-transplant diabetes was present in 7.8% of 18–49-year old recipients, in 23.2% of 50–59-year old recipients and in 33.2% of ≥ 60-year old recipients. It was a risk factor for patient survival only in 50–59-year old recipients of kidneys from18–49-year old (HR 2.24; 95% CI 1.33–3.79; $p = 0.003$) and from ≥50-year old (HR 2.43; 95% CI 1.56–3.80; $p < 0.001$) donors (**Figure 5**).

Impact of Time on Dialysis on Patient Survival in Different Donor-Recipient Ages Combinations

Significantly lower 5-year patient survival in patients with ≥ 10 years on dialysis was observed in transplants with all donor-recipient ages combinations, except in the case of recipients with ≥ 60 years that received a kidney from a 18–49-year old donor. The survival curves and the risk conferred by ≥ 10 years on dialysis in each donor-recipient age combination are presented in **Figure 6.**

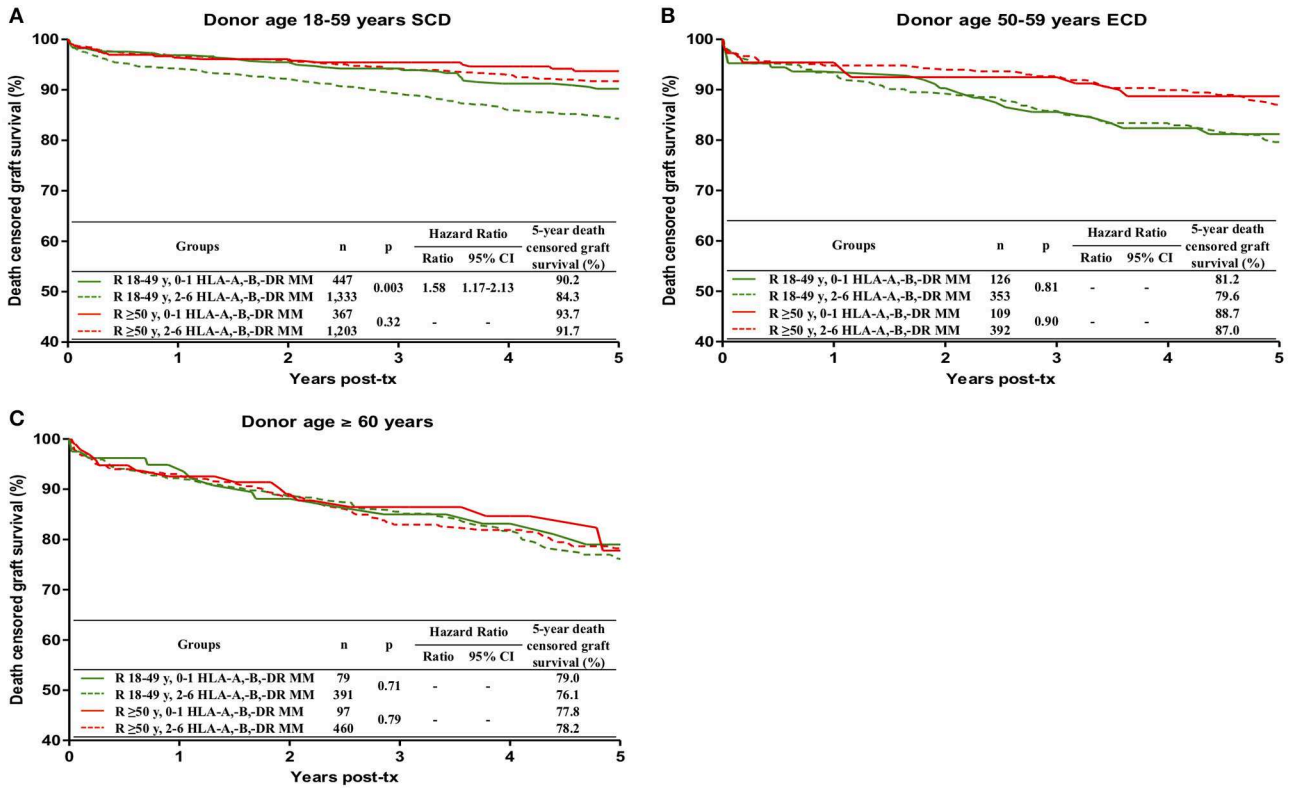

FIGURE 4 | Influence of the combination of recipient age and HLA-A, -B, -DR mismatches on death censored graft survival during the first 5 post-transplant years, stratified by donor age, **(A)** 18–59 years with standard criteria (SCD), **(B)** 50–59 years with extended criteria (ECD) and **(C)** ≥ 60 years. ECD were defined according to the United Network for Organ Sharing definition. Kaplan-Meier curves were compared with the log rank test.

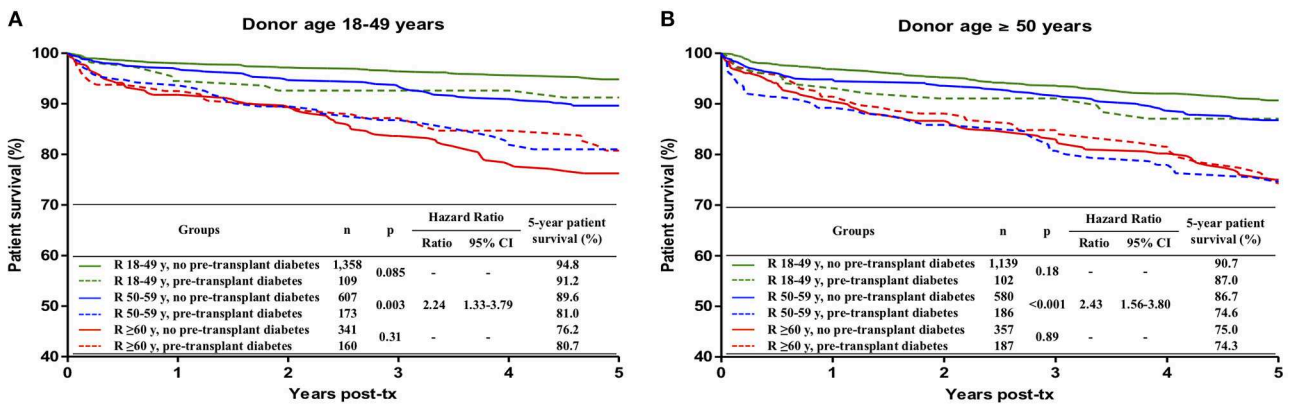

FIGURE 5 | Influence of the combination of recipient age and pre-transplant diabetes on patient survival during the first 5 post-transplant years, stratified by donor age: **(A)** 18-49 years, **(B)** ≥ 50 years. Kaplan-Meier curves were compared with the log rank test.

DISCUSSION

In the current scenario of kidney donor shortage, the use of older donors is unavoidably and thus it is important define/quantify the risks conferred by the advanced donor age that could be useful for allocation matters and for individualization of post-transplant care.

The purpose of this study was to assess the risks for death-censored graft survival and for patient survival conferred by older age of the donor in the context of the age of the recipient and of other possible or well-recognized risk factors for graft and/or patient survival. The investigation was conducted in a single-center cohort of 5,359 consecutive first kidney transplants with adult deceased

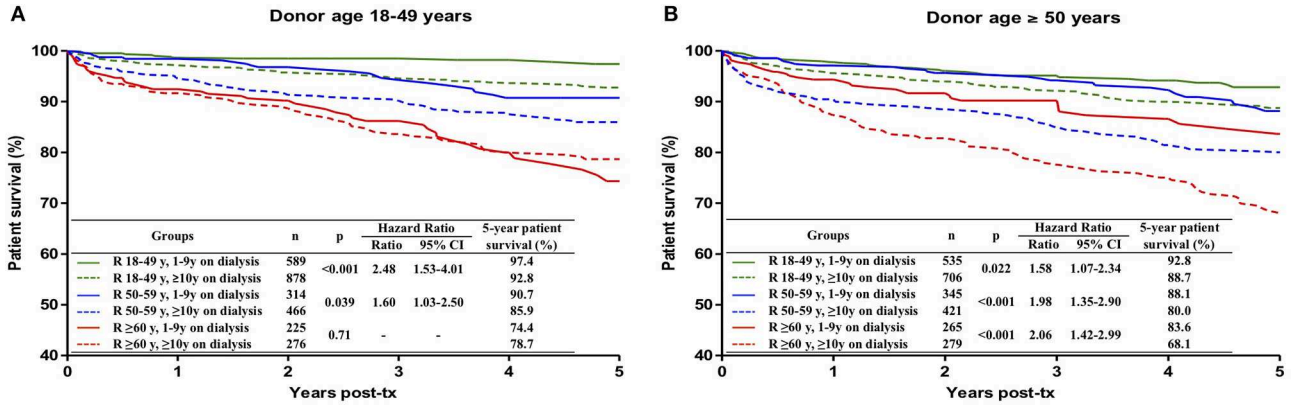

FIGURE 6 | Influence of the combination of recipient age and time on dialysis on patient survival during the first 5 post-transplant years, stratified by donor age: **(A)** 18–49 years, **(B)** ≥ 50 years. Kaplan-Meier curves were compared with the log rank test.

donors performed on non-prioritized adult recipients from January 1, 2002, to December 31, 2017. The end-points were death-censored graft survival and patient survival in the first 5 years post-transplant.

The univariate analysis showed that donor and recipient age influenced both graft and patient survival, cold ischemia time and HLA-A,B,DR mismatches had an impact on graft survival, and pre-transplant diabetes and time on dialysis influenced patient survival. All these associations, except for cold ischemia time, were confirmed in multivariate analyses.

Female recipients presented a tendency for higher survival ($p = 0.062$) in the univariate analysis, but this association was not significant in the multivariate analysis and thus was not further analyzed. We believe that our data do not allow a definitive conclusion about the influence of the sex of the recipient on patient survival. On the other hand, we did not find any indication for an impact of donor-recipient sex mismatch on transplant outcomes, corroborating the results of other studies (21, 22).

Increased donor age was associated with lower death-censored graft survival and with patient survival, as already described (4, 6, 8, 13–15). In our study, poorer graft survival started to be observed in transplants with 50–59-year old donors with extended criteria donors, while the impact on patient survival was already observed in transplants with 50–59-year old standard criteria donors.

Recipient age ≥ 50 years was associated with higher graft survival and with lower patient survival, confirming the findings of previous publications (16, 17). As it has been reported that younger recipients present a higher rate of rejection episodes (2, 16, 40), the lower graft survival in younger recipients is probably related to a more vigorous immune response, and perhaps also to a higher rate of non-adherence to treatment in this group of patients. On the other hand, the lower patient survival in older recipients is probably explained by the higher age *per se*, increased rate of co-morbidities and higher susceptibility to infections (41, 42).

Considering the opposite effects of recipient age on graft and on patient survival, we also calculated the overall graft survival, i.e., graft failure defined as death of the patient or return to dialysis, in relation to recipient age (data not shown). The results showed that 5-year overall graft survival was not statistically different ($p = 0.14$) between 18–49-year old (77.5%) and 50–59-year old (75.4%) recipients, but was significantly lower ($p = 0.002$) in ≥ 60-year old recipients (69.5%, HR of 1.28) in relation to 50–59-year old recipients.

An interesting observation was that there was no difference in graft survival in younger and older recipients when the donor was ≥ 60-year old, reinforcing the concept that kidneys from old donors should be preferentially allocated to old recipients. In the Eurotransplant Senior Program the ages of donor and the recipient were set at ≥ 65 years (2, 7, 10–12).

Regarding the interplay between donor age, recipient age and HLA incompatibilities, our data showed that 2-6 HLA-A,B,DR mismatches were significantly associated with lower graft survival only in transplants from 18–59-year old donors with standard criteria into younger (18–49-year old) recipients. The 5-year graft survival of 2–6 HLA mismatched transplants from these donors in younger recipients was 84.3%, in contrast with survivals of 90.2%, in 0-1 mismatched grafts in younger recipients, 93.7% in 0–1 mismatched grafts in ≥ 50-year old recipients, and 91.7% in 2–6 mismatched grafts in ≥ 50-year old recipients. The explanation for these results would be the more robust immune response of the younger recipient and the conclusion would be that mismatched grafts should be avoided in younger recipients. This subject deserves further analyses, not only to confirm these results but also to investigate which kind of HLA mismatch should be considered. For instance, would avoiding HLA-DR mismatches be sufficient?

Pre-transplant diabetes conferred a significant risk for the survival of 50–59-year old recipients, both in transplants from 18–49-year old donors (HR 2.24) and from ≥ 50-year old donors (HR 2.43). In 18–49-year old recipients, the survival of patients with pre-transplant diabetes was slightly inferior but the difference did not reach statistical significance, probably

because of the lower number of diabetic patients in this age group. On the other hand, among ≥ 60-year old recipients, no difference in patient survival was observed between cases with or without pre-transplant diabetes. The explanation for this finding could be that most patients with more severe diabetes-related comorbidities could not survive long enough to reach the transplant because of increased mortality in the waitlist.

The association of longer time on dialysis and inferior patient survival has already been repeatedly reported in the literature (29–31). In the present study, time on dialysis ≥ 10 years conferred risk for patient survival in all donor-recipient ages combinations, except in transplants from younger donors into ≥ 60-year recipients. The explanation for this exception is probably related to the better quality of the younger kidneys and the implicit selection for healthier recipients during the prolonged time on dialysis.

In summary, the main results of our study were: (1) association of increased age of the donor with lower graft and patient survivals; (2) association of increased age of the recipient with higher graft survival and with lower patient survival; (3) no difference in graft survival between transplants in younger and older recipients when the donor was ≥ 60-year old; (4) impact of HLA mismatches on death-censored graft survival only in transplants from younger donors to younger recipients; (5) association of pre-transplant diabetes with lower patient survival only in 50–59-year old recipients; (6) association of time on dialysis ≥ 10 years with lower patient survival in transplants with all donor-recipient ages combinations, except in recipients with ≥ 60 years that received a kidney from a 18–49-year old donor.

This study has the limitation of being a single-center retrospective study in a relatively small cohort of 5,359 kidney transplants and with a limited number of factors that could be analyzed. In addition, some important factors could not be included, as the PRA (panel reactive antibody) because different methodologies for antibody determination have been used during the period covered by this study, socioeconomic variables, which are especially relevant in developing countries (43, 44) and cardiovascular disease, a very important risk factor for patient survival (42, 45).

In conclusion, this study has disclosed interesting interactions between age of the donor, age of the recipient and other factors that influence the survival of the graft and of the patient. Future multicentric studies, with large number of transplants, are warranted to further explore the impact of combinations of donor age with other risk factors to better understand and predict the impact of the age of the donor on kidney transplant outcomes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available as the data are from a governmental state registry of transplantation data. Requests to access these datasets should be directed to Maria Gerbase-DeLima, gerbase@igen.org.br.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MG-D, KM, RM, FM, HT-S, and JM-P conceived and designed the study. MG-D and KM analyzed the data. MG-D and KM wrote the manuscript.

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Oral Preconditioning of Donors After Brain Death With Calcineurin Inhibitors vs. Inhibitors of Mammalian Target for Rapamycin in Pig Kidney Transplantation

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Background: The systemic inflammatory cascade triggered in donors after brain death enhances the ischemia-reperfusion injury after organ transplantation. Intravenous steroids are routinely used in the intensive care units for the donor preconditioning. Immunosuppressive medications could be potentially used for this purpose as well. Data regarding donor preconditioning with calcineurin inhibitors or inhibitors of mammalian target for Rapamycin is limited. The aim of this project is to investigate the effects of (oral) donor preconditioning with a calcineurin inhibitor (Cyclosporine) vs. an inhibitor of mammalian target for Rapamycin (Everolimus) compared to the conventional administration of steroid in the setting of donation after brain death in porcine renal transplantation.

Methods: Six hours after the induction of brain death, German landrace donor pigs (33.2 ± 3.9 kg) were randomly preconditioned with either Cyclosporine ($n = 9$) or Everolimus ($n = 9$) administered via nasogastric tube with a repeated dose just before organ procurement. Control donors received intravenous Methylprednisolone ($n = 8$). Kidneys were procured, cold-stored in Histidine-Tryptophane-Ketoglutarate solution at 4°C and transplanted in nephrectomized recipients after a mean cold ischemia time of 18 h. No post-transplant immunosuppression was given to avoid confounding bias. Blood samples were obtained at 4 h post reperfusion and daily until postoperative day 5 for complete blood count, blood urea nitrogen, creatinine, and electrolytes. Graft protocol biopsies were performed 4 h after reperfusion to assess early histological and immunohistochemical changes.

Results: There was no difference in the hemodynamic parameters, hemoglobin/hematocrit and electrolytes between the groups. Serum blood urea nitrogen and creatinine peaked on postoperative day 1 in all groups and went back to the

preoperative levels at the conclusion of the study on postoperative day 5. Histological assessment of the kidney grafts revealed no significant differences between the groups. TNF-α expression was significantly lower in the study groups compared with Methylprednisolone group ($p = 0.01$) Immunohistochemistry staining for cytochrome c showed no difference between the groups.

Conclusion: Oral preconditioning with Cyclosporine or Everolimus is feasible in donation after brain death pig kidney transplantation and reduces the expression of TNF-α. Future studies are needed to further delineate the role of oral donor preconditioning against ischemia-reperfusion injury.

Keywords: oral preconditioning, brain death donor, kidney transplantation, pig, calcineurin inhibitors, inhibitors of mammalian target for rapamycin, TNF-α

INTRODUCTION

During the process of organ transplantation, the ischemia reperfusion injury (IRI) together with the systemic inflammatory response to brain death causes infrastructural organ injury which could lead to initial poor function and ultimately primary non-function (1). The increased intracranial pressure and the absence of cerebral flow during brain death activates a full-blown neuronal, hemomdynamic and hormonal storm. The consequence is an inflammatory cascade which releases proinflamatory cytokines, chemokines and adhesion molecules and leads to infiltration of T-lymphocytes and macrophages in the organs (2). It has been shown that the treatment with methylprednisolone in donors after brain death (DBD) exerts protective effects against IRI in terms of decreased incidence of acute rejection (3).

Preconditioning with calcineurin inhibitors (CNIs) has been shown to have protective effects in a model of renal transplantation in rats compared to vehicle-treated animals (4). This renoprotective effect was seen with only one dose of CNI and was not different between cyclosporine and tacrolimus regarding measured outcomes. To the best of our knowledge, there is no data available regarding the donor preconditioning with CNI in a big animal (porcine) model. Everolimus (Certican), an inhibitor of mammalian target for Rapamycin (mTORi), inhibits the proliferation and the clonal expansion of antigen-activated T-cells, making it an interesting candidate for the pharmacologic preconditioning against IRI in the setting of DBD. Currently, there is very few data in the literature regarding this possible protective effect of Everolimus.

The aim of this study has been to investigate the feasibility and the effects of oral preconditioning of DBD donors with CNI (Cyclosporine A) vs. mTORi (Everolimus) vs. conventional administration of steroid in a porcine model of kidney transplantation.

Abbreviations: DBD, donors after brain death; IRI, ischemia-reperfusion injury; CNIs, calcineurin inhibitors; mTORI, inhibitors of mammalian target for Rapamycin; HTK, Histidine-Tryptophane-Ketoglutarate; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6; IL-10, interleukin 10; GFR, Glomerular filtration rate; POD, post-operative day; SD, Standard deviation; i.m., intramuscularly; i.v., intravenously; MAP, mean arterial pressure; CVP, central venous pressure; HR, heart rate; IVC, inferior vena cava; PS, proportion score; IS, intensity score; TS, total score; BUN, blood urea nitrogen; Cr, creatinine; ATI, acute tubular injury.

METHODS

German landrace pigs (weight: 33.2 ± 3.9 kg) were given access to standard laboratory chow (ssniff R/M-H, ssniff Spezialdiäten, Soest, Germany) and tap water before experiments. The study protocol was reviewed and approved by the responsible animal welfare state authority (Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany (file number: 35-9185.81/G-5/16) and were performed according to the institutional guidelines at the Ruprecht-Karls Univesity, Heidelberg, Germany in accordance with the guidelines of FELASA (Federation for Laboratory Animal Science Associations).

Experimental Design

All operations and investigations were performed under general anesthesia. After premedication (azaperone 6 mg/kg intramuscularly (i.m.), ketamine 10 mg/kg i.m., and midazolamine hydrochloride 0.5 mg/kg i.m.), anesthesia was induced with ketamine [1 mg/kg intravenously (i.v.)], midazolamine hydrochloride (0.1 mg/kg i.v.), and atropine (0.04 mg/kg i.v.). During the operation, anesthesia was maintained with 1.5–2% isoflurane. Ventilation was pressure-controlled in a half-closed system. The ventilation parameters included a tidal volume of 240 ml, frequency of 17/min, maximum pressure of 24 cmH_2O and positive end-expiratory pressure of 3–5 cmH_2O. The pH, HCO_3, pCO_2, and pO_2 concentrations were determined by routine analysis of arterial blood gases. The respiration parameters were then adapted to these values. During surgery, controlled infusion therapy was applied using 20 ml/kg/h Sterofundin (B. Braun, Melsungen, Germany). The mean arterial pressure (MAP) as well as the central venous pressure (CVP) and the heart rate (HR) were continuously monitored. For these reasons, the right common carotid artery and the jugular vein were first prepared, cannulated and connected to pressure transducers. The central venous catheter additionally served for volume substitution, for the administration of pharmaceuticals and for obtaining central venous blood samples.

Induction of Brain Death

Our standardized method for the induction of brain death in pigs has been published elsewhere (5, 6). Briefly, under general anesthesia, two burr holes (diameter, 10 mm) were placed epidurally in the left temporal (CODMAN® MICROSENSOR® Integra LifeSciences, Plainsboro, NJ, USA), right temporal (10-French Tiemann balloon catheter, B. Braun, Melsungen, Germany), and intraparenchymal in left frontal (thermal diffusion probe), regions. The slow inflation of the epidurally inserted Tiemann balloon catheter with a total of 6–13 mL NaCl 0.9% solution (running rate: 1 mL in 3 min) caused brain death within about 60 min. Brain death was confirmed after cessation of anesthesia by (1) the typical hemodynamic changes of brain death, (2) the absence of response to painful stimuli, and (3) the absence of pupillary and corneal reflexes. Ventilation and close monitoring of cardiovascular parameters such as heart rate and blood pressure were continued during organ procurement.

Preconditioning of Donor Animals

Six hours the after induction of brain death (i.e., 2 h prior to organ procurement) preconditioning was performed with the oral administration of Cyclosporine suspension (Novartis Pharma GmbH, Nuremberg, Germany) (10 mg/kg body weight) ($n = 9$) or Certican suspension (2 mg) ($n = 9$) (Novartis Pharma GmbH, Nuremberg, Germany) - via the nasogastric tube. Doses were analogous to usual administered doses in adult organ transplantation. A repeated dose was administered immediately before organ procurement. Control group ($n = 8$) received 250 mg intravenous bolus of Methylprednisolone (Urbason®, SANOFI-AVENTIS GmbH, Vienna, Austria) then continuously at a dose of 100 mg/h until procurement (**Figure 1**).

Organ Procurement and Preservation

A full-length midline laparotomy was performed and abdominal aorta and inferior vena cava (IVC) were dissected at the level of iliac bifurcation. Subsequently supratruncal aorta was prepared just below the diaphragm. After the administration of 200 IU/Kg heparin, the perfusion catheter was inserted into the aorta. Renal artery was checked for possible lower pole arteries. Slight mobilization of adrenal gland was done for better exposure of renal vein. The aorta was cross-clamped and the cold perfusion was performed with HTK (histidine tryptophan ketoglutarate) solution (Custodiol®, Dr. F. Köhler Chemie GmbH, Alsbach-Hähnlein, Germany) and the infrarenal IVC was vented. The renal artery was cut without a patch; renal veins were cut with a short IVC cuff. After the procurement, renal artery was catheterized by a soft cannula and perfused again. The kidney was subsequently cold-stored in HTK for 18 h.

