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Reconditioning DCD lungs through machine perfusion

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TRANSPLANT TRIAL WATCH
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Transplant Trial Watch

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Keywords: kidney transplant, randomised controlled trial, immunosuppresion, diabetes mellitus, tacrolimus

To keep the transplantation community informed about recently published level 1 evidence in organ transplantation ESOT and the Centre for Evidence in Transplantation have developed the Transplant Trial Watch. The Transplant Trial Watch is a monthly overview of 10 new randomised controlled trials (RCTs) and systematic reviews. This page of Transplant International offers commentaries on methodological issues and clinical implications on two articles of particular interest from the CET Transplant Trial Watch monthly selection. For all high quality evidence in solid organ transplantation, visit the Transplant Library: www.transplantlibrary.com.

RANDOMISED CONTROLLED TRIAL 1

Criteria for Prediabetes and Posttransplant Diabetes Mellitus After Kidney Transplantation: A 2-Year Diagnostic Accuracy Study of Participants From a Randomized Controlled Trial.

by Kurnikowski, A., et al. American Journal of Transplantation 2022 [record in progress].

Aims

This post-hoc study aimed to investigate the diagnostic ability of fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) against the oral glucose tolerance test (OGTT)-derived 2-h plasma glucose (2hPG) in kidney transplant recipients (KTRs) from the Insulin Therapy for the Prevention of New Onset Diabetes After Transplantation study (ITP-NODAT).

Interventions

Participants in the ITP-NODAT trial were randomised to either the basal insulin intervention group or the standard-of-care group.

Participants

263 kidney transplant recipients (KTRs) from the ITP-NODAT trial.

Outcomes

The main outcomes of interest were the evolution of posttransplant diabetes mellitus (PTDM), diagnostic accuracy of HbA1c and FPG criteria for PTDM and impaired glucose tolerance (IGT), and relationship of fasting plasma glucose and HbA1c versus 2hPG.

Follow-Up

24 months after transplantation.

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CET Conclusion
This interesting post-hoc study investigates diagnostic parameters for post-transplant diabetes in a cohort of patients from the ITP-NODAT study. The authors demonstrate that around 1/3 of patients switch glycaemic category (normal/impaired glucose tolerance/diabetes) in the 2 years post-transplant. Use of conventional HbA1C or fasting glucose thresholds for diagnosis missed up to 69% cases diagnosed by a formal 2-h oral glucose tolerance test (OGTT). There are some limitations to this post-hoc study, including a relatively small sample size with few patients with PTDM, and a lack of data on ethnicity. However, it does demonstrate the usefulness of a formal OGTT in diagnosing post-transplant diabetes.

Trial Registration
ClinicalTrials.gov—NCT03507829.

Funding Source
Non-industry funded.

RANDOMISED CONTROLLED TRIAL 2

Prolonged-Release