Kidney Transplantation

The details regarding operation procedures have been published elsewhere (7). Briefly, the recipient animals were first premedicated in the same way as the donor animals, anesthetized, ventilated and instrumented. Baseline blood samples were obtained. After a midline laparotomy, the pigs underwent nephrectomy followed by standard kidney transplantation. In summary, right sided kidney transplantation was started with an end-to-side venous anastomosis of the renal vein to IVC with 5-0 Prolene using a continuous suture technique. The arterial anastomosis was performed end-to-side on the aorta in an analogous manner. The kidney was re-perfused first by releasing the venous perfusion by removing the clamp on the vein and, as a second step, releasing the arterial perfusion by removing the clamp on the artery. Subsequently, the ureteroneocystostomy was performed using 5-0 PDS sutures

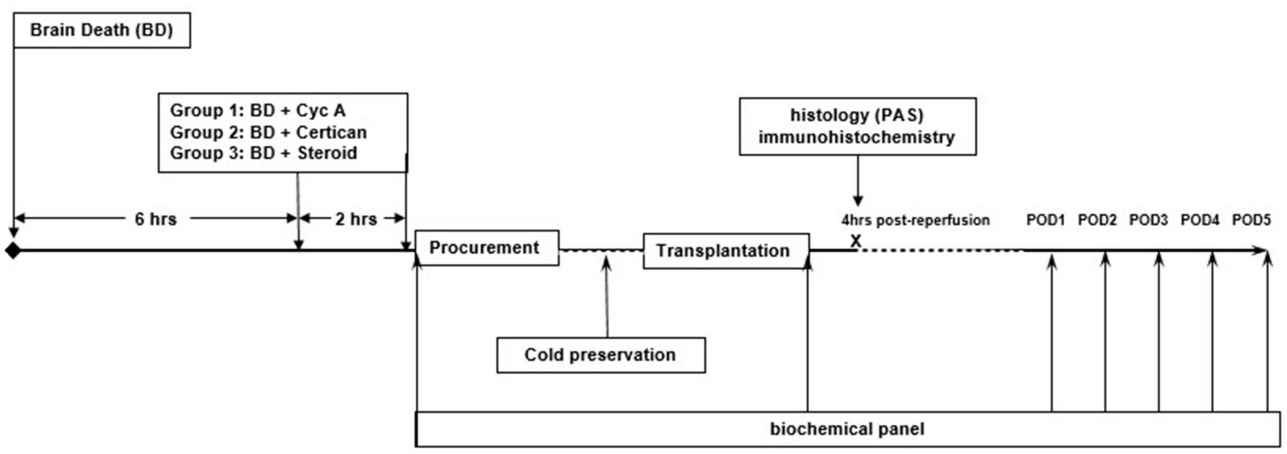

FIGURE 1 | Study design. Six hours after the induction of brain death, German landrace donor pigs (33.2 ± 3.9 kg) were randomly preconditioned with either Cyclosporine ($n = 9$) or Everolimus ($n = 9$) administered via nasogastric tube with a repeated dose just before organ procurement. Control donors received intravenous (i.v.) Methylprednisolone ($n = 8$). Kidneys were procured, cold-stored in HTK solution at 4°C and transplanted in nephrectomized recipients after a mean cold ischemia time of 19.32 ± 2.92 (SD) hours. No post-transplant immunosuppression was given to avoid confounding bias. Blood samples were obtained at 4 h post reperfusion and daily until postoperative day (POD) 5 for complete blood count, blood urea nitrogen (BUN), creatinine (Cr), and electrolytes. Graft protocol biopsies were performed 4 h after reperfusion to assess early histological and immunohistochemical changes.

continuously. The two recipient pigs in each recipient group were transplanted simultaneously using two kidneys from each donor pig.

Post-transplant Procedure

The recipients were monitored on the operating table for 4 h, after which blood samples and protocol biopsies were taken and the abdomen was closed. The indwelling central venous catheter was kept in order to draw blood samples as well as for the intravenous administration of analgesics, antibiotics and volume and substrate substitution. The catheter in the carotid artery was removed after surgery. The animals were then extubated and returned to the cage. Recipients received 0.05 mg/kg buprenorphine, 25–50 mg/kg Metamizole for analgesia as well as 200 mg ciprofloxacin and 125 mg metronidazole, over the remaining central venous catheter. Buprenorphine 0.02–0.05 mg/kg and Metamizole 25–50 mg/kg were given every 12 h for the first 48 h postoperatively.

When the animals were awake and had regained their physiological body temperature, they were taken to the holding area of the University's Interfaculty Biomedical Research Facility. The animals were under observation of the competent animal caretakers and veterinarians, immediately gaining free access to water. On the evening of the operating day, the animals received 500 ml glucose 5% + 500 ml lactated Ringer. On the 1st postoperative day, the animals received 1,000 mL glucose 10% + 1,000 mL ringer lactate. Solid food was allowed only after bowel sound was heard. Parenteral nutrition with Nutriflex peri was considered for animals unable to eat. After the surgery based on pigs' general performance, it was decided how they should be observed and kept. All the pigs were visited three times a day and checked in terms of weight change. Blood was drawn over the central venous catheter daily to measure complete blood count, blood urea nitrogen (BUN), creatinine (Cr) and electrolytes up to postoperative day (POD) 5. No immunosuppression was administered. Animals were sacrificed at the end of the study on POD 5 under deep anesthesia by intravenous injection of potassium chloride (2 mmol/kg).

Histopathology

To investigate early histopathological changes during kidney transplantation, wedge biopsies were obtained 4 h after reperfusion. Kidney samples were fixed in 10% buffered formalin, routinely embedded in paraffin, cut into 4 μm-thick sections for hematoxylin and eosin stain as well as for Periodic acid-Schiff reaction according to standard protocols. Qualitative assessment of samples was performed to determine and grade acute tubular injury (1 = mild, dilated tubules, partial brush border loss, 2 = moderate, dilated tubules, complete brush border loss, hyaline cylinders, 3 = severe, complete epithelial atrophy, tubule necrosis). Quantitative assessment of acute tubular damage was also performed and scored as quartiles (1 = 0–25, 2 = 26–50, 3 = 51–75, and 4 = 76–100%).

Immunohistochemistry

For immunohistochemical examination, sections were labeled with commercially available antibodies against cytochrome c (Abcam, Cambridge, UK, ab90529, dilution 1:200) and TNF-α (Abcam, Cambridge, UK, ab6671, dilution 1:50). After heat-induced antigen retrieval at pH 9 (Target Retrieval Solution, Agilent Technologies, Inc., Santa Clara, USA) for cytochrome c and pH 6 (Target Retrieval Solution, Agilent Technologies, Inc., Santa Clara, USA) for TNF-α, respectively, the slides were blocked with Dako REAL Peroxidase-Blocking Solution (Agilent Technologies, Inc., Santa Clara, USA) and incubated with the primary antibody. An anti-rabbit secondary antibody conjugated to HRP (Polyview plus HRP (anti-rabbit) reagent, ENZO Life Sciences GmbH, Lörrach, Germany) was applied. AEC solution (Dako REAL Substrate Solution, Agilent Technologies, Inc., Santa Clara, USA) was used to visualize the signal.

The immunohistochemical scoring was performed according to Allred et al. (8). The Proportion Score (PS) was the estimated percentage ratio of positive TNF-α-stained or cytochrome c-stained cells to the total number of cells, classified as: PS0 (0%), PS1 (>0–1%), PS2 (≥1–10%), PS3 (≥10–33%), PS4 (≥33–66%), and PS5 (≥66–100%). The Intensity Score (IS) was measured based on estimated staining intensity by visual assessment and was scored as: 0 (negative), 1+ (weak), 2+ (moderate), or 3+ (strong). The total score (TS) was calculated as the sum of the PS and IS and ranged from 0 to 8.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp. Released 2013. Armonk, NY). Continuous data are expressed as mean values ± standard deviation (SD) and differences between groups were analyzed using the one-way ANOVA test. Categorical data were compared using the chi-square test of association. Histopathological data were analyzed using the Kruskal-Wallis test followed by the Bonferroni *post-hoc* method. $P < 0.05$ were accepted as statistically significant differences.

RESULTS

There was no difference in preoperative hemodynamic parameters, hemoglobin/hematocrit, electrolytes as well as intraoperative blood loss between the groups (**Table 1**). The duration of brain death and the ischemia did not vary between the groups, either (**Table 2**). BUN and Cr increased posttransplant in all groups and returned to normal through POD 4 to 5 (**Figures 2**, **3**) and were not significantly different between the groups except for higher BUN after preconditioning with Everolimus compared to other groups on POD 2 (30 Cyclosporine vs. 43 in Everolimus vs. 24.5 in Methylprednisolone groups, $p = 0.01$) (**Figure 2**), and higher Cr after preconditioning with Cyclosporine on POD 1 (2.39 in Cyclosporine vs. 1.98 in Everolimus vs. 1.58 in Methylprednisolone, $p = 0.02$) (**Figure 3**). The electrolytes showed no difference between the groups throughout the study (**Figures 4**, **5**).

Histopathological Analysis

Histological assessment revealed no significant differences between the groups (**Table 3**). A various degree of acute tubular injury was shown in all groups, with a mean score of 1 (<25%

TABLE 1 | Baseline data.

Variables	Cyclosporine	Everolimus	Methylprednisolone	*p* value
Weight (kg)*	32.76 ± 1.88	34.07 ± 2.20	32.25 ± 1.28	0.130
BUN (mg/dl)*	18.67 ± 7.45	19.37 ± 5.55	25.87 ± 7.95	0.098
Cr (mg/dl)*	1.50 ± 0.34	1.39 ± 0.24	1.36 ± 0.26	0.581
K (mmol/L)*	5.19 ± 1.47	4.75 ± 2.14	3.89 ± 0.70	0.243
Ca (mmol/L)*	2.01 ± 0.27	2.20 ± 0.17	2.19 ± 0.15	0.122
Hemoglobin (g/dl)*	10.38 ± 1.85	11.03 ± 1.47	12.24 ± 1.61	0.087
mean arterial pressure (mmHg)**	62.78 ± 3.53	63.67 ± 3.24	65.00 ± 3.50	0.421
heart rate**	101.22 ± 4.92	97.56 ± 3.50	99.00 ± 5.26	0.255
Temperature (°C)**	35.33 ± 0.22	35.34 ± 0.19	35.47 ± 0.17	0.271
Blood loss (ml)	130 ± 27	137 ± 21	147 ± 17	0.318

*kg, kilogram; mg/dl, milligram per deciliter; mmol/L, millimole per liter; g/dl, gram per deciliter; mmHg, millimeter mercuri; C, centigrade, ml, milliliter; BUN, Blood Urea Nitrogen; Cr, creatinine; K, Kalium; Ca, Calcium, *, preoperative; **, before procurement.*

TABLE 2 | Operative times.

	BD duration [h]	CIT duration [h]	WIT duration [min]
Cyclosporine	6.6 ± 2.1	18.4 ± 2.0	48.9 ± 7.7
Everolimus	7.8 ± 0.8	20.9 ± 3.6	48.9 ± 10.5
Methylprednisolone	6.8 ± 1.6	18.5 ± 2.9	43.8 ± 8.3
All groups	7 ± 1.6	19.3 ± 2.9	47.7 ± 8.7
P-value	0.39	0.24	0.48

Data is presented as mean ± SD. BD, brain death; CIT, cold ischemia time; WIT, warm ischemia time; h, hour; min, minute.

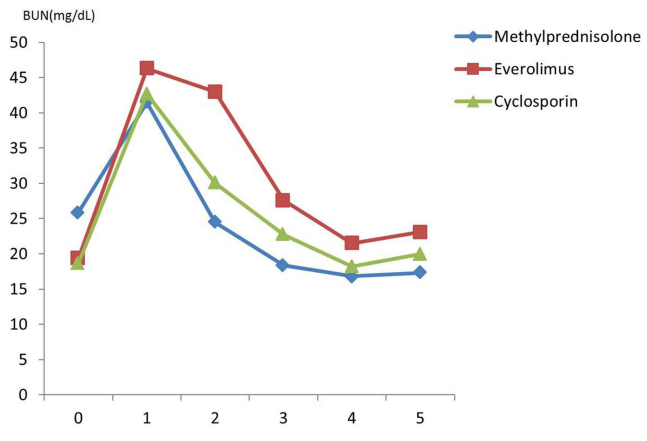

FIGURE 2 | Posttransplant blood urea nitrogen (BUN) in recipients in different study groups.

tubular damage) in Quantitative assessment, as well as a mean score of 1 (mild tubular injury) in qualitative assessment of acute tubular damage, attributable to post-explant ischemia. A significant difference could neither be shown regarding the severity, nor the quantity of ATI.

TABLE 3 | Quantitative and qualitative histopathological assessment of acute tubular injury.

	ATI quantitative*	ATI qualitative#
Cyclosporine	1 (1–2)	1 (1–2)
Everolimus	1 (1–2)	1 (1–2)
Methylprednisolone	1 (1–3)	1 (1–2)
p value	0.825	0.491

**Quantitative assessment of samples was performed to determining acute tubular necrosis as quartiles (1 = 0–25, 2 = 26–50, 3 = 51–75, and 4 = 76–100%).*
#Qualitative assessment of samples determined acute tubular injury (ATI) as quartiles (1 = mild, dilated tubules, partial brush border loss, 2 = moderate ATI, dilated tubules, complete brush border loss, hyaline cylinders, 3 = severe ATI, complete epithelial atrophy, tubule necrosis).

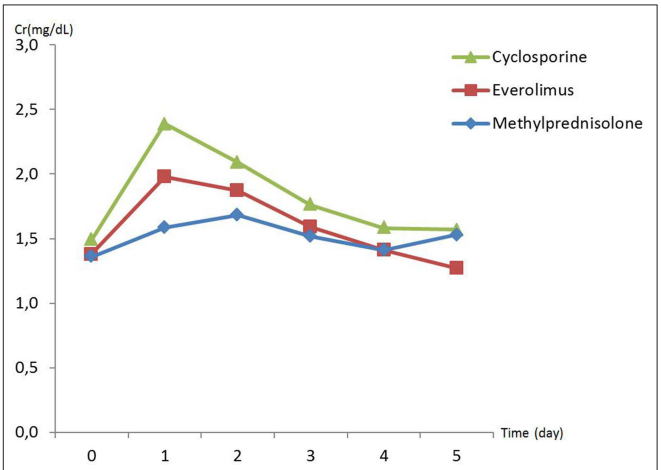

FIGURE 3 | Posttransplant creatinine (Cr) in recipients in different study groups.

Immunohistochemistry

Figures 6–11 show the immunohistochemical staining as well as the (semi)quantitative assessment of the expression of TNF-α and cytochrome c 4 h after reperfusion. TNF-α expression in the immunohistochemistry staining was significantly higher in the Methylprednisolone groups compared with the Everolimus and Cyclosporine groups ($p = 0.01$). This significance was seen in both PS and TS ($P < 0.01$, **Figure 12** A1 and A3). There was no difference in cytochrome c expression between the groups.

DISCUSSION

Brain death triggers an inflammatory response in the donor organs with T lymphocyte and macrophage infiltration and release of multiple proinflammatory cytokines, among all TNF-α, Interleukin-6, and Interleukin-10, which has been shown to enhance the immunogenicity of the organs and potentiate the deleterious effects of IRI after organ transplantation (9). The pharmacologic preconditioning of the donor has been shown to ameliorate the allo-immune response to

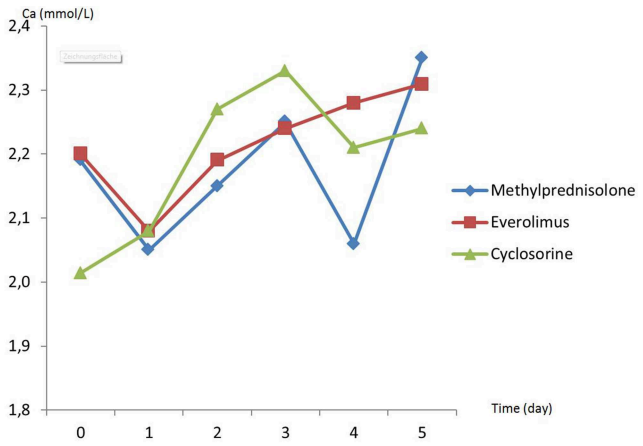

FIGURE 4 | Posttransplant calcium (Ca) in recipients in different study groups.

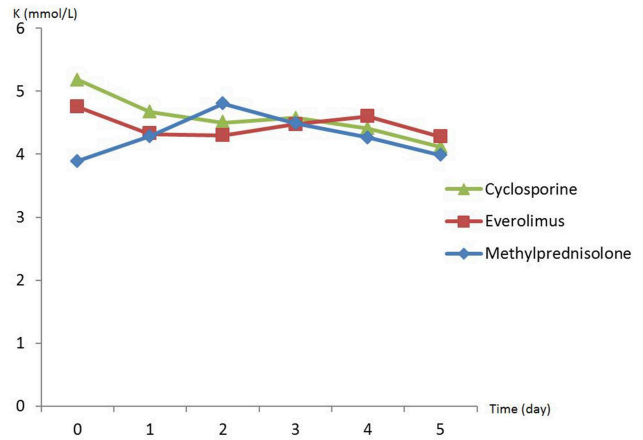

FIGURE 5 | Posttransplant potassium (K) in recipients in different study groups.

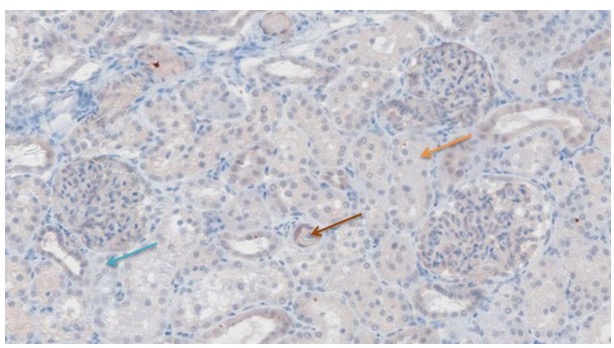

FIGURE 6 | TNF-α antibody staining after preconditioning with Cyclosporine. Arrows show different intensities; blue: intensity 0, orange: intensity 1, brown: intensity 2, and black: intensity 3.

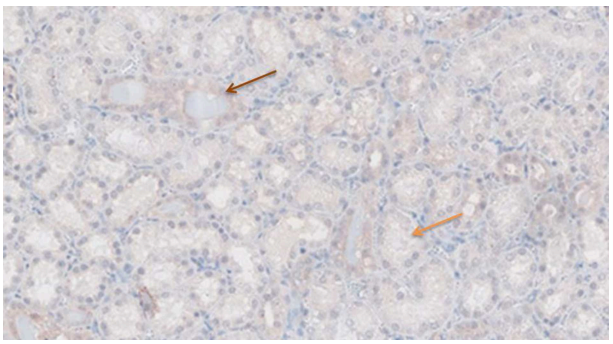

FIGURE 7 | TNF-α antibody staining after preconditioning with Everolimus. Arrows show different intensities; blue: intensity 0, orange: intensity 1, brown: intensity 2, and black: intensity 3.

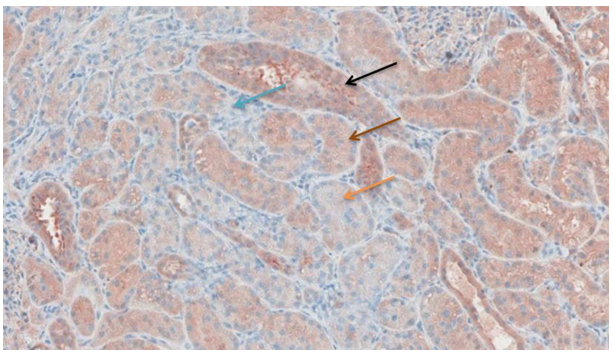

FIGURE 8 | TNF-α antibody staining after preconditioning with Methylprednisolone. Arrows show different intensities; blue: intensity 0, orange: intensity 1, brown: intensity 2, and black: intensity 3.

this enhanced immunogenicity after DBD (10–15). Few studies have investigated pharmacological preconditioning with Cyclosporine in rat kidneys (16, 17). In these studies, preconditioning with Cyclosporine led to improved renal function and histology, increased heat shock protein 70, and decreased expression of pro-inflammatory cytokines (Interleukin-1 and TNF-α) as well as amelioration of oxidative stress after IRI. In contrast, other studies observed aggravated IRI in rat kidney after Cyclosporine, as detected by increased renal dysfunction, decreased Glomerular filtration rate (GFR) and delayed tubular regeneration (18–20). Similarly, there have been reports on negative effects of sirolimus on IRI (including renal dysfunctions, delayed tubular regeneration and increased expression of heme oxygenase-1) (21), while others observed no negative effect of sirolimus pre-treatment on renal outcome after IRI (22). Moreover, data regarding oral donor preconditioning with immunosuppressive agents on the outcome of renal transplantation is scares. One study has shown that the oral donor pharmacological preconditioning with Everolimus or Cyclosporine does not reduce IRI in a rat kidney transplant model (23). We have previously shown that the oral administration of a preconditioning nutritional supplement is protective against IRI in pigs (24). The possible responsible mechanisms include the inactivation of hepatic Kupffer cells via cellular and molecular mechanisms

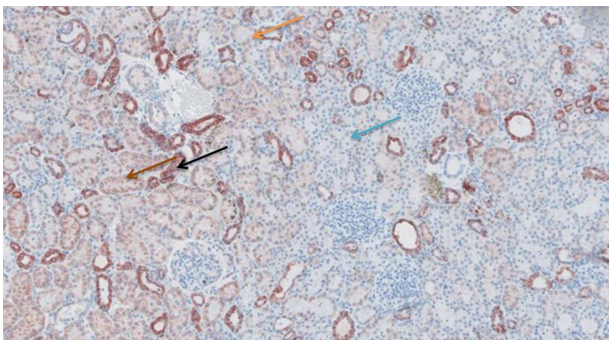

FIGURE 9 | Cytochrome c antibody staining after preconditioning with Cyclosporine. Arrows show different intensities; blue: intensity 0, orange: intensity 1, brown: intensity 2, and black: intensity 3.

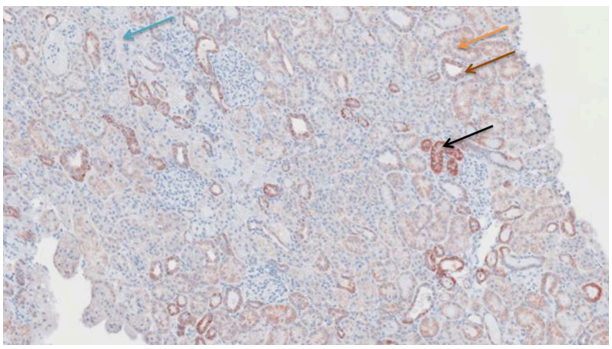

FIGURE 11 | Cytochrome c antibody staining after preconditioning with Methylprednisolone. Arrows show different intensities; blue: intensity 0, orange: intensity 1, brown: intensity 2, and black: intensity 3.

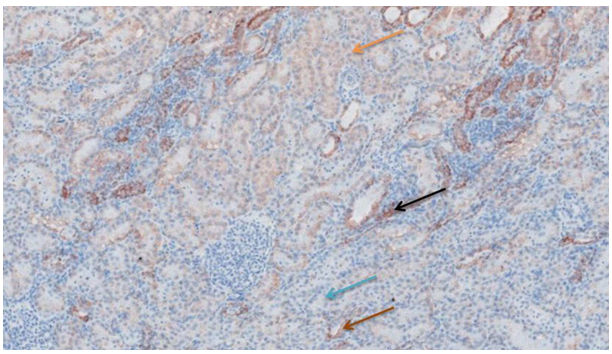

FIGURE 10 | Cytochrome c antibody staining after preconditioning with Everolimus. Arrows show different intensities; blue: intensity 0, orange: intensity 1, brown: intensity 2, and black: intensity 3.

including bacterial translocation and lipopolysaccharide release that prevents the systemic cytokine release, adhesion molecules, leukocyte infiltration and subsequent histological changes. There is a pivotal interaction between the intestinal epithelium, the enteric antigen-presenting cells (e.g., gut dendritic cells), portal circulation, and hepatic kupffer cells, so that the tackling of the IRI via pharmacologic oral preconditioning may significantly modulate the ultimate immune response of the host (25). As for standard application in human kidney transplantation, the absorption phase for CSA occurs over the first 4 h after oral administration. Oral everolimus is absorbed rapidly, and reaches peak concentration after 1.3–1.8 h. For this reason, we administrated the oral CSA and Everolimus only few hours before organ procurement.

To our knowledge, there has been no study on the oral preconditioning of DBD donor in a big animal transplant model. Our present work showed that oral preconditioning with Cyclosporine or Everolimus in DBD pig kidney transplantation is feasible and down-regulates TNF-α expression. The reduction in TNF-α expression seems to be plausible in our model, as an increase of intragraft TNF-α expression is documented in the organs of DBD donors, and after organ reperfusion. TNF-α aggravates the adherence of leukocytes to vascular endothelium leading to enhancement of IRI and acceleration of acute allograft rejection after organ transplantation (26–30). The observed reduction of TNF-α expression might be a hint to suggest an IRI-reducing effect of the preconditioning with Cyclosporine and Everolimus.Cytochrome c, on the other hand, is a hemeprotein in the inner mitochondrial membrane. It has been shown that IRI leads to membrane depolarization by calcium overload in mitochondria, leading to opening of the mitochondrial permeability transition pore. As a result, cytochrome c is released into cytosol and activates the caspase family, leading to apoptosis (31). Our immunohistochemistry stains of cytochrome c showed, however, no difference between the groups. The release of cytochrome c into cytosol might have occured later than 4 h after reperfusion.

In the present work, in order to stimulate the actual clinical practice, we induced hypotensive brain death in our donors, and allowed 6 h' time for the inflammatory response following brain death to develop. Moreover, we kept an average of 18 h cold ischemia time to enhance IRI. No posttransplant immunosuppression therapy in recipients was administered to avoid confounding bias.

In order to detect the early allograft changes after IRI, the protocol biopsies were performed 4 h after reperfusion. Kusaka et al. have shown that the early changes including the expression of the inflammatory proteins could take place as early as 1 h after the implantation of the kidneys after DBD (32). Although we could show a difference in the expression of TNF-α between the study groups after four hours, our conventional histopathological studies were not different between the different groups, which might imply that the interval being too short to observe the histological changes of tubular damage. Although we have followed the recipients until POD 5 for the quality controlling of the transplants, we did not look for POD 5 biopsies as the findings would have not been specifically attributable to the immunological effects after DBD, and rather acute rejection.

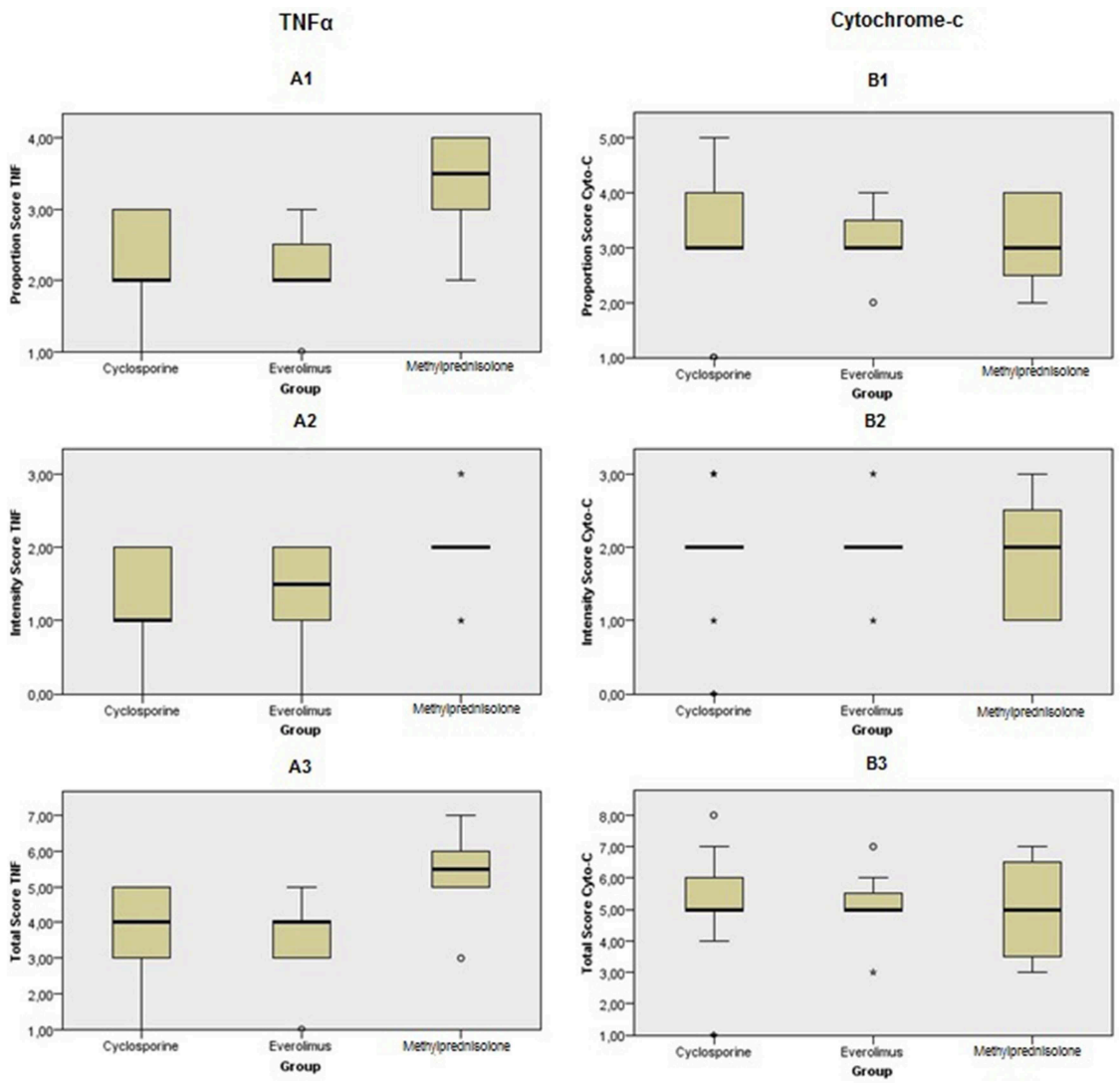

FIGURE 12 | Immunohistochemical scoring after preconditioning with Cyclosporine, Everolimus, and Methylprednisolone 4 h following kidney transplantation. (A1) PS in TNF-α, (A2) IS in TNF-α, (A3) TS in TNF-α, (B1) PS in cytochrome c, (B2) IS in cytochrome c and (B3) TS in cytochrome c. *P* values were calculated for comparison between intervention groups (Everolimus and Cyclosporine) and Methylprednisolone group. PS and TS were significantly different in TNF-α staining between groups. It shows that apoptosis process was started sooner in Methylprednisolone groups rather than the others.

This was to avoid the acute rejection as a confounding bias. The clinical as well as lab data through POD 5 including the laboratory tests showed no relevant difference between the groups.

The present work has its own limitations. Although we administered the routine immunosuppressive doses, data on the appropriate oral doses of Cyclosporine and Everolimus for the purpose of oral preconditioning is lacking. Furthermore, the best time points for the administration of the oral preconditioning agents as well as the frequency of medication are not known and vary widely among different studies. Furthermore, the best time point to look for the early innate host immune response triggered synergistically by IRI and DBD in allografts is still unclear.

In summary, our findings suggest the feasibility of the oral preconditioning with CNI or mTORi in DBD donors in pig kidney transplantation. A reduced expression of TNF-α in transplanted organs in the early post-transplant phase was seen after oral preconditioning with these agents. Our data can serve as a platform for future experimental and clinical studies to evaluate the protecting role of donor oral preconditioning against IRI and its clinical relevance.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany (file number: 35-9185.81/G-5/16).

AUTHOR CONTRIBUTIONS

AN, AMe, and SA developed the original concept of the study. AN, SA, MN, OG, and EK developed the design and methodology. SA, OG, EK, MN, AA, SM, AMa, MS, MK, and AY participated in the operations and data collection. CE and TP participated in the pathological assessment. MN, EK, OG, and AA performed the statistical assessments and developed the analysis plan. SA, AN, MN, EK, OG, and AA contributed to drafting the article. AN, NG, MG, SM, AMa, MS, MK, AY, and AMe contributed to the revision of the final report. All authors read and approved the final manuscript.

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Non-ischemic Heart Preservation via Hypothermic Cardioplegic Perfusion Induces Immunodepletion of Donor Hearts Resulting in Diminished Graft Infiltration Following Transplantation

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Introduction: Many donor organs contain significant leukocyte reservoirs which upon transplantation activate recipient leukocytes to initiate acute rejection. We aimed to assess whether non-ischemic heart preservation via *ex vivo* perfusion promotes immunodepletion and alters the inflammatory status of the donor organ prior to transplantation.

Methods: Isolated porcine hearts underwent *ex vivo* hypothermic, cardioplegic perfusion for 8 h. Leukocyte populations were quantified in left ventricle samples by flow cytometry. Cell-free DNA, cytokines, and chemokines were quantified in the perfusate. Tissue integrity was profiled by targeted proteomics and a histological assessment was performed. Heterotopic transplants comparing *ex vivo* hypothermic preservation and static cold storage were utilized to assess graft infiltration as a solid clinical endpoint.

Results: *Ex vivo* perfusion significantly immunodepleted myocardial tissue. The perfusate displayed a selective, pro-inflammatory cytokine/chemokine pattern dominated by IFN-γ. The tissue molecular profile was improved following perfusion by diminished expression of nine pro-apoptotic and six ischemia-associated proteins. Histologically, no evidence of tissue damage was observed and cardiac troponin I was low throughout perfusion. Cell-free DNA was detected, the source of which may be necrotic/apoptotic leukocytes. Post-transplant graft infiltration was markedly reduced in terms of both leucocyte distribution and intensity of foci.

Conclusions: These findings demonstrate that *ex vivo* perfusion significantly reduced donor heart immunogenicity via loss of resident leukocytes. Despite the pro-inflammatory

cytokine pattern observed, a pro-survival and reduced ischemia-related profile was observed, indicating an improvement in graft viability by perfusion. Diminished graft infiltration was observed in perfused hearts compared with those preserved by static cold storage following 48 h of transplantation.

Keywords: heart transplantation, acute rejection, heart preservation, hypothermic cardioplegic *ex vivo* heart perfusion, passenger leukocytes

INTRODUCTION

Heart transplantation represents the only effective treatment option for end stage heart failure, but is limited by a lack of suitable donor organs. Standard donor heart preservation utilizes static storage on ice (1), which inherently causes ischemic injury, limiting the duration for which the heart can be stored before transplantation. In an effort to address this problem and increase the donor pool, our group have developed a method of non-ischemic heart preservation using hypothermic cardioplegic *ex vivo* heart perfusion (HCP). This technology can safely extend preservation times to 24 h with stable function following transplantation (2), and has been successfully used to safely perfuse and transplant a heart by the clinical transplant team from Lund in 2017. HCP combines the protective effect of minimized metabolic demand with optimal nutritional support and oxygenation. Whilst this has clear implications for improved donor organ preservation, the potential for auxiliary benefits following transplantation have not been explored, particularly with regard to acute graft rejection.

Acute graft rejection represents a major barrier to successful transplantation requiring permanent immunosuppression, which predominantly target recipient T cells. However, little attention is paid to the donor immune compartment which can orchestrate acute rejection of the transplanted heart (3). Depletion of donor dendritic cells is sufficient to prevent rejection of transplanted lungs in mice (4) and reintroduction of donor dendritic cells restores the immune response following rat kidney transplantation (5). We have previously demonstrated that *ex vivo* perfusion is sufficient to alter immunogenicity of the donor lung and kidney via removal of passenger leukocytes, and this significantly reduces recipient T cell recruitment at 24 h post-transplantation (6, 7).

In this study, we aimed to explore the impact of HCP on the donor immune compartment and provide early pilot data to indicate how this may alter clinical outcome following transplantation.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the local Ethics Committee for Experimental Research. Animals were treated in compliance with the "Guide for the Care and Use of Laboratory Animals" published by NIH (Eight Edition, revised 2011) and the European Directive 2010/63/EU "On the protection of animals used for scientific purposes."

PERFUSION STUDY

Donor Organ Retrieval and HCP

Six healthy 6 month old Swedish pigs were used. All pigs were free of pericardial exudates and observable cardiac pathology during harvesting. Anesthesia and donor organ retrieval were performed as previously described in detail (8). HCP was performed as described previously although with continuous rather than intermittent perfusion (8). All organs were perfused at a constant 20 mmHg perfusion pressure with the aim of 100 ml/min minimum coronary flow. This fixed pressure system enables the organ to regulate its own coronary flow without forcing perfusate through at excessive pressure.

Biopsy Processing

Left ventricle tissue was obtained by surgical dissection from the apical region before and after 8 h of HCP and split into 3 sections. Tissue weighing 30–100 mg was dissected and homogenized in 25 ml Hank's buffered salt solution (Sigma-Aldrich, Dorset, UK). Homogenates underwent serial filtration through 500, 250, and 40 μm strainers. Cells were washed and flow cytometry performed. The second section was snap frozen in liquid nitrogen and stored at −80°C. The final section was fixed in 10% buffered formalin and paraffin embedded.

Perfusate Collection

Perfusate was collected at baseline and every 2 h throughout perfusion. The final sample was taken immediately prior to retrieval of the final myocardial sample. Samples were centrifuged at 2,000 g for 10 min and plasma stored at −80°C.

Leukocyte Filter Processing

Following perfusion, the leukocyte filter was removed and trypsinized at 37°C for 15 min. Filter contents were assessed using flow cytometry.

Inflammatory Profiling

Thirteen cytokines were quantified in undiluted perfusate supernatant using a porcine Luminex assay (Merck Millipore, Billerica, MA, USA) and analyzed using a Bio-Plex 200 system (Bio-Rad, Herts, UK).

Abbreviations: HCP, Hypothermic cardioplegic *ex vivo* heart perfusion; DNA, Deoxyribonucleaic acid; IFN, Interferon; CD, Cluster of differentiation; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; STAT, Signal transducer and activator of transcription.

Chemokine Quantification

ELISA kits were used to quantify CCL2, CCL4, CCL5, CXCL9, CXCL10 (Insight Biotechnology, Wembley, UK) and CXCL11 (2BScientific, Oxfordshire, UK) in perfusate supernatant without dilution. Absorbance was read using a Tecan infinite 200 PRO system (Tecan Group, Männedorf).

Flow Cytometry

Using an Attune flow cytometer (ThermoFisher, Massachusetts, US), a single cell suspension obtained by homogenization of the left ventricle was analyzed to quantify immature neutrophils (6D10+2B2–), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10–2B2+), helper T cells (CD4α+CD8β-), cytotoxic T cells (CD4α –CD8β+), NK cells (CD335+), B cells (CD21+), classical monocytes (CD14+CD163–), non-classical monocytes (CD14+CD163+), intermediate monocytes ($CD14^{dim}$ $CD163^{bright}$), and macrophages (CD203a+). SLA-DR expression was assessed as a marker of antigen presentation. Toll-like receptor 4 expression was assessed on each population. Viability was assessed using propidium iodide. All gating strategies and absolute cell counts were determined using Attune Cytometric software. Cell counts were normalized per milligram of starting tissue.

Quantitative PCR

Primers were designed to detect mitochondrial DNA (cytochrome b) and genomic DNA (GAPDH) (Sigma Aldrich, Dorset, UK) using Primer Express® Software v3.0.1 (LifeTech, Paisley, UK) and homology assessed using BLAST (see **Supplementary Methods**). qPCR analysis was performed with a QuantStudio 12K Flex system using a Power SYBR green PCR master mix (LifeTech, Paisley, UK).

Phosphokinase and Apoptosis Signaling

Tissue biopsies were obtained from each pig before and after perfusion. Phosphokinase and apoptosis antibody proteome profile arrays were used according to manufacturer's instructions (R&D systems, Abingdon, UK). A separate membrane was utilized for each sample. Chemiluminescence detection was performed using a ChemiDoc MP imaging system (Bio-Rad, Herts, UK). Pixel density analysis was performed using ImageJ (NIH, USA).

Cardiac Tissue Viability

Troponin I was quantified in undiluted perfusate supernatant to detect cardiac injury by ELISA (Abbexa, Cambridge, UK). Absorbance was read at 450 nm using a Tecan infinite 200 PRO system (Tecan Group, Männedorf).

Evaluation of Tissue Integrity

Histological assessment was performed using formalin-fixed tissue obtained before and after perfusion. Sections were cut at 4 μm, de-paraffinized and stained with hematoxylin and eosin. Separate sections were stained for caspase-3 expression as a marker of apoptosis (see **Supplementary Methods**). All samples were blinded from the consultant histopathologist.

HETEROTOPIC HEART TRANSPLANT PILOT STUDY

Heterotopic Transplant Procedure

In order to determine whether immunodepletion by HCP altered clinical outcome we performed six heterotopic heart transplants. Six donor organs were harvested as above from 6 month old pigs. Three organs were preserved by 2 h of static cold storage and three organs preserved by 8 h of HCP. Recipient pigs (6 months old, weighing 58–64 kg) were selected based on a blood group cross match with the donor, and received anesthesia via intramuscular ketamine hydrochloride (25 mg/kg; Pfizer, Sweden) and xylasin (4 mg/kg; Bayer, Sweden). Recipient pigs were ventilated throughout the procedure. Once anesthetized, a longitudinal incision was made to the left of the linea alba. At implantation, the aorta of the donor heart was sutured end-to-side to the infrarenal aorta and the pulmonary artery was connected end-to-side to the vena cava. Reperfusion was commenced at the earliest opportunity and the hearts were defibrillated if sinus rhythm was not spontaneously established. Once the donor heart had achieved sinus rhythm, the incision was closed and the pig was awakened. Recipient pigs were maintained without immunosuppression for 48 h. Following euthanasia, biopsies were collected from the heart for histological analysis.

Histological Evaluation of Graft Infiltration

Histological assessment of the donor heart was performed. 4 μm sections were de-paraffinized and stained with hematoxylin and eosin. Samples were prepared and assessed by a consultant histopathologist who reported intensity of leukocyte infiltration on an ordinal scale of severity (0 = no infiltration, 1 = mild infiltration, 2 = moderate infiltration, and 3 = severe infiltration). The distribution of infiltration was analyzed and presented as a percentage of the field of view affected.

Statistical Analysis

Prism software version 7.00 (GraphPad, LaJolla, California, USA) was used to perform all statistical analyses. Data are expressed as mean ± standard deviation if normally distributed or as median [interquartile range] if non-normally distributed. Paired samples *T*-tests or related samples Wilcoxon signed rank tests were utilized to assess the changes in leukocyte content and protein expression profile between pre and post perfusion tissue samples depending upon data distribution. The related samples Friedman's two-way ANOVA by ranks was utilized to assess changes in the perfusate over time. Statistical significance was accepted when $p \leq 0.05$.

RESULTS

Baseline Myocardial Leukocyte Content

We first profiled the donor heart immune repertoire by flow cytometry to generate a baseline reference using a single cell suspension from left ventricle tissue. We demonstrate a significant cardiac-resident immune repertoire including large

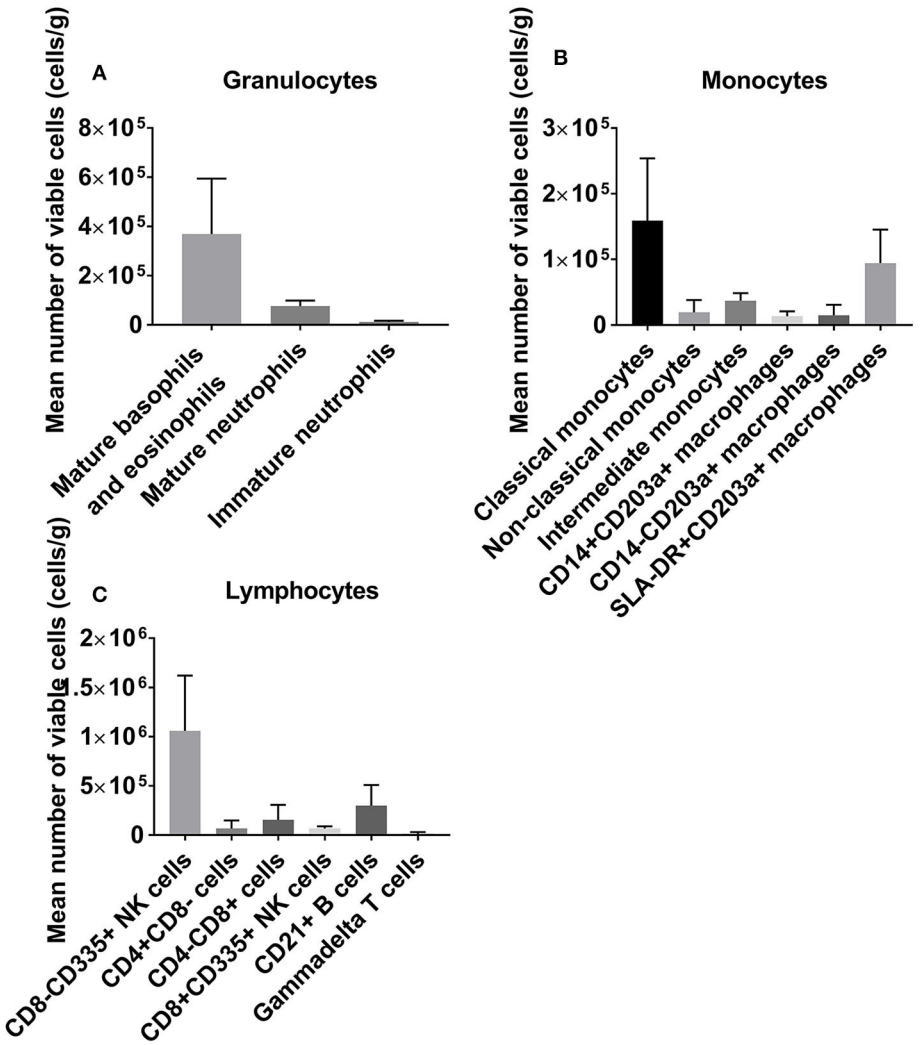

FIGURE 1 | A baseline reference of the leukocyte repertoire resident within the donor heart, categorized into granulocytes **(A)**, monocytes/macrophages **(B)**, and lymphocytes **(C)**. NK cells are abundant, with large granulocyte populations and B cells also observed.

populations of both innate and adaptive cells. NK cells represent the largest immune phenotype detected in the tissue (**Figure 1**).

Perfusion-Associated Variables

Clinically relevant parameters associated with organ retrieval and HCP were recorded. A mean cold ischemic time of 18.5 ± 7.66 min between retrieval and perfusion was recorded. Coronary flow ranged from 100 to 200 mL/min depending on organ weight.

HCP Induces Donor Heart Immunodepletion

There was a significant loss of viable leukocytes from the tissue following perfusion (**Figure 2**), including mature neutrophils (−85%, $p = 0.003$), mature basophils/eosinophils (−84%, $p = 0.023$), classical monocytes (−72%, $p = 0.024$), and B cells (−60%, $p = 0.042$). Depletion of immature neutrophils ($p = 0.011$), CD14+CD203a+ and CD14–CD203a+ macrophages (both $p = 0.043$) and CD8+ NK cells ($p = 0.003$) was also observed. Non-classical monocytes ($p = 0.117$) and γδ T cells ($p = 0.119$) were reduced for each sample pair but this did not reach significance. CD8– NK cells were markedly reduced in all but one heart although this was not statistically significant ($p = 0.129$). Intermediate monocytes ($p = 0.225$), SLA-DR+CD203a+ macrophages ($p = 0.500$), helper T cells ($p = 0.409$), and cytotoxic T cells ($p = 0.140$) were not altered by perfusion. Toll-like receptor 4 expression did not change significantly on any population except for mature neutrophils (see **Figure S1**).

Immunodepletion Using Leukocyte Filtration

The content of the in-line leukocyte filter comprised predominantly NK cells, classical monocytes, mature basophils/eosinophils, and T cells. Whilst B cells represented a

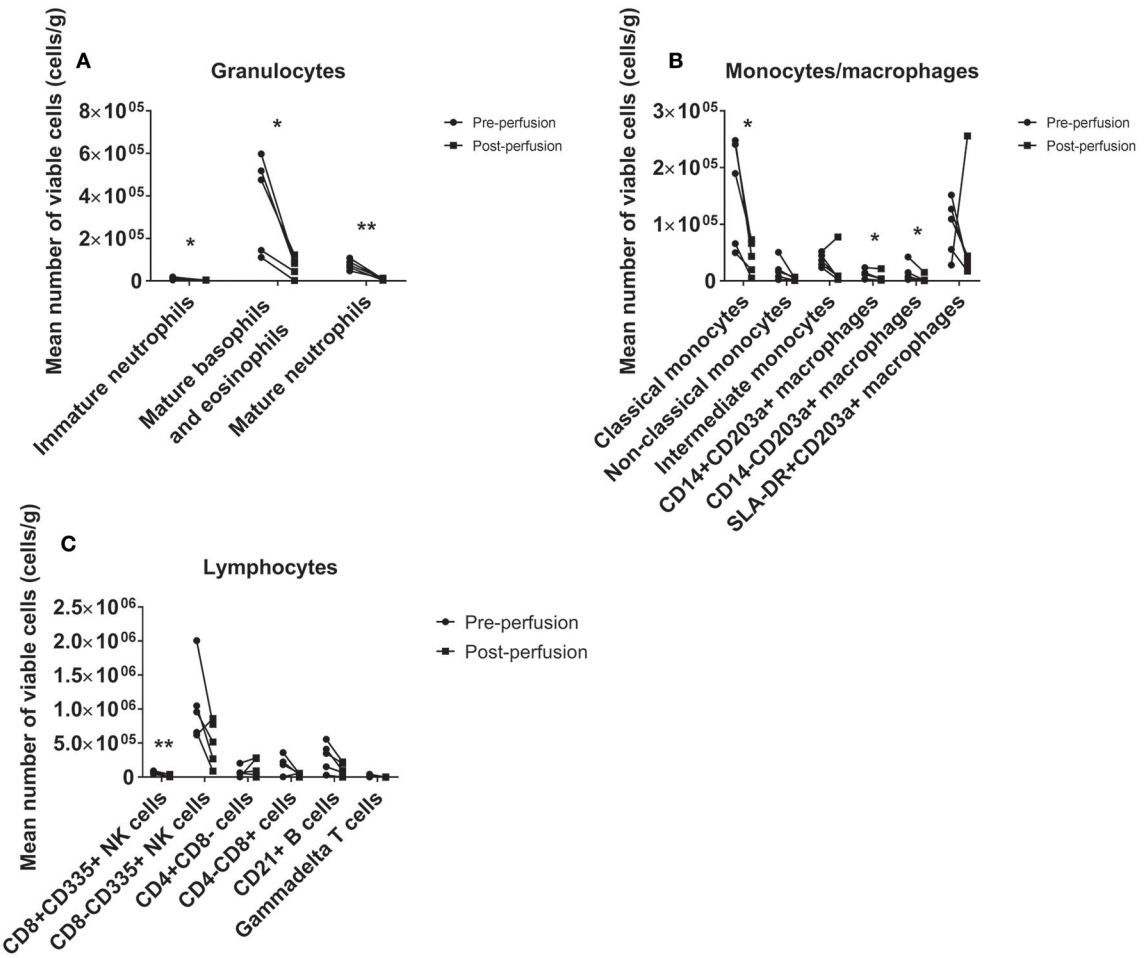

FIGURE 2 | Immunodepletion of the donor heart via HCP. We observed significant leukocyte loss from the tissue across a range of phenotypes, including granulocytes **(A)**, monocytes/macrophages **(B)**, and lymphocytes **(C)**. All granulocyte populations were markedly reduced, in particular mature neutrophils and mature basophils/eosinophils (86 and 84% reductions, respectively). $^{*}p < 0.05$, $^{**}p < 0.01$.

large population in the tissue and were significantly depleted by perfusion, they were not well-retained by the filter suggesting some other mechanism of loss. Collectively the leukocyte filter did not account for all cells lost (**Figure S2**).

HCP Mediates an Inflammatory Storm Dominated by Interferon-γ

Of Twelve cytokines analyzed, only 4 were detected. Interferon-γ (IFN-γ) increased markedly and dominated the cytokine profile (peaking at 7,610 pg/ml, $p = 0.003$, **Figure 3A**). Significant increases in granulocyte-macrophage colony-stimulating factor (GM-CSF) (peaking at 50 pg/ml, $p = 0.021$, **Figure 3B**), interleukin (IL)-18 (peaking at 120p pg/ml, $p = 0.001$, **Figure 3C**) and tumor-necrosis factor (TNF)-α (peaking at 55 pg/ml, $p = 0.001$, **Figure 3D**) were also detected as perfusion progressed.

Chemokine Release Is Induced by HCP

To determine whether leukocyte migration occurred due to specific chemotactic signals, we quantified 7 chemokines within the perfusate (**Figure 4**). Due to the high IFN-γ concentration we focused on chemokines responsive to IFN-γ stimulation. CCL5 and CXCL11 were not detectable during perfusion. CXCL8 concentration increased significantly over time, peaking at 4 h but remaining elevated until 8 h ($p = 0.001$). A small increase in CCL2 ($p = 0.021$) and a large increase in CXCL9 ($p < 0.001$) were observed over time. CCL4 and CXCL10 were detected in the perfusate from 4/6 to 2/6 pigs, respectively, and thus demonstrated no significant changes over time ($p = 0.184$ and $p = 0.255$, respectively).

Impact of Ischemia-Reperfusion Injury Following HCP

We profiled the immunodepleted tissue to assess whether HCP altered phosphorylation status of a broad range of protein kinases. Six proteins intrinsically linked to ischemia-reperfusion injury (IRI) were diminished following HCP including ^{689}Y-phospho STAT2 (fold change: 0.88, $p = 0.044$), ^{694}Y/^{699}Y-phospho STAT5a/b (fold change: 0.82, $p = 0.011$), ^{694}Y-phospho

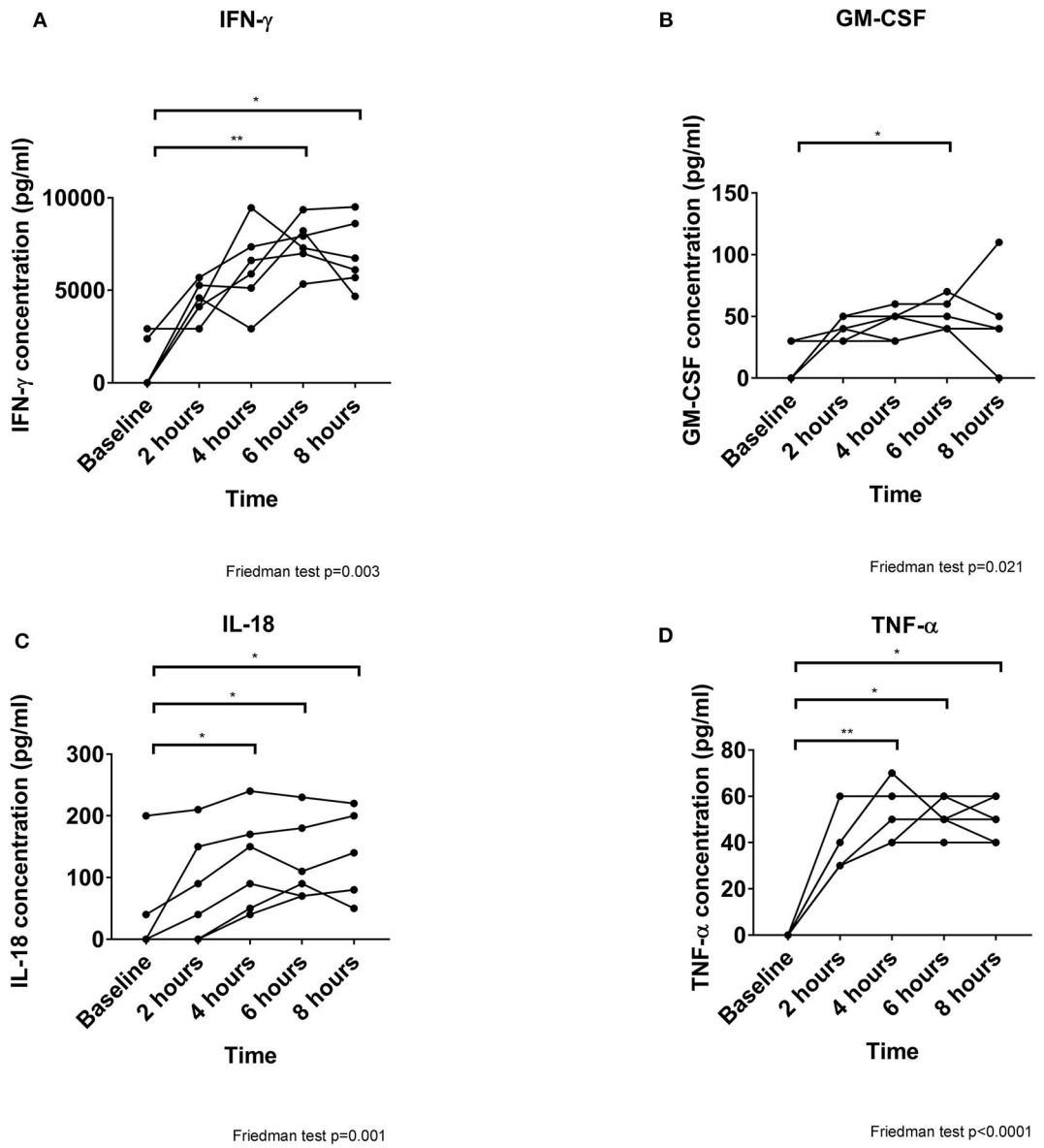

FIGURE 3 | Cytokine secretion increases over time during perfusion. All 4 cytokines detected are increased significantly as perfusion progresses, although IFN-γ **(A)** is released at markedly greater concentrations than GM-CSF **(B)**, IL-18 **(C)**, and TNF-α **(D)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

STAT5a (fold change: 0.78, $p = 0.028$), ^{641}Y-phospho STAT6 (fold change: 0.87, $p = 0.009$), ^{133}S-phospho CREB (fold change: 0.62, $p = 0.045$), and ^{60}T-phospho WNK1 (fold change: 0.61, $p = 0.022$).

HCP Alters Cell Death Pathways

Nine cell death proteins were diminished following HCP, including ^{46}S-phospho p53 (fold change: 0.85, $p = 0.046$), TNF receptor 1 (fold change: 0.86, $p = 0.009$), death receptor 5 (fold change: 0.87, $p = 0.001$), heme oxygenase 1 (fold change: 0.88, $p = 0.015$), Bad (fold change: 0.85, $p = 0.034$), Bcl-x (fold change: 0.70, $p = 0.041$), pro-caspase-3 (fold change: 0.83, $p = 0.019$), claspin (fold change: 0.85, $p = 0.045$), and clusterin (fold change: 0.78, $p = 0.018$, **Figure 5**).

Cell-Free DNA Is Released During Perfusion

Steadily increasing release of both mitochondrial ($p = 0.063$) and genomic DNA ($p = 0.037$) was observed during HCP (**Figure 6**). Genomic DNA was consistently released at a significantly higher concentration than mitochondrial DNA ($p = 0.009$).

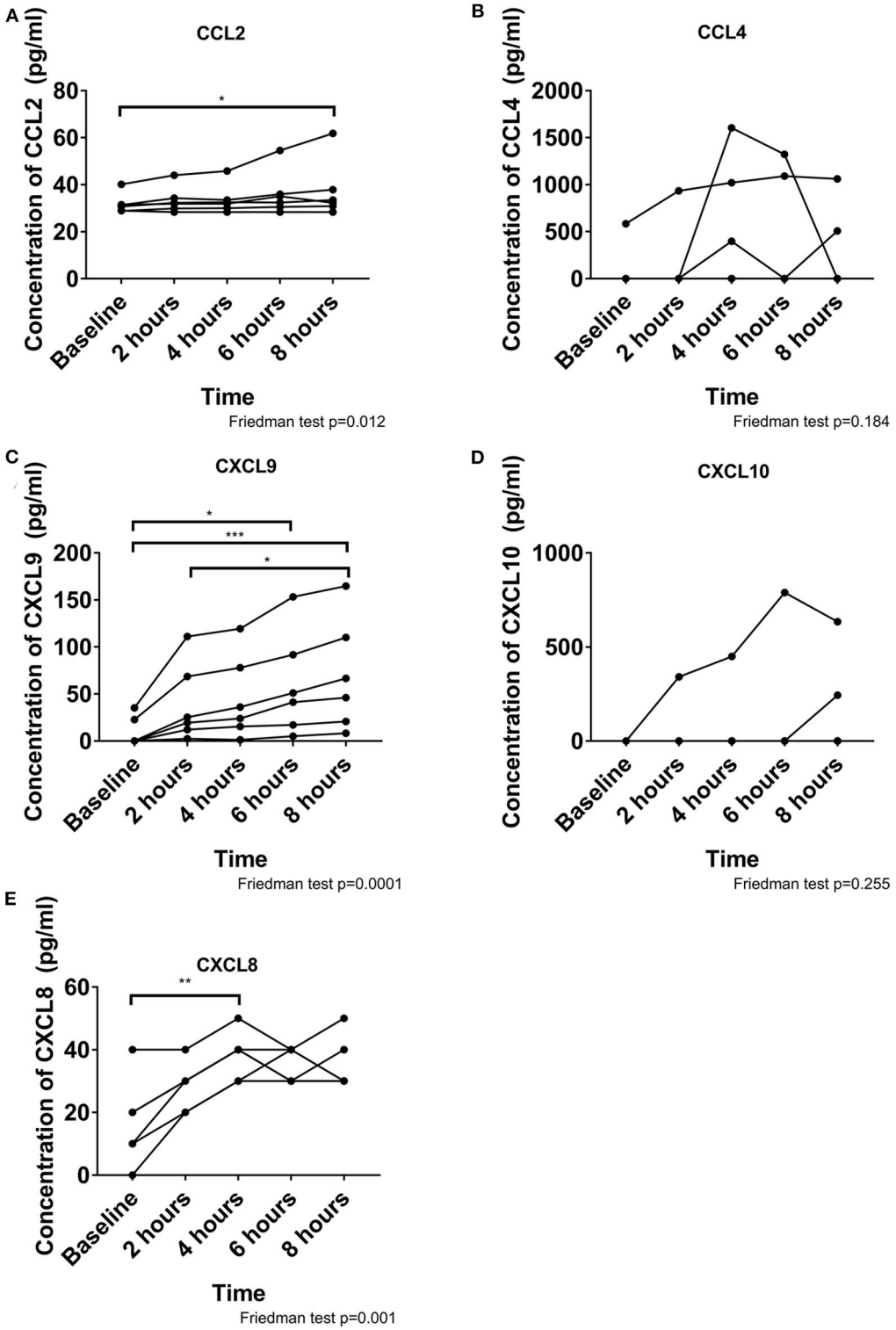

FIGURE 4 | Chemokine release during perfusion is dominated by CXCL9, CXCL8, and CCL2. A small increase is observed over time for CCL2 **(A)**. CCL4 does not change over time and was inconsistent between pigs **(B)**. CXCL9 consistently increased over time **(C)**. CXCL10 was not detected in all pigs and did not change over time **(D)**. CXCL8 increased significantly by 4 hours and remained elevated throughout **(E)**. These chemokines may contribute to the migration of leukocytes out of the heart. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

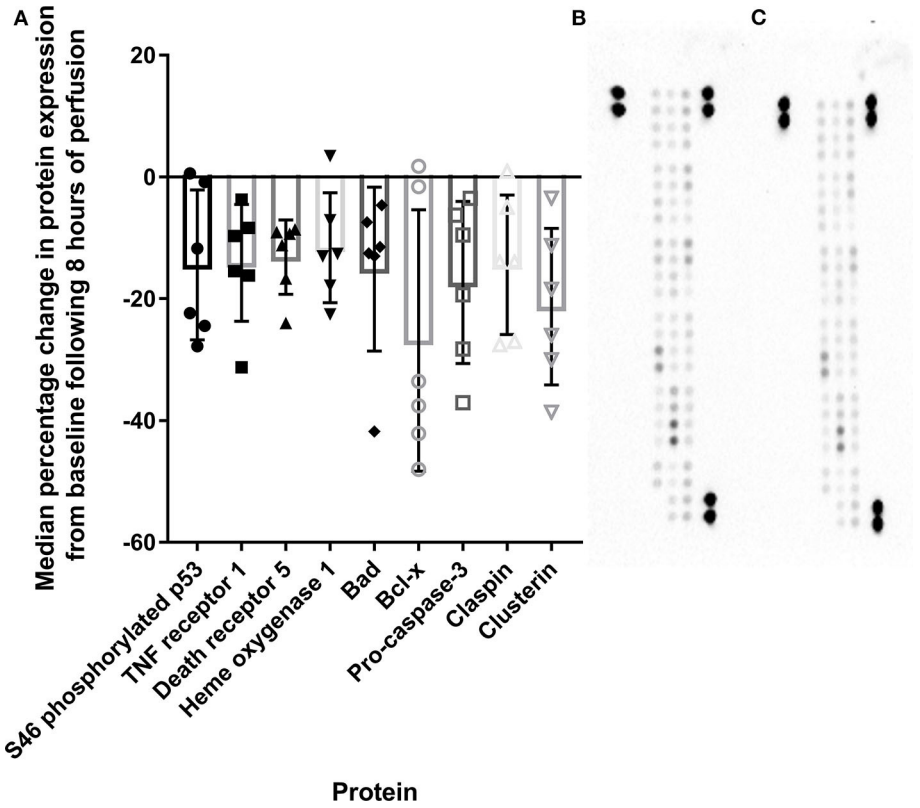

FIGURE 5 | Apoptosis-related protein expression is diminished compared to baseline tissue following 8 h of HCP **(A)**. Representative array blots are provided for pre-perfusion **(B)** and post-perfusion **(C)**. TNF, Tumor necrosis factor; S46, serine 46; Bcl, B cell lymphoma.

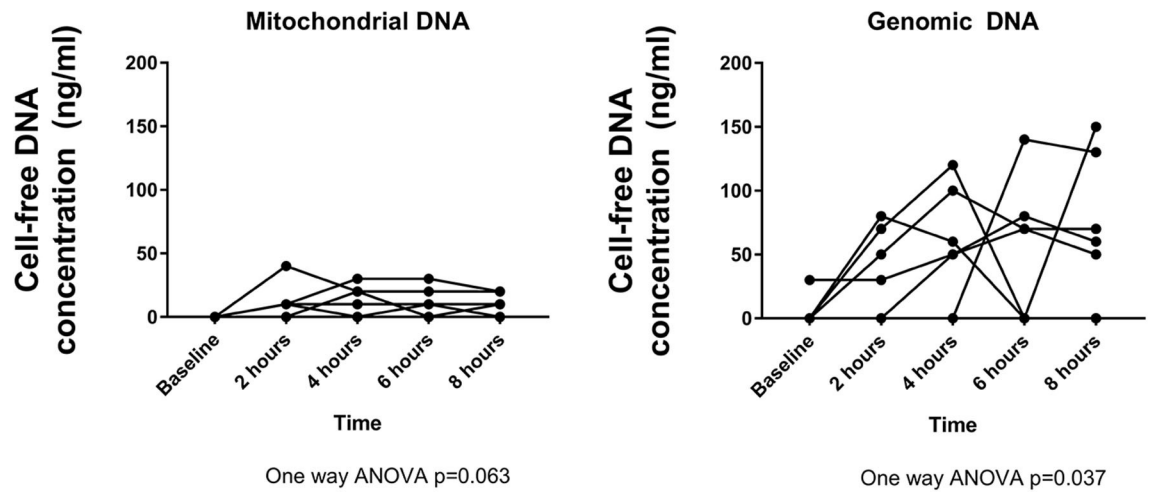

FIGURE 6 | Cell-free DNA is released into the perfusate at increasing concentrations over time. Mitochondrial DNA peaks at ~4 h, whereas genomic DNA peaks at 6 h. Genomic DNA is detected at higher concentrations than mitochondrial DNA.

Tissue Viability Is Maintained Throughout Perfusion

As a clinically relevant end-point, a blinded histological analysis of tissue architecture was performed. HCP preserved the myocardium without ischemia or endothelial disruption (**Figure 7**) and caspase-3 expression remained undetectable in the muscle, endothelium, and fibroblasts, although apoptotic leukocytes were observed

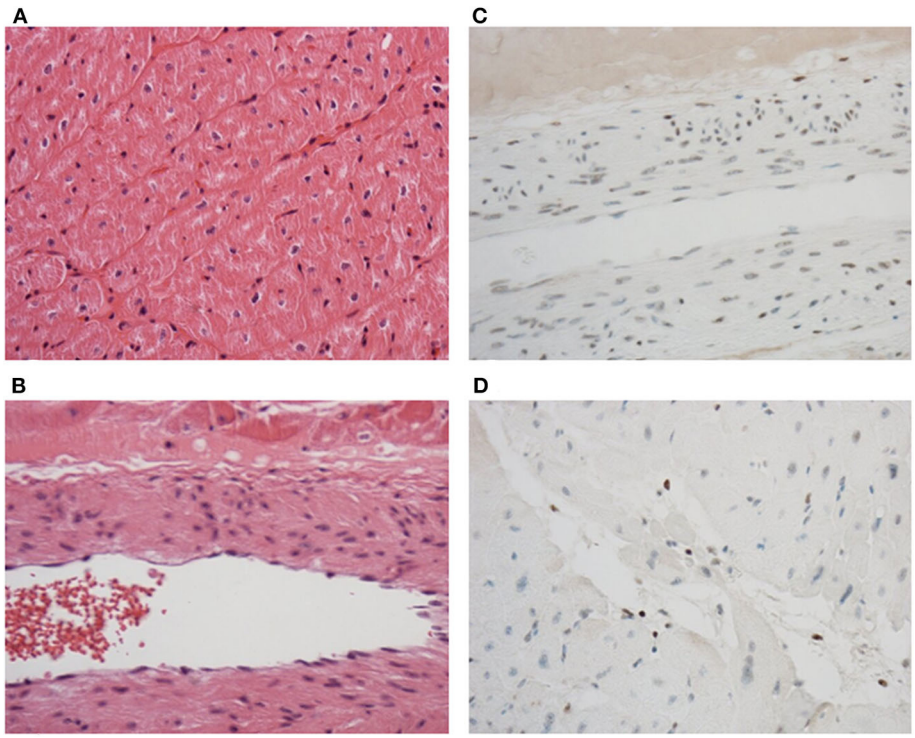

FIGURE 7 | Tissue architecture and structural integrity are maintained throughout perfusion. No edema or damage to muscle **(A)** or endothelial cells **(B)** were observed after perfusion in sections stained with hematoxylin and eosin. No caspase-3 induction was observed in the muscle, endothelium, or fibroblasts **(C)**, but was detected in leukocytes **(D)** in sections stained by immunohistochemistry to detect caspase-3. Discrete brown staining in **(D)** indicates caspase-3 positive leukocytes. Original magnification was 200 × **(A)** and 400 × **(B–D)**.

(**Figure 7**). All hearts were deemed suitable for transplant following perfusion.

HCP Is Not Associated With Myocardial Injury

Cardiac troponin I remained stable during perfusion [median (IQR); baseline: 0.00 (71.16), 2: 0.00 (53.83), 4: 27.31 (61.56), 6: 0.00 (78.01), 8: 0.00 (86.75) pg/ml, $p = 0.930$] and undetectable at 8 h in 4/6 hearts.

HCP Diminishes Post-transplant Graft Infiltration

A series of six heterotopic transplants were performed to determine if perfusion ($n = 3$ transplants) reduces recipient leukocyte recruitment into the graft post-transplantation compared with static cold storage ($n = 3$ transplants). HCP was associated with diminished graft infiltration compared to static cold storage as determined by percentage of the total cardiac tissue affected (cold stored vs. perfused: 30.7% ± 13.4 vs. 10.7% ± 2.1, $p = 0.06$, **Figure 8**). This was true for distribution of leukocytes within the coronary arteries (cold stored vs. perfused: 43.3% ± 23.1 vs. 14.0% ± 10.4), left ventricle (cold stored vs. perfused: 35.0% ± 39.1 vs. 6.7% ± 2.9), right ventricle (cold stored vs. perfused: 26.7% ± 22.5 vs. 11.7 ± 11.6), and septum (cold stored vs. perfused: 18.3% ± 5.8 vs. 11.7% ± 7.6). Alongside the effect on tissue distribution, the intensity of the infiltration was also diminished by HCP. Overall intensity of infiltration for perfused donor hearts was mild whereas overall intensity in the cold stored hearts was moderate.

DISCUSSION

Allograft rejection occurs via recipient T cell priming and infiltration of the heart. Whilst current therapies predominantly target recipient T cells, immunomodulation at an earlier stage may be advantageous. We have previously demonstrated a significant role for passenger leukocytes in the induction of T cell alloreactivity following lung transplantation (7). However, it remained unclear whether similar benefits would be observed in other organs with less well-defined resident immune repertoires.

We describe herein that the donor heart possesses a broad leukocyte compartment which could contribute to acute rejection. Donor leukocytes traffic to recipient lymph nodes upon revascularization and prime recipient allospecific T cells as part of direct allorecognition. Previous studies of cardiac donor immunodepletion have provided distinct benefits suggesting that removal of passenger leukocytes is broadly advantageous. In animal models, the specific depletion of donor CD4 T helper cells by anti-CD4 antibody or irradiation is associated with ameliorated recipient alloresponse as these cells were shown to

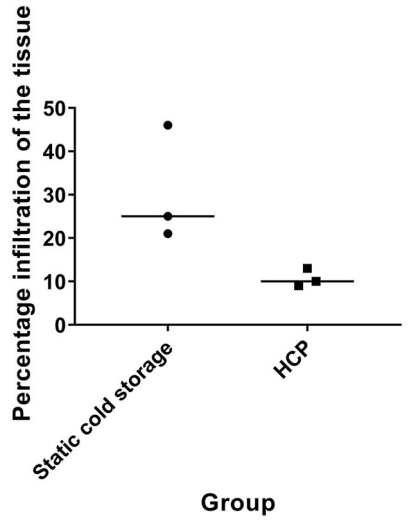

FIGURE 8 | Cold cardioplegic perfusion is associated with a reduction in leukocyte infiltration at 48 h. Intensity of leukocyte infiltration is also reduced by HCP. Donor hearts preserved by cold cardioplegic perfusion were deemed to have only mild infiltration across the heart, whereas those preserved by static cold storage were considered to have moderate infiltration. Data is displayed for $n = 3$ transplants in each group.

contribute to cardiac allograft vasculopathy (9). This effect was further supported by subsequent work from the same group (10). A murine cardiac transplant study also demonstrated a role for donor passenger lymphocytes in augmenting the alloresponse to the cardiac graft, resulting in early graft failure and vasculopathy (11). In our study, we demonstrate that perfusion is sufficient to induce the depletion of a significant proportion of the donor immune repertoire prior to transplantation. Prior studies have demonstrated that donor-derived regulatory T cells are beneficial in prolonging allograft survival and thus their removal may be undesirable (12). Whilst we were unable to evaluate regulatory cells in the current study, it is likely that these cells will also have been lost from the graft. However, HCP was associated with a clinically relevant reduction in recipient leukocyte recruitment of the donor heart until at least 48 h post-transplantation in our pilot study, without any immunosuppression when compared to storage on ice. Indeed, the level of graft infiltration was diminished by ∼65% relative to that in the control group, suggesting a marked beneficial effect despite this potential loss of regulatory cells. It is important to note that whilst there was consistently lower levels of infiltration in the HCP group, this only reached statistical significance at the 10% level due to the low number involved in the pilot arm of the study. This provides novel evidence that HCP drives immunodepletion and alters alloreactivity without the requirement for immunosuppression. HCP allows continuous coronary flow that promotes the clearance of passenger leukocytes from the donor organ. The extent of this flow is important in maintaining healthy myocardial tissue, and no perfused organs in this study displayed any indications of ischemia or other damage to suggest interrupted or insufficient flow. Whilst we did not evaluate the extent to which the level of coronary flow (either during perfusion or post-transplant) corresponds to intensity of subsequent graft infiltration, it is apparent that some level of coronary flow during storage is necessary for the benefits we observed. Our method therefore provides the dual-benefits of removal of passenger leukocytes alongside the extended safe storage of the donor organ as described previously.

The cause of this immunodepletion is unclear but may be in response to the inflammatory milieu in the perfusate, particularly IFN-γ. High IFN-γ concentrations were unexpected from an isolated donor heart, but further emphasize the role of the donor immune response in the immediate events following transplantation. Potentially, HCP may "therapeutically exhaust" this IFN-γ response, reducing inflammation post-transplantation. IFN-γ is not directly chemotactic but induces CCL2, CXCL8, and CXCL9 secretion (13). The release of such a milieu of IFN-γ associated proteins during HCP suggests a prominent role for this signaling network in mediating leukocyte migration from the heart. This is particularly true for CXCL9 which if neutralized, prevents IFN-γ secretion and is essential for donor specific T cell reactivity (14).

The distinct cytokine pattern observed here is interesting as many of these proteins have been previously implicated in transplant rejection. Prior analysis of cytokine levels following transplantation has demonstrated that IFN-γ is highly upregulated during rejection (15) and allograft survival is prolonged when its production is suppressed (16). Further, NK cells devoid of IFN-γ have diminished ability to induce lesions as part of antibody-mediated chronic allograft vasculopathy, illustrating the importance of minimizing transfer of this cytokine to the recipient (17). IL-18 has a well-documented role in promoting the lymphocytic production of IFN-γ, and as such IL-18 elevation in the perfusate is consistent with the massive IFN-γ levels observed (18). Neutralization of IL-18 provides protection for the cardiac allograft in murine models, as indicated by significantly prolonged survival, suggesting a key role (19). Both GM-CSF and TNF-α have been shown to be elevated during rejection (15) and GM-CSF is known to promote the differentiation of pro-inflammatory dendritic cells, which could potentially enhance alloantigen presentation (20). If the perfusate provides an approximate indication of the levels of cytokines that could be released from passenger leukocytes post-transplant then there are obvious benefits to ensuring that these are released in the circuit rather than in the recipient.

Aside from the impact of HCP on donor immunity, we also report that perfusion maintained tissue viability with no observable edema or endothelial damage. This was accompanied by minimal cardiac troponin I release. Moreover, tissue obtained post-perfusion displayed a molecular signature indicative of reduced apoptosis and IRI compared with corresponding tissue taken at retrieval following cardioplegia. HCP alone reduced STAT5 and STAT6 pathway activation which contributes to myocardial injury following ischemia and reperfusion (21, 22). Hypothermic preservation of rat donor hearts with continuous perfusion of mesenchymal stem cell conditioned medium was previously shown to protect against IRI (23). This was associated

with diminished pro-inflammatory cytokine expression and increased levels of the anti-oxidant superoxide dismutase-2. It is difficult to compare these results with those presented in our study due to differences in perfusate used. However, it does provide insight into potential additional factors that could be supplemented to further bolster the beneficial effects of hypothermic perfusion. Whilst the changes in protein expression induced by HCP in our study are beneficial immediately prior to transplant, it is difficult to discern whether this altered profile reflects changes in protein expression on the myocardium or reflects loss of signals due to immunodepletion. However, caspase-3 was not evident histologically in endothelium, cardiomyocytes, smooth muscle, or fibroblasts, but was identified in remaining tissue leukocytes, supporting leukocyte death as the source of cell-free DNA during HCP.

Principally designed as a method of extending the safe storage of the donor heart, it has now become apparent that there are auxiliary benefits to organ preservation in this manner. Further studies are necessary to determine whether these proposed benefits are translated into the clinic and such a trial is currently underway. There are also additional uses that may be further explored including the possibility of organ reconditioning during perfusion, which has been postulated to occur in other systems (2, 24, 25). The device may also be utilized as a platform for delivery of therapeutic agents, which could be added for the duration of storage but flushed out from the vasculature prior to transplantation if necessary.

LIMITATIONS

The donor animals were healthy and did not undergo brain or circulatory death as would be the case for standard donation. This may increase the immune content of the organ prior to retrieval. Due to the lack of porcine specific antibodies, we could not investigate the impact of perfusion on these phenotypes nor identify leukocytes within the tissues using immunohistochemistry. Furthermore, we could not determine the source of cell-free DNA within the perfusate. However, we suggest that leukocyte apoptosis/necrosis may be a major contributor as we observe no damage to the graft but do note significant leukocyte loss from the tissue combined with observable caspase-3 expression in leukocytes. The number of transplantations performed in the pilot arm of the study was low in keeping with NC3Rs principles, meaning a low power to detect a statistically significant difference, although the numerical difference detected between the groups was profound. We also limited survival to 48 h in the interests of the welfare of the animals.

CONCLUSION

Collectively, this study reinforces the importance of the donor as a therapeutic target for immunomodulation. It also provides evidence that HCP alters the immune content and molecular signature of the donor heart, which in turn reduces recipient T cell recruitment up to 48 h following transplantation in the absence of immunosuppression. Incorporating HCP into clinical practice could potentially allow the use of more immunosuppression-sparing regimens. These exciting findings require translation with discarded human tissue prior to incorporation into clinical practice, although the technique clearly holds great promise for revolutionizing donor heart storage.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Malmö/Lunds regionala djurförsöksetiska nämnd (REB). Ethic approval M174-15 Affiliation: Department of Cardiothoracic Surgery, Lund University and Igelösa Life Science AB.

AUTHOR CONTRIBUTIONS

WC, JS, and HS participated in research design, writing of the paper, performance of the research, and data analysis. QL, GQ, and IR participated in performance of the research. AT participated in research design and writing of the paper. TS, SS, and JF participated in generating the original concept, research design, writing of the paper, performance of the research, and data analysis. 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/fimmu.2020.01621/full#supplementary-material

Figure S1 | Toll-like receptor 4 expression is significantly reduced on the surface of mature neutrophils following HCP compared to pre-perfusion values ($p = 0.004$). No changes in surface expression of toll-like receptor 4 were observed for mature basophils/eosinophils ($p = 0.500$), immature neutrophils ($p = 0.500$), CD335+CD8– NK cells ($p = 0.893$), CD335+CD8+ NK cells ($p = 0.933$), B cells ($p = 0.074$), helper T cells ($p = 0.526$), cytotoxic T cells ($p = 0.938$), γδ T cells ($p = 0.715$), classical monocytes ($p = 0.819$), non-classical monocytes ($p = 0.152$), intermediate monocytes ($p = 0.434$), CD14+CD203a+ macrophages ($p = 0.686$), CD14–CD203a+ macrophages ($p = 0.579$), or SLA-DR+CD203a+ macrophages ($p = 0.801$).

Figure S2 | Immune populations are sequestered by the leukocyte filter. The leukocyte pattern detected in the filter reflects that observed in the baseline reference of the donor heart and includes granulocytes **(A)**, monocytes/macrophages **(B)**, and lymphocytes **(C)**.

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The Graz Liver Allocation Strategy—Impact of Extended Criteria Grafts on Outcome Considering Immunological Aspects

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Background: Transplant centers are forced to use livers of extended criteria donors for transplantation due to a dramatic organ shortage. The outcome effect of extended donor criteria (EDCs) remains unclear. Thus, this study was designed to assess the impact of EDCs on outcome including immunological aspects after liver transplantation (LT).

Patients and Methods: Between November 2016 and March 2018, 49 patients (85.7% male) with a mean age of 57 ± 11 years underwent LT. The impact of EDCs on outcome after LT was assessed retrospectively using both MedOcs and ENIS (Eurotransplant Network Information System).

Results: About 80% of grafts derived from extended criteria donors. Alanine aminotransferase/aspartate aminotransferase (AST/ALT) levels elevated more than three times above normal values in organ donors was the only significant risk factor for primary dysfunction (PDF) and primary non-function (PNF)/Re-LT and early non-anastomotic biliary strictures (NAS). Balance of risk (BAR) score did not differ between EDC and non-EDC recipients. PDF (14.3% of all patients) and PNF (6.1% of all patients) occurred in 23.1% of EDC-graft recipients and in 10.0% of non-EDC-graft recipients (RR 2.31, $p = 0.663$). The 90-day mortality was 3.6%. There was no difference of early non-anastomotic biliary tract complications and biopsy proven rejections (BPR). There was no correlation of PDF/PNF with BPR and NAS, respectively; however, 66.7% of the patients with BPR also developed early NAS ($p < 0.001$).

Conclusion: With the Graz liver allocation strategy, excellent survival can be achieved selecting livers with no more than 2 not outcome-relevant EDCs for patients with MELD >20. Further, BPR is associated with biliary complications.

Keywords: extended donor criteria, immunological aspects, liver transplantation, liver allocation, outcome

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INTRODUCTION

Organ shortage has driven transplant centers to extend their criteria for organ acceptance. Donors have become increasingly older and multi-morbid. Allocation strategies for liver transplantation (LT) as well as the acceptance criteria for donor organs in order to expand the entire pool of available organs (1, 2) are continuously being adapted. Various extended donor criteria (EDC)

for each organ have been defined; however, the impact of these criteria on outcome after LT is still under debate. Apart from that, there is no definite answer to the question of how to measure the advantages and disadvantages of LT with EDC-grafts. Is it more adequate to judge the waitlist mortality, or the cumulative patient and graft survival, when assessing a LT program? What is the primary aim of LT? To make a long answer short: it is the utility, the generation of a maximum of life years through an optimized allocation of this scarce resource.

To better predict the mortality risk of patients on the waiting list, the model of end-stage liver disease (MELD) system, which is based on three laboratory values including serum creatinine, bilirubin, and international normalized ratio (INR) (laboratory (lab)MELD score), was introduced and adopted by many LT programs worldwide in order to prioritize patients for transplantation by urgency. This "sickest first" allocation policy shows conflicting results, and it has induced medical, ethical, and socio-economic debates. Several risk scores combining donor and/or recipient risk factors predicting outcome after LT have been developed, like the donor risk index (DRI, Eurotransplant [ET]-DRI), and balance of risk (BAR) score including 6 variables (donor age, recipient age, recipient MELD score, re-transplantation, pretransplant life support, and cold ischemic time), University of California, Los Angeles (UCLA), acute-on-chronic liver failure (ACLF), survival outcome following LT score (SOFT) using 18 risk factors, Pedi-SOFT and D-MELD (donor age × recipient MELD) scores, which are models for matching EDC grafts with low-risk recipients and vice versa in order to find a balance between urgency and utility and benefit. Allocation of an EDC graft to a high-risk recipient with a high MELD score should be avoided because of the risk of short-term mortality. Those patients were shown to benefit from high-quality grafts (3). Comorbidities that are not categorically evaluated in the above mentioned scores should also be exceptionally considered to accurately predict post-LT outcome, as a combination of comorbidities like age and aggravation of comorbidities like cardiovascular disease, and frailty can potentially lead to deleterious outcomes after LT.

Abbreviations: EDC, extended donor criteria; LT, liver transplantation; AST, alanine aminotransferase; ALT, aspartate aminotransferase; HBV, hepatitis B virus anti-HBc antibody, anti-hepatitis B core antibody HBs antigen, hepatitis B surface antigen; PDF, primary dysfunction; PNF, primary non-function; BPR, biopsy proven rejections; NAS, non-anastomotic biliary strictures; UCLA, University of California, Los Angeles; INR, international normalized ratio; MELD, model of end stage liver disease; labMELD, laboratory model of end stage liver disease; DRI, donor risk index; ET-DRI, Eurotransplant donor risk index; BAR score, balance of risk score; ACLF, acute-on-chronic liver failure; SOFT, survival outcome following liver transplantation score; Pedi-SOFT, pediatric survival outcome following liver transplantation score; BMI, body mass index; CIT, cold ischemic time; AP, alkaline phosphatase; GGT, γ-glutamyl transferase; HAT, hepatic artery thrombosis; IC, informed consent; DCD, donation after cardiac death; AIH, autoimmune hepatitis; ALF, acute liver failure; ELTR, European Liver Transplant Registry; EASL CPG, European association of the study of the liver Clinical Practice Guidelines; IS, immunosuppression; ITBL, ischemic type biliary lesions; ICU, intensive care unit; IRI, ischemia reperfusion injury; BÄK, German Medical Association; RA, rescue allocation; CTS, Collaborative Transplant Study; KT, kidney transplantation; DSA, donor specific antibodies; DBD, donation after brain death; WIT, warm ischemic time; DC, dendritic cells.

Apart from that, a score can never replace subjective surgical experience when inspecting a graft during organ retrieval and directly prior to transplantation after having reviewed a particular recipient's condition at the time of transplant.

EDC-grafts have been widely used in the Eurotransplant (ET) region. Good results can be achieved using such liver grafts. An increased risk for biliary tract complications, primarily non-anastomotic biliary strictures (NAS), as well as vascular complications associated with the various types of EDC, as well as an potential increase of early malfunction, i.e., primary dysfunction (PPF) and primary non-function (PNF), have been reported after LT using EDC-grafts (4). Implications on acute and chronic graft rejection have been proposed (5), representing a link between the degree of ischemia reperfusion injury (IRI) and activation of innate immunity (6). EDC in LT is a hot topic. Various EDC have a different impact on outcome after LT.

Here the impact of the Graz allocation strategy (no acceptance of potentially outcome-relevant EDCs in >20 MELD recipients; i.e., >3-fold elevation of normal ranges of aspartate aminotransferase (AST) or alanine aminotransferase (ALT) or cold ischemic time (CIT > 10.5 h) on outcome after LT has been assessed considering immunological aspects in a low volume transplant center (≤40 LT/year) in a non-MELD based allocation system.

PATIENTS AND METHODS

All clinical, demographic, surgical, and post-surgical follow-up data were analyzed from all consecutive primary LT performed between November 2016 and March 2018 in a single transplant center. Based on the definition of EDC by the executive committee of the German Federal Medical Society and the ET definition the following donor criteria were assessed as EDC: donor age >65 years, ventilation >7 days, >3-fold elevation of normal ranges AST or ALT, bilirubin >3 mg/dl, peak serum sodium >165 mmol/l, biopsy-proven macrovesicular steatosis >40%, prolonged hypotensive episodes in the donor (>1 h, <60 mmHg, inotropic drug use, e.g., dopamine >14 μg/kg/min) or donor cardiac arrest, body mass index (BMI) >30, CIT >14 h, history of extrahepatic malignancy, previous drug abuse, positive hepatitis B serology (anti-hepatitis B core [HBc] antibody and/or hepatitis B surface [HBs] antigen positive) and donation after cardiac death (DCD) grafts. The concept of EDC LT was explained to the patients on the wait list for LT, and IC was obtained in all patients prior to LT except in high urgent recipients. The presence of any EDC was assessed as well as the number of EDC, if present. The following recipient criteria were assessed: demographics, indication for LT, labMELD score, post-LT laboratory parameters for liver function and liver injury (AST, ALT, alkaline phosphatase [AP], bilirubin, γ-glutamyl transferase [GGT]), PDF, PNF, ICU stay, re-LT, biliary complications within the first 3 post-operative months, vascular complications including hepatic artery thrombosis (HAT), portal vein or hepatic vein thrombosis, bleeding requiring further surgical interventions, and rejection episodes. Primary dysfunction (PDF) and primary non-function (PNF) were defined as AST and

ALT >1,500 U/l and AST >2,500 U/l, respectively, during the first 72 h after LT or re-LT/graft failure (7).

The surgical technique of LT included cavo-caval end-to-side (Piggyback technique) or side-to-side anastomosis (Belghiti modified Piggyback technique). The immunosuppressive regimen was tacrolimus based together with mycophenolic acid and a cortison taper for 3 months. Induction therapy was administered in patients <40 years of age, patients suffering from autoimmune hepatitis (AIH), patients with renal insufficiency with a glomerular filtration rate of <60 ml/min, grafts from donors after cardiac death (DCD).

This retrospective analysis was based on both MedOcs and ENIS (ET Network Information System) electronic data. The study protocol has been approved by the local ethics committee, Medical University of Graz, Austria (Ethic Committee number 30-426 ex 17/18).

Statistical Analyses

Continuous data are presented as mean ± standard deviation or median and range, as appropriate. Categorical data are presented as absolute and relative frequencies. For continuous data differences between groups were analyzed using *t*-test, Mann Whitney *U*-test. Differences in the distribution of categorical data were analyzed using χ^2 -test or Fisher's exact test. For risk factor analysis relative risks and corresponding 95% confidence intervals (95%CI) were calculated. R version 3.4.4 and SPSS 26.0.0.0 (IBM Corp., Armonk, NY, USA) were used for these analyses.

RESULTS

General Data

Forty-nine patients (85.7% male) with a mean age of 57 ± 11 years underwent LT for alcoholic liver cirrhosis (45%), hepatocellular carcinoma (HCC) (41%), primary sclerosing cholangitis (PSC) or AIH (10%), HBV-associated liver cirrhosis (2%), and acute liver failure (ALF) (2%) (**Table 1**). The median labMELD score of the patients was 15 (range 7–32), and 24.5% of patients presented with a labMELD score of <10, 61.2% of cases have shown a labMELD score of 10–20, 10.2% were identified with a labMELD score of 21–30, and 4.1% of patients were documented with a labMELD score of >30 (labMELD categories 1–4, **Figure 1**). Three patients underwent re-LT, one of which for PNF and the other 2 cases for HAT. Re-LT were excluded from further analysis, and 79.6% of grafts have shown up to three EDCs (**Figure 2**); the categories of EDCs are shown in **Table 2**. Of those, 51.3% had 1 EDC, 38.5% had 2 EDCs, and 10.2% had 3 or 4 EDCs (**Table 3**). No EDC existed for 20.4% of grafts. Patients were classified in EDC and non-EDC recipients. These 2 groups were comparable based on demographics, indication for LT, and labMELD score. In labMELD category 1 and 2, 16.7% of the patients (MELD score ≤ 20) received a non-EDC organ, 83.3% received an EDC organ; 57.1% of the patients in labMELD category 3 and 4 (labMELD score >20) received a non-EDC organ, 42.9% received an EDC organ with no more than 2 EDC categories.

Donor/Recipient Match

BAR-score was 6.1 (±2.4) in EDC and 7.6 (±3.2) in non-EDC recipients (n.s.) (**Table 4**).

Survival

Median follow-up time of the patients was 22 months [range 13–31 months]. One-year patient survival was 96.4% with a 90-day mortality of 3.6%. While one patient died after acute pulmonary embolism on post-operative day (POD) 7 the other cause of death

TABLE 1 | Recipient characteristics.

	all LT	EDC	Non-EDC
Sex			
Male	42	34/42 (80.9%)	8/42 (19.1%)
Female	7	5/7 (71.4%)	2/7 (28.6%)
Age at LT [years]	56.8 ± 11.4	59.0 ± 8.4	48.2 ± 17.0
Indication for LT			
Alcoholic liver disease	22	17/22 (77.2%)	5/22 (22.8%)
HCC	20	18/20 (90.0%)	2/20 (10.0%)
PSC/AIH	5	3/5 (60.0%)	2/5 (40.0%)
viral (HCV/HBV), ALF	2	1/2 (50%)	1/2 (50%)
MELD (labMELD)	15.2 ± 6.3	13.9 ± 4.9	20.3 ± 8.5
MELD ≤ 20	42	35/42 (83.3%)	7/42 (16.7%)
MELD > 20	7	3/7 (42.9%)	4/7 (57.1%)
HU			
Yes	1	0/1	1/1
No	48	39/48	9/48
BMI	25.9 ± 3.6	25.8 ± 3.5	26.5 ± 4.1

Data are presented as total numbers or as means ± standard deviation.
LT, liver transplantation; EDC, extended donor criteria; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma (either primary etiology or concomitant); ALF, acute liver failure; PSC, primary sclerosing cholangitis; AIH, autoimmune hepatitis; MELD, Model of End-stage Liver Disease; HU, high urgent.

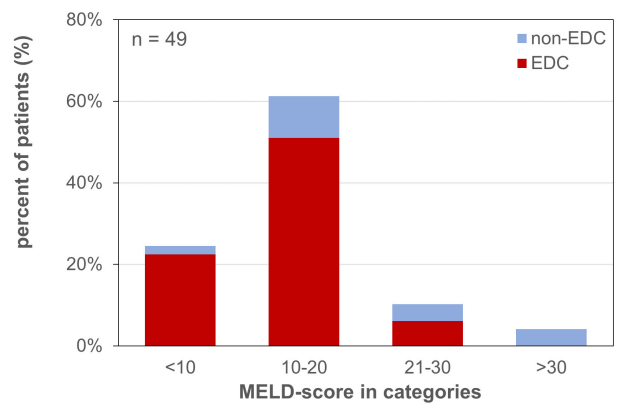

FIGURE 1 | MELD-score categories of patients waiting for liver transplantation. MELD-score was categorized into 4 groups: 1: MELD <10, 2: MELD 10–20, 3: MELD 21–30, 4: MELD >30. EDC (extended donor criteria) vs. non-EDC (non-extended donor criteria) graft recipients.

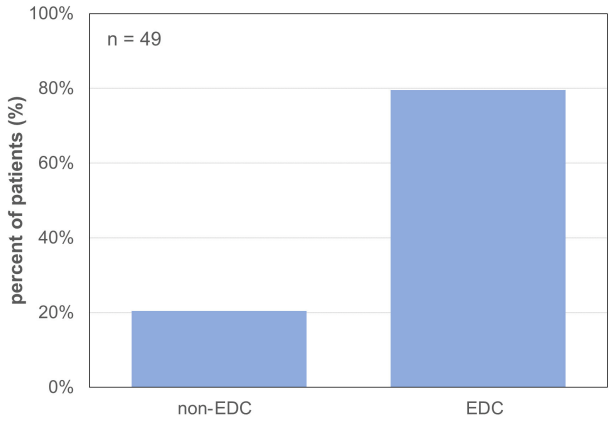

FIGURE 2 | Percentage of grafts with extended donor criteria and non-extended donor criteria. 79.6% EDC (extended donor criteria) grafts and 20.4% non-EDC (non-extended donor criteria) grafts.

TABLE 2 | Donor characteristics.

Extended donor criteria *n* [%]	All LT
1) Age >65 years	19 [38.8]
2) Serum transaminases (AST, ALT) >3 times normal	13 [26.5]
3) ICU/MV >7 days prior to organ procurement	9 [18.4]
4) Cardiac arrest	9 [18.4]
5) BMI >30 kg/m2	5 [10.2]
6) CIT>14 h	3 [6.1]
7) Serum Na^+ >165 mmol/L	3 [6.1]
8) History of extrahepatic malignancy*	3 [6.1]
9) DCD	3 [6.1]
10) Positive hepatitis serology*	1 [2]

Categories of extended donor criteria.
Na^+, serum sodium; BMI, body mass index; ICU, duration of intensive care unit stay before organ procurement; MV, duration of mechanical ventilation of the donor before organ procurement; CIT, cold ischemic time.
**Not relevant for post-transplant graft function.*

TABLE 3 | Number of functionally relevant extended donor criteria per graft.

EDC (*n*)	*n* = 0	*n* = 1	*n* = 2	*n* = 3/4
Grafts [%]	20.4	40.8	30.6	8.2
PNF [%]	2	4.1	0	0

EDC, extended donor criteria; PNF, primary non-function.

TABLE 4 | Balance of risk (BAR) score in extended donor criteria (EDC)/Non-EDC recipients.

	EDC	Non-EDC
BAR-score	6.1 (±2.4)	7.6 (±3.2)

BAR-score, Balance of risk score: including 6 variables (donor age, recipient age, recipient MELD score, re-transplantation, pretransplant life support, and cold ischemic time); EDC, extended donor criteria.

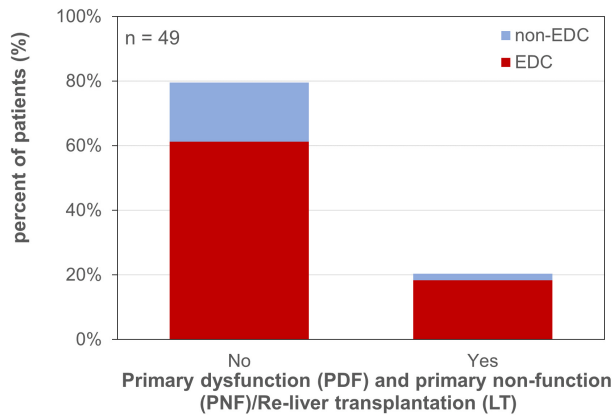

FIGURE 3 | Primary dysfunction and primary non-function/re-liver transplantation. EDC (extended donor criteria) vs. non-EDC (non-extended donor criteria) graft recipients.

was due to septic multi-organ failure. Both deaths occurred after EDC LT.

Post-operative Data

Laboratory findings reflecting both graft injury and graft function were comparable between groups. All early (first post-operative 3 months) but one (HAT after non-EDC LT), and all late surgical re-interventions due to bleeding, vascular complication, and incisional hernia (IH) repair were performed in EDC-graft recipients. Of all EDC-graft recipients, 20.5% had 1 re-intervention, 5.1% had 2 re-interventions, and 2.6% had 3 re-interventions.

EDC LT had no significant impact on both the ICU stay and ventilation time. The median ICU stay was 2 days in both groups; however, the range of ventilation time with 1–60 days was higher in EDC-graft recipients as compared to 1–9 days after non-EDC LT; 25.6% of EDC-graft recipients requiring more than 4 days of ICU in contrast to 10.0% after non-EDC LT (RR 1.19; $p = 0.419$).

While the median ventilation time after LT was comparable after both EDC and non-EDC LT with 11 and 15 h, respectively; the range was higher after EDC LT with 5–179 h as compared to 8–65 h after non-EDC LT. Only 7.9% of EDC-graft recipients required a post-operative ventilation of more than 24 h. This is in contrast to 22.2% after non-EDC LT (RR 0.72; $p = 0.240$).

Temporary post-operative hemodialysis was necessary in 12.2% of all patients with no difference between groups (EDC-graft recipients: 12.8%, non-EDC-graft recipients: 10.0%; RR of 1.28; $p = 1$).

Early Graft Function

PDF (14.3% of all patients), PNF and the necessity for re-LT (PNF/Re-LT; 6.1% of all patients) occurred in 23.1% of cases after EDC LT and in 10.0% after non-EDC LT (RR 2.31; $p = 0.663$; **Figure 3**).

Biliary Complications

Early NAS (12.2% of all patients during the first 3 months) occurred in 12.8% of EDC-graft recipients and in 10.0% after non-EDC LT (RR of 1.28; $p = 1$; **Figure 4**).

Biopsy-Proven Acute and Chronic Rejections (BPR)

Six patients developed BPR after a median follow up of 106.5 days (6–177 days) post-LT. BPR occurred in 12.8% in EDC recipients (grafts with 1 EDC: 3 patients; grafts with 3 or 4 EDCs: 2 patients, respectively) and in 10.0% in non-EDC recipients (RR of 1.28; $p = 1$; **Figure 5**). There was a coincidence of BPR with PDF/PNF in 33.3% of cases ($p = 0.588$), and 66.7% of the patients with BPR also developed early NAS ($p < 0.001$).

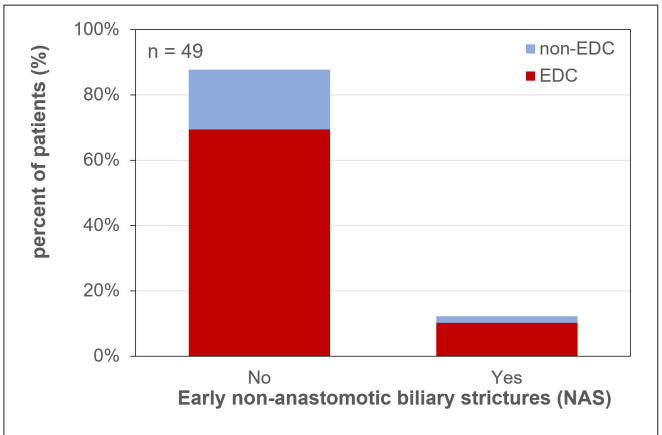

FIGURE 4 | Early non-anastomotic biliary strictures after liver transplantation. EDC (extended donor criteria) vs. non-EDC (non-extended donor criteria) graft recipients.

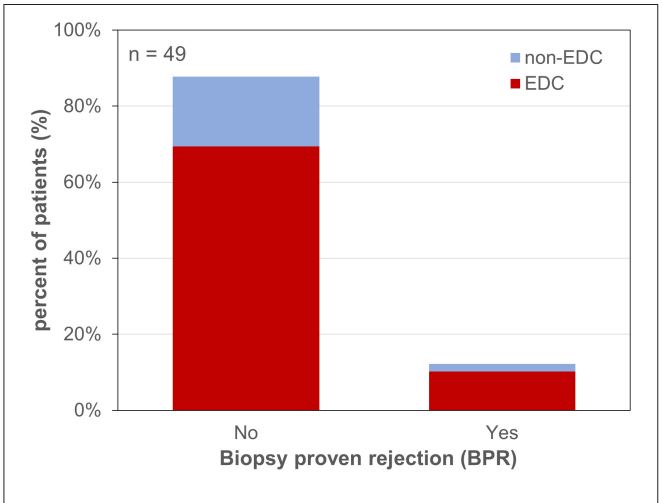

FIGURE 5 | Biopsy proven rejection after liver transplantation. EDC (extended donor criteria) vs. non-EDC (non-extended donor criteria) graft recipients.

Immunosuppression

Intra-patient tacrolimus trough level variability within the first post-LT year did not differ between EDC- and non-EDC-graft recipients (42.5 ± 1.9% vs. 49.9 ± 10.8%, respectively; $p = 1$).

Risk Factor Analysis

AST/ALT serum levels in organ donors more than three times increased above normal limits was a significant risk factor for PDF and PNF/Re-LT (RR 4.15, 95%CI 1.39–12.41; $p = 0.024$) as well as for early NAS (RR 5.54, 95%CI 1.62–18.99; $p = 0.003$). A CIT of > 10.5 h was the second strongest risk factor for PDF and PNF/Re-LT (RR 3.33, 95%CI 0.95–11.71) and for early NAS (RR 2.08, 95%CI 0.64–6.77).

All patients with a labMELD score >20 received either non-EDC grafts or EDC grafts with no more than 2 EDCs which did not include increased AST/ALT levels or prolonged CIT (**Figures 6**, **7**).

DISCUSSION

Due to the increasing lack of organs, the criteria that define donor organs suitable for LT are constantly being expanded. While older donor age or resuscitation of the donor, for example, were absolute contraindications for LT 30 years ago, today this is at most a relative contraindication. Nevertheless, survival after LT has steadily increased over the years. One-year survival is currently more than 90%, 5-year survival 80%, and 10-year survival more than 70% according to the European Liver Transplant Registry (ELTR) (8), EASL Clinical Practice Guidelines (CPG) LT (9). What are the current challenges? These are primarily early functional disorders of the graft such as PDF and PNF in 2–15% of cases (10, 11), as well as the long-term consequences of the immunosuppression (IS). The primary aim of LT is the generation of a maximum of life years through an optimized allocation of this scarce resource (12).

EDC Definition

There is no unique definition for EDC. But, there are two categories: (i) factors directly influencing post-transplant-function and (ii) factors not influencing post-transplant-function. There was a consensus conference in 2007 on extended donor criteria, and those were defined as donor age, macrosteatosis, elevated liver enzymes, hemodynamic instability of the donor, hypernatremia, CIT, DCD, split LT, transmission of malignancy, and infections (13). Other attempts to sum up the main EDC criteria are the ET score, the German Medical Association (BÄK) score, and the UNOS definition score (14–16).

Concerning donor factors potentially influencing post-transplant graft function, one of the most important challenges is the fact that the age of donors is constantly increasing. Potential risks of LT using aged grafts are higher rates of transplant failure PNF and PDF with potentially increased mortality, and a higher degree of ischemia reperfusion injury (IRI) due to less potential to regenerate. The risk in hepatitis C positive aged grafts is even higher, as well as the damage due to longer CIT in combination with aged grafts. Some studies confirmed the negative consequences of such grafts (17–19), especially in

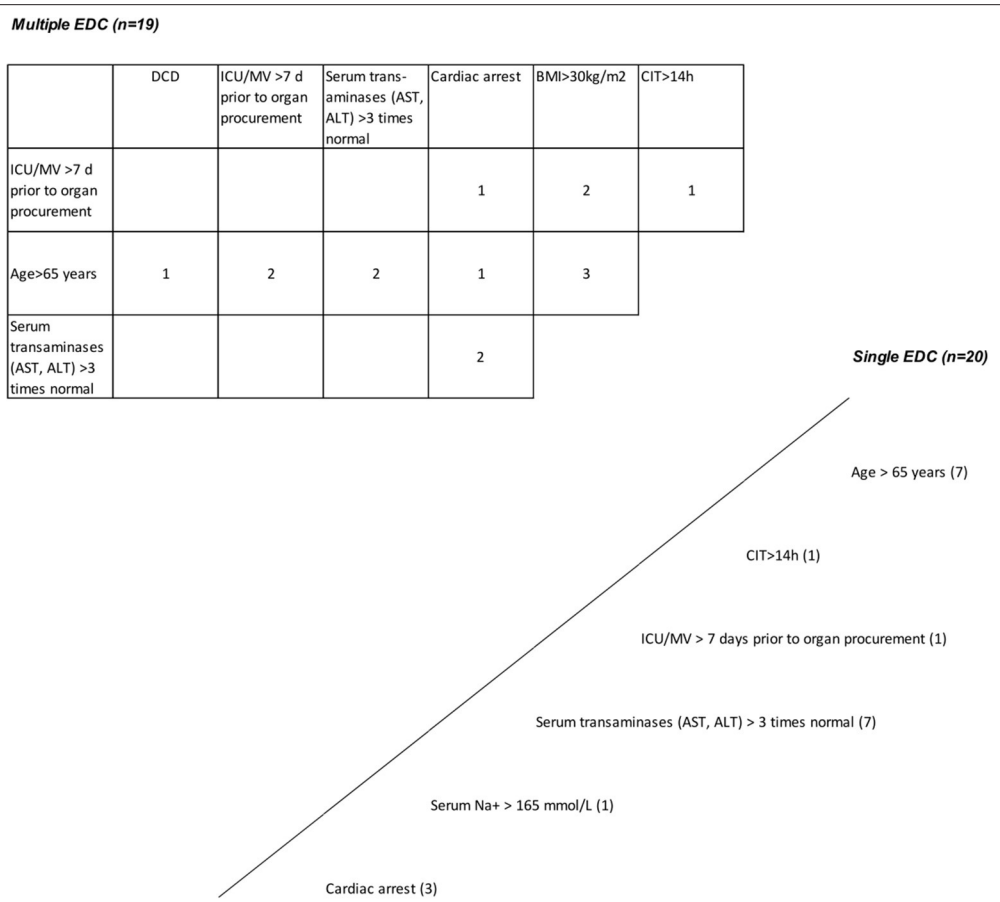

FIGURE 6 | Grafts with extended donor criteria (EDCs). Single EDC—multiple EDC. A total of 49 primary cadaveric LTs were analyzed. Up to four EDCs were present in 79.6% of all grafts. Grafts with single EDC are shown below and right to the oblique line. Nineteen grafts had more than one extended criterion (table above and left to the oblique line; multiple EDCs [$n = 19$]). The number within the boxes represents how many grafts had the corresponding combined EDC. Prolonged cold ischemia time >14 h and ICU stay with ventilation >7 days, and cardiac arrest and alanine aminotransferase/aspartate aminotransferase (AST/ALT) levels >3 times normal were present in 2 grafts from donation after cardiac death (DCD) donors >65 years; AST/ALT levels >3 times normal in combination with cardiac arrest and positive hepatitis B virus (HBV) serology*, and in combination with hypernatremia >165 mmol/l and intensive care unit (ICU) stay with ventilation >7 days were present in 2 grafts; a history of extrahepatic malignancy* in combination with donor age >65 years, cardiac arrest and hypernatremia >165 mmol/l was present in 1 graft, in combination with ICU stay with ventilation >7 days and a prolonged cold ischemic time (CIT) in another (4 grafts with 3 or more functionally relevant EDCs). *Not relevant for post-transplant graft function.

the context of hepatitis C positivity, but many studies to date have confirmed no disadvantages for patients receiving aged liver grafts in large cohorts (up to 23,763 patients) (20, 21). Macrosteatosis of >60% of the donor liver is an unacceptable risk for graft failure, while 30–60% macrosteatosis can achieve acceptable outcomes in select donor-recipient combinations (9). The balance of risk score (BAR) is one attempt to combine the strongest donor and recipient risk predictors to generate a risk score predicting less survival for a BAR score of >18 (22). Elevated liver enzymes AST and ALT of donor livers were shown to achieve good results after LT (23), whereas elevated GGT and INR were shown to be associated with inferior results (13). Hypotensive episodes in organ donors as well as donor resuscitation were associated with non-inferior LT results (24). The need of catecholamines (norepinephrine, dopamine >10 mcg/kg/min) was shown to be a risk factor for graft failure (25). Lower patient and graft survival with donor hypernatremia >155 mEq/l was reported in several studies (26, 27), whereas most recent studies on donor hypernatremia showed no influence on patient and graft survival (28, 29). CIT of more than 8 h leads to impaired 5-year-survival after LT (7), and with each additional hour of CIT the risk for PNF increases by 1% (30).

LT after DCD has steadily increased over the years, with a DCD rate of >20% in the UK and around 6% in the United States (31). Associated risks with DCD LT include biliary tract complications (i.e., ischemic type biliary lesions [ITBL]), vascular complications like HAT, as well as PDF and PNF potentially necessitating re-LT. An increased rate of biliary tract complications of more than 30% was reported by various groups (32, 33), whereas similar 1-, 3-, and 5-year survival was reported by Kollmann et al. (34), and similar 1- and 10-year survival, but inferior 5-year-survival by Blok et al. (35) analyzing ET data. One

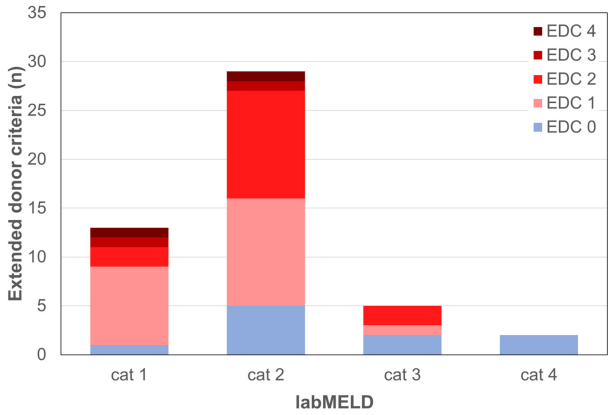

FIGURE 7 | Extended donor criteria (EDCs)/laboratory model of end stage liver disease (labMELD) categories (cat 1-4). Distribution of grafts with no EDCs/EDCs (n=0-4) among recipients at different risk/labMELD category 1 (labMELD <10), category 2 (labMELD 10–20), category 3 (labMELD 21–30), and category 4 (labMELD >30). In category 3 and 4 grafts with >3-fold increased AST/ALT levels and CIT > 10.5 h were not used.

recent study even showed better results in DCD LT with donor age <50 years and CIT <6 h than in DBD LT using grafts from donors >60 years (36).

The other donor criteria which were defined as "extended" like split LT, transmission of infections, and malignancy do not directly have a potential impact on graft function, PDF, and PNF.

Donor/Recipient Matching

The experienced transplant surgeon is responsible for accepting the best possible match. General rules include that EDC organs shall not be used for the sickest patients, since these patients do not have any reserves to survive primary dysfunction or primary non-function. Further, according to the literature, the combination of 3 or more than 3 EDC factors decreases outcome quality after transplantation (37). The number of EDCs was higher in patients with lower labMELD scores, which is based on the opinion that a recipient in a good clinical condition can better tolerate an EDC graft than a patient with a higher labMELD score. This is in line with other publications (4, 38, 39). Hence, according to our data and other reports in the literature, patient and graft outcomes were not different (1, 2, 4, 12). The BAR-score, which is available before decision making of accepting or not an organ for a specific recipient, was reported to have the potential to detect unfavorable combinations of donor and recipient factors (22). It was also applied in this patient cohort. In this small volume center within a non-MELD-based allocation system, the MELD scores were generally low among patients on the waiting list for LT with only 14.3% of the patients with a labMELD score of >20, as were the BAR scores (6.1 [±2.4] in EDC and 7.6 [±3.2] in non-EDC recipients). According to findings in the literature (2, 4, 13, 27, 40, 41) the Graz allocation system was established avoiding outcome-relevant EDCs for high risk patients; patients with labMELD scores >20 received grafts with no more than 2 EDCs excluding >3-fold increased AST/ALT levels or prolonged CIT > 10.5 h which were most relevant for outcome after LT. Risk factor analysis revealed that AST/ALT levels elevated more than three times above normal values in organ donors was the only significant risk factor for primary dysfunction (PDF) and primary non-function (PNF)/Re-LT and early non-anastomotic biliary strictures (NAS).

EDC Transplantation / Immunological Risk

In EDC-kidney transplantation (KT), Aubert et al. found an EDC-graft survival comparable to that of patients receiving a SDC transplant in KT recipients, whereby patients receiving EDC transplants who presented with circulating donor specific antibodies (DSA) at the time of transplantation had significantly worse allograft survival after 7 years than patients receiving EDC kidneys without circulating DSA at transplantation (44 vs. 85%). Recipients of EDC kidneys with circulating DSA showed a 5.6-fold increased risk of graft loss compared with all other transplant therapies [$p < 0.001$; (42)]. According to this large KT analysis including 6,891 patients allocation policies to avoid DSA and CIT could promote wider implementation of EDC transplantation in the context of organ shortage and improve its prognosis. No comparable results are available from LT cohorts, whereas allocation policies for EDC liver grafts have been modified accordingly. The so-called rescue allocation (RA) is one strategy for LT that has been implemented within the ET area mainly for this reason. Liver grafts are considered for RA when the regular organ allocation is declined by at least 3 centers or is averted because of donor instability or other unfavorable logistical reasons. Thus, such a donor enters a competitive or a single-recipient rescue organ offer procedure, respectively. The accepting center has the advantage to select a recipient from its own waiting list for these RA grafts (1), which is not common practice in all countries within ET.

According to the Collaborative Transplant Study (CTS) positive lymphocytotoxic T-cell crossmatches have been shown to be associated with significantly decreased graft survival in first kidney transplants performed from 1990 to 1999, but not from 2000 to 2007, in kidney re-transplants regardless of transplant period and in heart and liver transplants. Positive B-cell crossmatches were associated with significantly decreased kidney and heart, but not liver transplant survival (43). According to consensus guidelines on the testing and clinical management associated with HLA and non-HLA antibodies, a KT can be performed in the absence of a prospective crossmatch if single-antigen bead screening for antibodies to all class I and II HLA loci is negative. The presence of DSA HLA antibodies should be avoided in heart and lung transplantation and considered a risk factor for liver, intestinal, and islet cell transplantation (44).

Biliary Complications/Immunological Link

Biliary complications after LT have a constant incidence of 10–15%. Anastomotic biliary strictures (AS) are more related to technical aspects as bile leaks, or HAT, whereas NAS are related to risk factors including immunologic, IRI, or consequences of infectious complications (45). ITBL (46) is

one of the major post-operative complications accounting for up to 38% of morbidity and mortality rates of all biliary complications.

In the longer term, NAS potentially result from the use of grafts with various EDC and can be a consequence of profound IRI, as well as an increased incidence of acute and chronic rejection (4–6, 47). EDC-liver grafts are more susceptible to cold and warm IRI and develop more easily ITBL than normal livers (48), as ischemic cholangiopathy is more common with the use of DCD grafts and prolonged warm ischemic time (WIT) (49). Several studies link ITBL to various immunologically mediated processes such as AB0-incompatible liver transplants, PSC, PBC, and AIH (50).

Immunological risk factors like PBC, crossmatch positivity, and acute and chronic rejection were found to be important variables associated with the development of biliary strictures after LT in a retrospective analysis of 273 DBD LT (45), independent from IRI. An immunological component causing ITBL could be confirmed by the detection of DSA HLA antibodies in LT recipients (51).

EDC Transplantation / Rejection

Organ age has been linked to higher acute rejection rates (52). Experimental data show that age-associated epigenetic changes that result in hypermethylation of the CpG regions or hypomethylation of the non-CpG regions (53) may increase the immunogenicity of the DNA; hypomethylation of aged DNA has been reported to induce a stronger activation of dendritic cells (DCs) compared to DNA from young donors (54). Old DCs have also been shown to secrete more inflammatory cytokines upon stimulation, possibly via decreased activation of PI3K-signaling pathways and reduced suppression of p38-MAPK activation (55). Although immunosenescence leads to an overall decline of immune function, enhanced antigen-presenting capacities have been reported (56). Older endothelial cells express higher levels of VCAM-1 and MCP-1, facilitating leukocyte adhesion and infiltration and thereby contribute to enhanced immunogenicity (57).

The compromised repairing capacity of aged organs may also play an important role for an aggravated immune response. Cell death via apoptosis is a physiological part of the aging process and older grafts contain more apoptotic cells representing a significant source of local inflammation (54, 55). As a consequence of impaired repairing capacity, old parenchymal cells express more MHC molecules (58).

Non-specific injuries like IRI, and a mechanical trauma during explantation, induce a proinflammatory milieu which can activate the innate immune response and initiate the adaptive immune response. This can be aggravated by longer CIT, also potentially leading to an increased rate of acute rejection (59, 60).

Activation and recruitment of recipient's dendritic cells (DCs) into the graft activating recipient's T cells via the indirect pathway, together with increased apoptosis and antigen presentation augmenting the immune response, represents an important link between injury and immune response (56). It has been shown that IRI enhances the immunogenicity of allograft-derived DCs via toll-like receptor 4 and nuclear factor-kappa B activation (59).

In steatotic livers, the increased volume of the hepatocytes leads to microcirculatory impairment and thereby to an increased susceptibility to IRI with an immunological impact as mentioned above.

In conclusion, the immune response against steatotic grafts and older grafts can be enhanced relative to younger grafts with cryptic self-antigens exposed during necrotic cell death involved (56).

CONCLUSION

Results of our data are in accordance with previous findings, that excellent survival can be achieved with careful selection of EDC-liver grafts and appropriate recipient matching (EDC grafts for low-risk recipients and vice versa). However, there is an increased risk for biliary complications associated with the various types of EDC, and there is an indication that there may be implications in rejection, but without increased mortality risk. We also found no significant difference with respect to biliary complications, PDF/PNF, and rejection between EDC- and non-EDC-graft recipients. Altogether, the Graz allocation strategy has been proven to be safe and effective within a non-MELD based allocation system.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available. Requests to access the datasets should be directed to judith.kahn@medunigraz.at.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics commission of the Medical University of Graz. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JK performed the study and wrote the manuscript. GP and AA analyzed the data. PS designed and performed the study, and wrote the manuscript. DK and HM edited the manuscript. All authors contributed to the article and approved the submitted version.

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Preliminary results were presented at the Austrotransplant congress 2018 with an abstract published in *Transplant International* (Proceedings) (61).

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Pre-transplant HLA Antibodies and Delayed Graft Function in the Current Era of Kidney Transplantation

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Delayed graft function (DGF) occurs in a significant proportion of deceased donor kidney transplant recipients and was associated with graft injury and inferior clinical outcome. The aim of the present multi-center study was to identify the immunological and non-immunological predictors of DGF and to determine its influence on outcome in the presence and absence of human leukocyte antigen (HLA) antibodies. 1,724 patients who received a deceased donor kidney transplant during 2008–2017 and on whom a pre-transplant serum sample was available were studied. Graft survival during the first 3 post-transplant years was analyzed by multivariable Cox regression. Pre-transplant predictors of DGF and influence of DGF and pre-transplant HLA antibodies on biopsy-proven rejections in the first 3 post-transplant months were determined by multivariable logistic regression. Donor age ≥50 years, simultaneous pre-transplant presence of HLA class I and II antibodies, diabetes mellitus as cause of end-stage renal disease, cold ischemia time ≥18 h, and time on dialysis >5 years were associated with increased risk of DGF, while the risk was reduced if gender of donor or recipient was female or the reason for death of donor was trauma. DGF alone doubled the risk for graft loss, more due to impaired death-censored graft than patient survival. In DGF patients, the risk of death-censored graft loss increased further if HLA antibodies (hazard ratio HR=4.75, $P < 0.001$) or donor-specific HLA antibodies (DSA, HR=7.39, $P < 0.001$) were present pre-transplant. In the presence of HLA antibodies or DSA, the incidence of biopsy-proven rejections, including antibody-mediated rejections, increased significantly in patients with as well as without DGF. Recipients without DGF and without biopsy-proven rejections

during the first 3 months had the highest fraction of patients with good kidney function at year 1, whereas patients with both DGF and rejection showed the lowest rate of good kidney function, especially when organs from ≥65-year-old donors were used. In this new era of transplantation, besides non-immunological factors, also the pre-transplant presence of HLA class I and II antibodies increase the risk of DGF. Measures to prevent the strong negative impact of DGF on outcome are necessary, especially during organ allocation for presensitized patients.

Keywords: renal transplantation, HLA antibodies, donor-specific antibodies, delayed graft function, biopsy-proven rejections, antibody-mediated rejections

INTRODUCTION

Acute renal injury early after transplantation can lead to delayed graft function (DGF), increase the immunogenicity of the tissue and result in immunological rejection episodes requiring treatment (1).

The reported incidence of DGF after deceased donor kidney transplantation varies between 5 and 50% and continues to grow as kidneys from elderly donors are increasingly used due to organ shortage (2–5). DGF was reported to have a negative impact on 12-month graft function (6) and longterm graft survival, almost doubling the risk of 5-year graft loss according to a recent study (7). Interventions to reduce the incidence of DGF, such as donor dopamine infusion or machine perfusion during organ removal and transport, are still experimental and there is no approved therapy to reduce or treat DGF (8). Therefore, there is a great interest in the early detection of procurement-, donor- and recipient-related risk factors of DGF to ensure optimal treatment for patients at risk. In addition to non-immunological factors, such as donor brain death, prolonged cold ischemia time and donor and recipient age, involvement of immunological factors has also been reported in the development of DGF (9, 10). Earlier data from the Serum Study of the Collaborative Transplant Study (CTS) indicated that adverse events in deceased donor kidney transplantation, such as no immediate function and rejection episodes during the first 3 months post-transplant, are associated with pre-transplant presence of alloantibodies against human leukocyte antigens (HLA) (11). Patients with these early adverse events showed significantly impaired graft survival rates. In the meantime, small single-center studies indicated that donor-specific HLA antibodies (DSA) and rejection episodes are particularly detrimental in patients with DGF, while more recent large-scale studies on an involvement of DSA in DGF are lacking (12, 13).

Sensitive antibody detection techniques have become routine since 2008 and this might have diminished the involvement of overlooked HLA antibodies in DGF. On the other hand, the risk of DGF is expected to have increased due to the growing use of kidneys from elderly donors. The aim of the current study was to identify the immunological and non-immunological predictors of DGF and to determine the alloantibody-dependent influence of DGF on post-transplant outcomes in a large cohort of patients transplanted at 8 different transplant centers in the recent 2008–2017 period.

MATERIALS AND METHODS

Study Population

The eight participating centers provided a pre-transplant serum on patients enrolled in the prospectively designed CTS Serum Study (www.ctstransplant.org) and completed a questionnaire 3 months post-transplant which contained the following queries: immediate function within the first 24 h after transplantation (e.g., >500 ml transplant urine), dialysis during the first post-transplant week (except for single dialysis for hyperkalemia), biopsy-proven rejection during the first 3 months, including the time and type of first rejection (borderline, T-cell-mediated, antibody-mediated or mixed T-cell- and antibody-mediated). The work of the CTS is approved by the Ethics Committee of the Medical Faculty of Heidelberg University (No. 083/2005) and performed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles in the currently valid version (14).

The HLA antibody screening was performed centrally in Heidelberg, using the AbScreen I and II ELISA kits of Biotest (Dreieich, Germany) which detected HLA class I and class II antibodies of the IgG isotype. Based on previous findings, an optical density (OD) of more than or equal to 300 was used as cut-off for anti-HLA positivity (15). As this kit was discontinued by the manufacturer, the LABScreen™ Mixed kit of Thermofisher/OneLambda (West Hills, CA, US) was used in 30% (513/1724) of the sera for detection of IgG HLA antibodies, following adjustment of the positivity cut-off to the normalized background ratio of ≥20 which resembles the positivity level of AbScreen ELISA.

DGF was defined as either no graft function during the first 24 h and/or dialysis during the first week (except for single dialysis for hyperkalemia) after transplantation (16). Adult patients (≥18 years) on whom we obtained a pre-transplant serum and a complete 3-months questionnaire and who received a kidney-only transplant from a deceased donor between January 1, 2008 and December 31, 2017 and had a functioning graft ≥8 days post-transplant were analyzed. The information obtained from the questionnaires was entered into the CTS database and connected with additional information on the transplants. In 757 cases (44%), we obtained from the participating centers information on the presence or absence of pre-transplant DSA as determined by single antigen bead technique.

Statistical Analysis

All cause graft, death-censored graft, and patient survival were analyzed from day 8 to the end of year 3 after transplantation. Multivariable Cox regression analysis was performed to account for the possible influence of the following confounders on graft survival: transplant year, transplant number, recipient age, recipient and donor sex and combination, diabetes mellitus as cause of end-stage renal disease, donor age, cold ischemia time, time on dialysis, HLA A+B+DR mismatches, general evaluation of the patient by the physician, latest panel-reactive antibody, donor history of hypertension, trauma as cause of donor death, donation after cardiac death, other causes of marginal donor, e.g., increased serum creatinine, antibody induction therapy, pre-transplant HLA class I and II antibodies and their combination, pre-transplant DSA, and DGF. Survival rates were illustrated using the Kaplan-Meier method.

Significant predictors of DGF and the influence of DGF together with HLA antibodies or DSA on biopsy-proven rejections during days 8–90 post-transplant were determined by multivariable logistic regression analysis, using the same confounders as in the Cox regression analysis. A stepwise backwards elimination of non-significant confounders was applied in the multivariable regression analysis. The software package IBM SPSS Statistics 25 (SPSS Inc, Chicago, IL, US) was used.

RESULTS

Predictors of DGF

A total of 1,724 patients from 8 centers who received a deceased donor kidney transplant between 2008 and 2017 and on whom a pre-transplant serum sample and a 3-months questionnaire on early adverse events was obtained in the framework of the prospective Serum Study of CTS (www.ctstransplant.org) was analyzed. These patients represented a random sample and a graft survival rate which was identical with that observed in 1,692 patients who were not included in the study, but received a deceased donor kidney transplant over the same time period at the same centers (**Figure 1**).

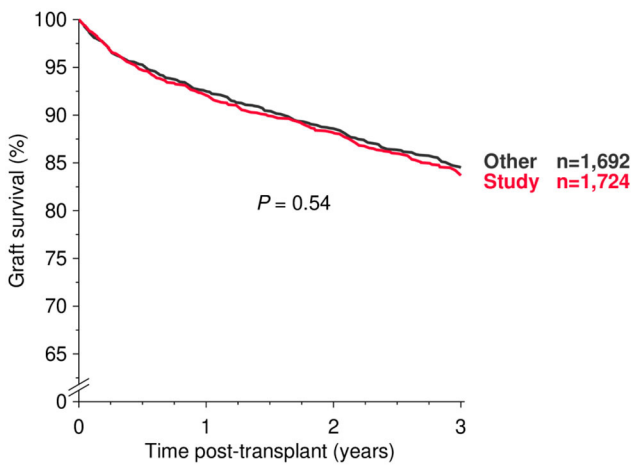

FIGURE 1 | All-cause graft survival during the first 3 post-transplant years in study patients and all other patients who received a deceased-donor kidney transplant at the participating centers during 2008–2017 (log rank *P* value is shown).

Table 1 shows the demographics of the study cohort. The patients were stratified according to whether they had DGF (n = 482, 28.0%) or not (n = 1,242, 72.0%). DGF was more frequent in

TABLE 1 | Demographics of study patients, *n* (%) or mean ± SD.

Characteristic	Unknown (%)	No DGF n = 1,242	With DGF n = 482	*P* value
TRANSPLANT YEAR	–			**<0.001**
2008–2012		734 (59)	242 (50)	
2013–2017		508 (41)	240 (50)	
TRANSPLANT NUMBER	–			0.17
First transplant		1,059 (85)	398 (83)	
Re-transplant		183 (15)	84 (17)	
RECIPIENT GENDER	–			**0.009**
Female		492 (40)	158 (33)	
Male		750 (60)	324 (67)	
RECIPIENT AGE (YEARS)	–	54.2 ± 13.0	56.1 ± 12.5	**0.004**
DONOR GENDER	–			0.11
Female		618 (50)	219 (45)	
Male		624 (50)	263 (55)	
DONOR AGE (YEARS)	–	52.4 ± 16.0	56.9 ± 14.3	**<0.001**
COLD ISCHEMIA TIME (HOURS)	–	14.0 ± 4.7	14.7 ± 5.7	**0.042**
TIME ON DIALYSIS (YEARS)	–	6.0 ± 4.1	6.8 ± 4.6	**<0.001**
DIABETES MELLITUS AS CAUSE OF ESRD		85 (7)	51 (11)	**0.010**
HLA-A+B+DR MISMATCHES	–			**0.019**
0–1		243 (20)	78 (16)	
2–4		840 (68)	324 (67)	
5–6		159 (13)	80 (17)	
CYTOTOXIC PRA	–			0.66
≤5%		1,132 (91)	436 (90)	
>5%		110 (9)	46 (10)	
PRE-TRANSPLANT HLA ANTIBODIES*	–			0.066
I neg, II neg		1,034 (83)	393 (82)	
I neg, II pos		61 (5)	21 (4)	
I pos, II neg		76 (6)	24 (5)	
I pos, II pos		71 (6)	44 (9)	
PRE-TRANSPLANT DSA	56			0.27
No		481 (85)	157 (82)	
Yes		84 (15)	35 (18)	

**ELISA or LABScreen Mixed.*

DGF, delayed graft function; ESRD, end-stage renal disease; PRA, panel-reactive antibodies; DSA, donor-specific HLA antibodies. Bold means statistically significant.

the more recent 2013–2017 than in the earlier 2008–2012 period (240/748, 32.1 vs. 242/976, 24.8%, $P < 0.001$). The mean of donor age was higher (56.9 vs. 52.4 years, $P < 0.001$) and the mean of cold ischemia time was longer (14.7 vs. 14.0 h, $P = 0.042$) in patients with DGF than in patients without DGF. Patients who developed DGF were more likely to be male (67.2 vs. 60.4%, $P = 0.009$) and older (56.1 vs. 54.2 years, $P = 0.004$). Furthermore, they had a longer dialysis time (6.8 vs. 6.0 years, $P < 0.001$) and more frequently a poor HLA match (5–6 HLA-A+B+DR mismatches: 16.6% vs. 12.8%, $P = 0.019$) and diabetes mellitus as cause of ESRD (10.6 vs. 6.8%, $P = 0.010$).

In the multivariable logistic regression analysis, donor age ≥70 years and simultaneous presence of HLA class I and II antibodies before transplantation were the strongest predictors of DGF (odds ratio [OR]=2.32 and 1.93, $P < 0.001$ and 0.002, respectively; **Table 2**). They were followed by donor age 60–69 years (OR = 1.64, $P = 0.001$), diabetes mellitus as cause of end-stage renal disease (OR = 1.62, $P = 0.012$), cold ischemia time ≥18 h (OR = 1.60, $P < 0.001$), pre-transplant time on dialysis >5 years (OR = 1.48, $P < 0.001$) and donor age 50–59 years (OR = 1.46, $P = 0.009$). A reduced risk of DGF was found when the cause of donor death was trauma (OR = 0.61, $P = 0.002$) or when recipient or donor gender was female (OR = 0.73, $P = 0.008$, and OR = 0.74, $P = 0.007$, respectively).

Influence of DGF and Pre-transplant HLA Antibodies on 3-Year Graft and Patient Survival

Figure 2 shows the influence of DGF on 3-year graft survival in patients with and without pretransplant HLA antibodies (**Figures 2A,B**) or donor-specific HLA antibodies (DSA, **Figures 2C,D**).

DGF was observed more frequently in patients who received a kidney transplant from ≥65- than <65 year-old-donors (153/432, 35.4% vs. 329/1,292, 25.5%; $P < 0.001$). Only 8.5% (13/153) of DGF patients who received a transplant from a ≥65-year-old donor had HLA antibodies prior to transplantation, as compared to the much higher 23.1% rate (76/329, $P < 0.001$) in patients with transplants from a <65-year-old donor. Because of this ambiguous distribution of variables in the different donor age groups, we stratified the univariate results according to donor age (**Figures 2A,C**: <65-year-old donor, **Figures 2B,D**: ≥65-year-old donor). Of note, due to a strong correlation of donor and recipient age, presumably as a result of age matching, e.g., in the Eurotransplant Senior Program, recipients of organs from <65-year-old donors were with a median of 52 years (interquartile range [IQR] 43–59 years) significantly younger than recipients of a graft from a ≥65 year-old donor (median 68 years, IQR 65–70 years).

For both donor age groups (<65- and ≥65-year-old), overall graft survival in patients without DGF was equally good, regardless of whether or not these patients had HLA antibodies (**Figures 2A,B**) or even DSA (**Figures 2C,D**) prior to transplantation. In contrast, the 3-year graft survival was significantly reduced in patients with DGF, even in the absence of pre-transplant HLA antibodies or DSA. The worst graft survival was observed in patients who had HLA antibodies or DSA before transplantation and developed DGF.

These results were confirmed in multivariable Cox regression analyses (**Table 3**). The overall graft survival was significantly reduced in patients with DGF, more due to impaired death-censored graft than patient survival. Compared to DGF-negative patients, the risk for death-censored graft loss was 2.37-fold higher in DGF-positive patients in the absence and 4.75-fold higher in the presence of pretransplant HLA antibodies ($P < 0.001$ for both). An even stronger increase of risk from 2.97- to 7.39-fold was observed in DGF-positive patients with pre-transplant DSA ($P < 0.001$ for both).

TABLE 2 | Significant predictors of delayed graft function as result of multivariable logistic regression.

Predictor	OR	95 % CI	*P*
Female recipient	0.73	0.58–0.92	**0.008**
Female donor	0.74	0.59–0.92	**0.007**
Donor 50–59 years	1.46	1.10–1.95	**0.009**
Donor 60–69 years	1.64	1.22–2.22	**0.001**
Donor ≥70 years	2.32	1.65–3.26	**<0.001**
Trauma as cause of donor death	0.61	0.45–0.83	**0.002**
Cold ischemia time ≥18 h	1.60	1.23–2.09	**<0.001**
Diabetes mellitus as cause of ESRD	1.62	1.11–2.37	**0.012**
Time on dialysis >5 years	1.48	1.18–1.86	**<0.001**
HLA class I and II AB pos	1.93	1.28–2.92	**0.002**

Odds ratios (OR) with 95%-confidence intervals (CI) are shown.
ESRD, end-stage renal disease; AB pos, antibody-positive. Bold means statistically significant.

Influence of DGF and Pre-transplant HLA Antibodies on Biopsy-Proven Rejection Episodes During the First 3 Post-transplant Months

Figure 3 illustrates the incidence of biopsy-proven rejection episodes from day 8 to 90 post-transplant for patients who received a kidney from a <65-year-old donor. Due to low patient numbers, generation of robust results for donors aged ≥65 years was not possible. Irrespective of whether the patients developed DGF or not, significantly higher rates of rejections, especially antibody-mediated rejections, were seen in patients with pre-transplant HLA antibodies or DSA than in patients without such antibodies. The multivariable analysis confirmed the univariate results with higher ORs for development of rejection in patients with pre-transplant HLA antibodies or DSA (**Table 4**). This association was statistically significant for all HLA antibody-positive groups and there was also a trend toward significance for DSA-positive patients with DGF (OR = 2.18, $P = 0.053$). DGF alone had no significant effect on the occurrence of rejections from day 8 to 90 after transplantation. To avoid

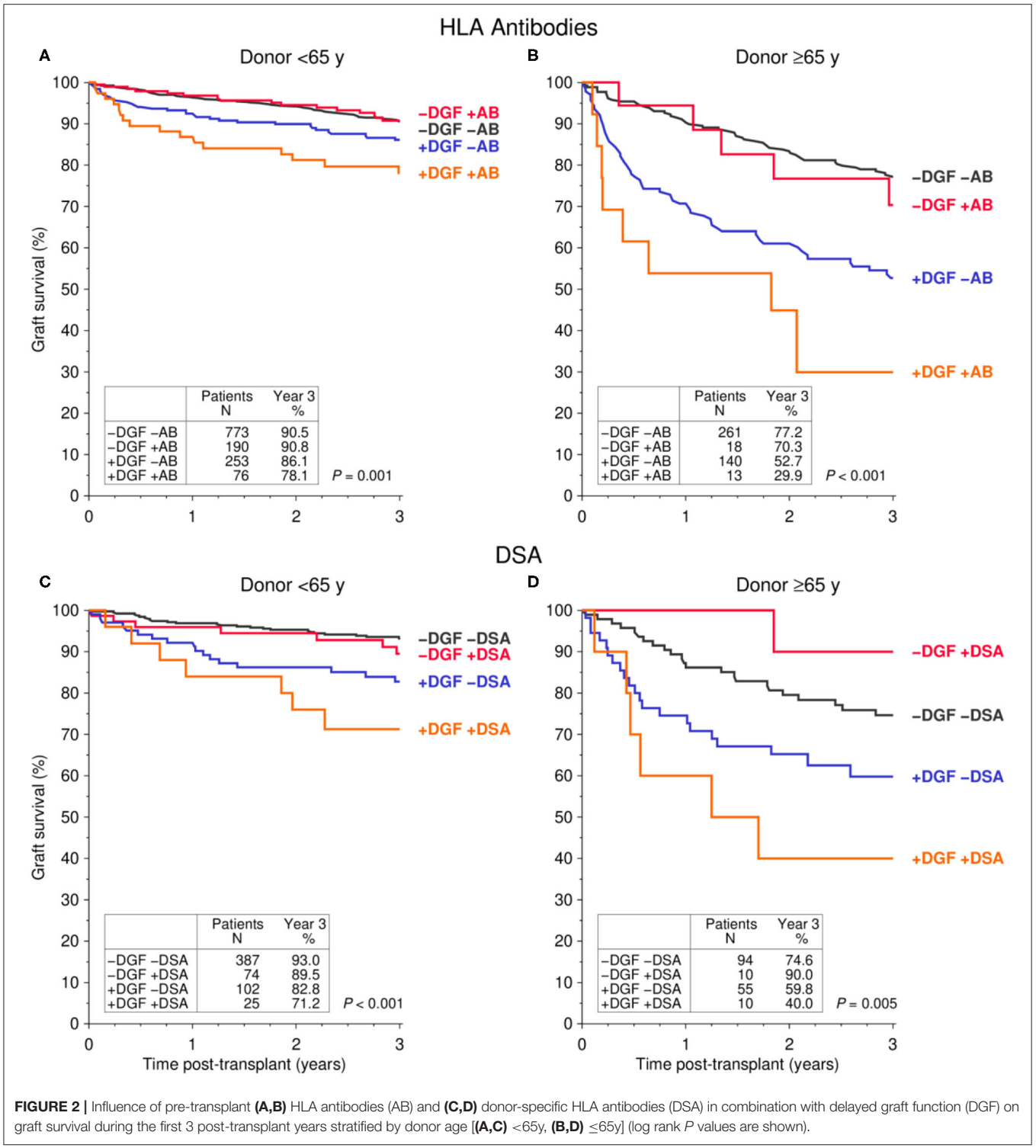

FIGURE 2 | Influence of pre-transplant **(A,B)** HLA antibodies (AB) and **(C,D)** donor-specific HLA antibodies (DSA) in combination with delayed graft function (DGF) on graft survival during the first 3 post-transplant years stratified by donor age [**(A,C)** <65y, **(B,D)** ≤65y] (log rank *P* values are shown).

a statistical bias, rejections during the first 7 days were not considered, most probably resulting in an underestimation of the proportion of rejections in patients with DGF. Indeed, 35% and 30% of rejections in DGF-patients with or without HLA antibodies, respectively, were observed during the first 7 days post-transplant, as compared to the much lower 25 and 18% rates in DGF-negative patients with and without HLA antibodies, respectively.

TABLE 3 | Results of multivariable Cox regression for the influence of delayed graft function (DGF), HLA antibodies (AB), and donor-specific antibodies (DSA) on survival during first 3 post-transplant years.

Confounder	N	HR	95 % CI	*P*
ALL CAUSE GRAFT SURVIVAL				
HLA Antibodies				
–DGF –AB	1,034	ref.		
–DGF +AB	208	1.13	0.71–1.80	0.62
+DGF –AB	393	2.02	1.55–2.65	**<0.001**
+DGF +AB	89	3.44	2.20–5.36	**<0.001**
DSA				
–DGF –DSA	481	ref.		
–DGF +DSA	84	1.04	0.49–2.21	0.92
+DGF –DSA	157	2.16	1.40–3.33	**<0.001**
+DGF +DSA	35	3.94	2.13–7.30	**<0.001**
DEATH-CENSORED GRAFT SURVIVAL				
HLA Antibodies				
–DGF –AB	1,034	ref.		
–DGF +AB	208	1.43	0.80–2.58	0.23
+DGF –AB	393	2.37	1.64–3.42	**<0.001**
+DGF +AB	89	4.75	2.74–8.22	**<0.001**
DSA				
–DGF –DSA	481	ref.		
–DGF +DSA	84	1.32	0.49–3.57	0.59
+DGF –DSA	157	2.97	1.59–5.55	**<0.001**
+DGF +DSA	35	7.39	3.50–15.6	**<0.001**
PATIENT SURVIVAL				
HLA Antibodies				
–DGF –AB	1,034	ref.		
–DGF +AB	208	1.05	0.54–2.06	0.88
+DGF –AB	393	1.78	1.24–2.57	**0.002**
+DGF +AB	89	1.75	0.79–3.84	0.17
DSA				
–DGF –DSA	481	ref.		
–DGF +DSA	84	0.95	0.33–2.74	0.92
+DGF –DSA	157	1.66	0.92–2.98	0.093
+DGF +DSA	35	1.35	0.40–4.51	0.63

Hazard ratios (HR) with 95%-confidence intervals (CI) are shown. Bold means statistically significant.

One-Year Kidney Graft Function Depending on DGF and Biopsy-Proven Rejection Episodes During the First 3 Post-transplant Months

The impact of DGF and rejections on serum creatinine at year 1 post-transplant, as stratified by donor age, is shown in **Figure 4**. Recipients of kidney allografts from <65-year-old deceased donors without DGF and without rejections during days 8–90 post-transplant had with 55.6% the highest fraction of patients with good kidney function at year 1 (creatinine <130 μmol/L) followed by patients with only DGF (37.6%) and only rejections (37.0%). Among patients with both DGF and rejections (REJ), the percentage of patients with good kidney function was an extremely low 27.5%; accompanied by a high graft failure rate of 11.0% during the first post-transplant year (**Figure 4A**).

When recipients of kidneys from ≥65-year-old deceased donors were analyzed, the fraction of patients with good kidney function at year 1 post-transplant was, overall, strikingly low with 20.1% in –DGF/–REJ, 10.6% in +DGF/–REJ, 6.7% in –DGF/+REJ, and 6.1% in +DGF/+REJ cases. Conversely, the rate of graft failure during the first post-transplant year was as high as 32.7 and 29.8% in DGF patients with and without rejections, respectively (**Figure 4B**).

DISCUSSION

The results obtained in this large multicenter cohort of more than 1,700 deceased-donor kidney transplant recipients indicate that, in addition to well-known non-immunological factors, a broad level of sensitization prior to transplantation as reflected by the co-presence of HLA class I and class II antibodies in patient's serum increases the risk of DGF development despite the currently applied sensitive antibody testing. Patients who developed DGF demonstrated impaired graft survival in the absence, and more strongly, in the presence of pre-transplant HLA antibodies or DSA. The potentiating effect of pre-transplant alloantibodies on the impact of DGF was not evident when patient survival was analyzed. In contrast, a strong influence of DGF was observed on death-censored graft loss when alloantibodies were present prior to transplantation, most probably due to additional immunological injury in an already damaged organ. This assumption was further supported by the high rate of diagnosed biopsy rejection episodes during days 8–90 after transplantation in pre-sensitized patients who had developed DGF up to day 7 post-transplant.

Mainly non-immunological donor-specific factors, such as age and brain death, and cold ischemia time have been associated with the development of DGF. In some previous studies, however, a significantly increased rate of DGF was found also in patients with pre-transplant HLA antibodies, whereas Quiroga et al. could not confirm such an association (17–20). Gibney et al. reported higher rates of primary non-function and DGF in 136 patients with pre-transplant DSA and we found in an independent previous series of 1,134 CTS Serum Study patients that no immediate function of the allograft was associated with the pre-transplant presence of especially HLA class I antibodies, whereas the association of this early adverse event with HLA class II antibodies reached statistical significance only in the univariate, but not in the multivariable analysis (18). The impact of double positivity for class I and class II on DGF development was not analyzed in this study. In two independent series of 4,136 and 5,315 kidney transplantations, the co-presence of class I and class II antibodies was found to be associated with strongly impaired graft survival (15, 21). Otten et al. reported a similar observation by analyzing the impact of pre-transplant DSA (22). The association of HLA antibodies with DGF was, however,

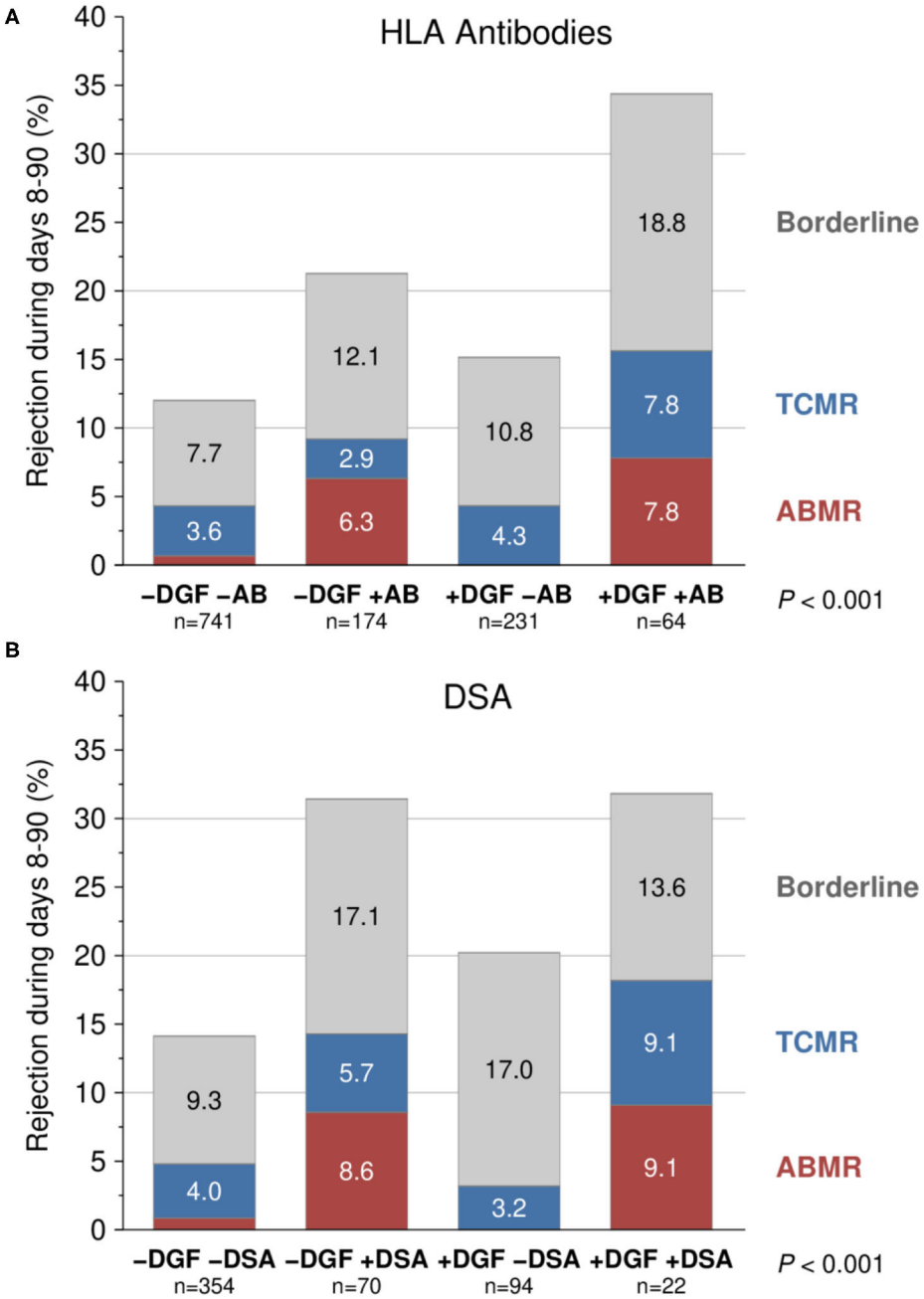

FIGURE 3 | Influence of pre-transplant **(A)** HLA antibodies (AB) and **(B)** donor-specific HLA antibodies (DSA) in combination with delayed graft function (DGF) on biopsy-proven rejection episodes during days 8–90 post-transplant (*P* value of chi-squared test is shown). Transplantations from <65-year-old donors were analyzed. Only the pairwise differences regarding DGF are not significant (1st column vs. 3rd column $P = 0.26$ and 0.15, 2nd column vs. 4th column $P = 0.14$ and 0.94; 1st vs. 2nd column $P < 0.001$; 3rd vs. 4th column $P < 0.001$ and 0.015). TCMR, T-cell-mediated rejection; ABMR, antibody-mediated rejection.

not studied in these three studies. Peräsaari et al. analyzed 771 patients from Helsinki and found that the risk of DGF was twice as high in patients with pretransplant DSA, while pre-transplant non-DSA had no significant effect (12). In the same study the risk of DGF was increased also with broadness of sensitization, number of DSA and cumulative antibody strength. Similarly, broad pre-transplant sensitization, as indicated by the simultaneous presence of HLA class I and II antibodies, was a strong predictor that almost doubled the risk of DGF in our study, whereas, most probably due to the currently applied sensitive antibody testing, the presence of only HLA class I or only class II antibody showed no significant effect. It is assumable that the co-presence of both HLA antibody classes is reflective of a generally increased alloreactivity which,

under the currently applied potent immunosuppression, can cause subclinical rejections that may go undetected in the early post-transplant phase. The rejection-mediated endothelial injury in transplant arteries could lead to a vasoconstriction, ultimately presenting the clinical picture of DGF.

In non-sensitized patients with DGF, the risk of all cause graft loss and death-censored graft loss was more than twice as high compared to the risk in patients without DGF. The risk of death-censored graft loss further increased to more than 7-fold when DGF-patients had detectable DSA pretransplant, most likely due to an increased rate of rejection episodes. Indeed, rejection was seen significantly more often and with greater severity in antibody-positive groups than in antibody-negative groups, irrespective of whether the patients developed DGF or not, while DGF alone resulted in only a small and non-significant increase in rejection episodes. Interestingly, 8 and 11% of DGF patients with or without rejection, respectively, had already lost their graft 1 year after transplantation when the donor organ was <65 years old. For recipients of an organ from a ≥65-year old donor, these figures rose to a striking 30 and 33%, respectively. This is all the more remarkable because transplant failures in the first 3 months after transplantation were not included in this calculation. Taken together, our results indicate that rejection in pre-sensitized patients is particularly harmful if they receive a pre-damaged organ from an elderly donor.

Compared to patients with no DSA and no DGF, Haller et al. found an insignificant increase of graft loss in patients with either DSA or DGF, while the same risk was 3 times and significantly higher in patients with pre-transplant DSA who developed DGF. They hypothesized that inferior graft survival in DSA-positive DGF-patients may either be due to more extensive effector functions of DSA, such as complement-activation in the inflammatory environment of DGF-patients compared to patients without DGF, or overlooked rejection episodes during the DGF process causing increased harm to the allograft (13). According to our data, a complementary explanation for the observed inferior outcomes in DSA-positive patients with DGF might be the occurrence of rejections in

TABLE 4 | Results of logistic regression for the influence of HLA antibodies (AB) and donor-specific HLA antibodies (DSA) in combination with delayed graft function (DGF) on biopsy-proven rejections during days 8–90 post-transplant.

Predictor	N	OR	95 % CI	*P*
HLA ANTIBODIES				
–DGF –AB	1,034	ref.		
–DGF +AB	208	1.76	1.20–2.59	**0.004**
+DGF –AB	393	1.29	0.93–1.77	0.12
+DGF +AB	89	2.41	1.45–4.01	**<0.001**
DSA				
–DGF –DSA	481	ref.		
–DGF +DSA	84	2.56	1.51–4.36	**<0.001**
+DGF –DSA	157	1.53	0.97–2.43	0.068
+DGF +DSA	35	2.18	0.99–4.80	0.053

Odds ratios (OR) with 95%-confidence intervals (CI) are shown. Bold means statistically significant.

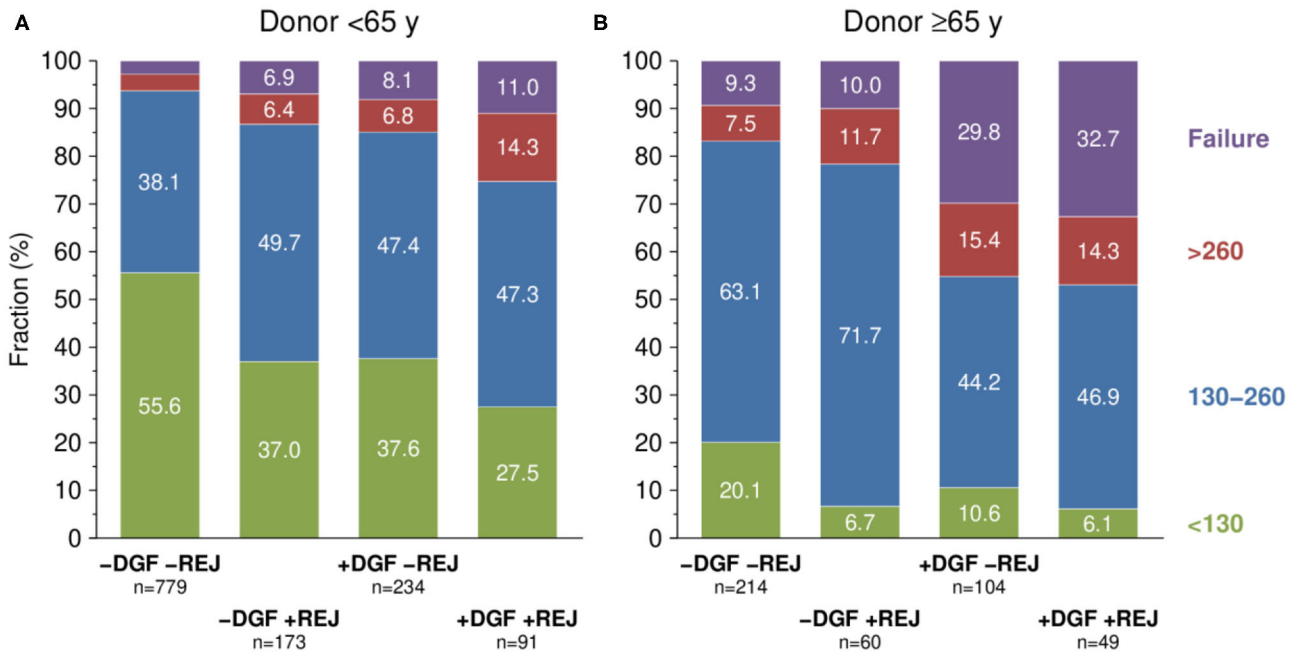

FIGURE 4 | Serum creatinine at 1 year post-transplant (μmol/L) depending on delayed graft function (DGF) and biopsy-proven rejection during first 3 months (REJ) stratified by donor age [**(A)** <65y, **(B)** ≤65y]. *P* values of chi-squared test with trend <0.001. Y, years of age.

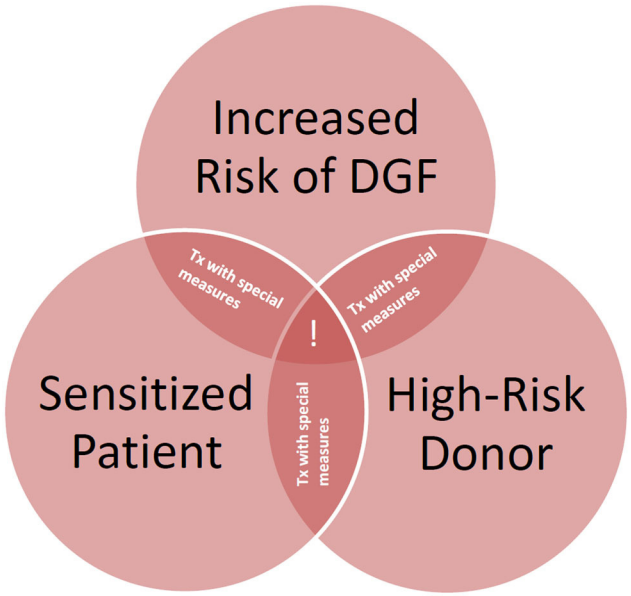

FIGURE 5 | Need for measures to improve graft survival in high-risk recipients of deceased donor kidney transplants. Special measures = avoidance of prolonged cold ischemia, avoidance of non-acceptable human leukocyte antigen (HLA) mismatches also in elderly recipients, desensitization if pre-sensitized, post-transplant HLA antibody monitoring. !, consideration of alternative options; Tx, transplantation.

an allograft that already has been damaged by DGF. DGF-associated damage can predispose the graft to an increased risk of immune attack by upregulating major histocompatibility complex as well as non-major histocompatibility complex alloantigens in the graft. Furthermore, graft injury caused by brain death or early damage due to DGF can lead to the production of chemokines that attract immune cells into the graft and eventually result in rejections. Given the high 36% rate of DGF in patients who received an organ from a ≥65-year-old donor in our study and the inferior outcomes, careful selection of recipients of these organs during organ allocation is mandatory, especially when they are presensitized.

The strength of the study is, besides the high patient number, the existence of relevant non-immunological and immunological variables, in all patients as, due to study design, only patients on whom these variables were available were analyzed. Limitations of the study are the multicenter approach, which forced us to reduce the number of variables that could be asked to the most relevant ones, and the missing information on the presence of pre-transplant DSA in 56% of the patients. Moreover, in these patients the DSA information was delivered by the participating centers and there is heterogeneity not only in the determination but also in consideration of acceptable levels of DSA. Single antigen tests used for DSA testing stem from two different suppliers with slight differences in the sensitivity and composition of detected HLA antibody specificities and there are technical variations between the laboratories, e.g., in pretreatment of sera to eliminate the prozone effect. In addition, the centers are using different algorithms for the determination of unacceptable HLA antigen mismatches, and depending on the algorithm, more or fewer organ offers are excluded for patients with a similar antibody profile (23, 24). Overall, despite these limitations, this is the first large-scale study that demonstrates the alloantibody-dependent detrimental influence of DGF on post-transplant outcomes in the modern era of transplantation.

In conclusion, DGF has a strong influence on graft survival, also in the absence of pre-transplant HLA alloantibodies. However, pre-transplant HLA alloantibodies are a predisposing factor for DGF, and the presence of alloantibodies, especially that of DSA, together with DGF are associated with strongly impaired graft outcome. Adequate measures to prevent DGF in sensitized patients should be in place, especially during the allocation and transplantation of organs from elderly donors (**Figure 5**).

DATA AVAILABILITY STATEMENT

The raw data are available upon request to the Collaborative Transplant Study in accordance with the consents of the patients, the participating transplant centers and registries.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

CS, BD, and CM designed the study, analyzed the data and wrote the paper. BD performed the statistical analysis. AR contributed to data acquisition. FK, LS, FEc, VS, SŽ-Ć, NK, DK, PB, MH, MZi, MN, FEm, PP, HK, RW, AM, and MZe delivered the sera and clinical data and contributed to the writing of the paper. CS, TT, and SS participated in testing of sera from the Heidelberg transplant center. The study received no external funding.

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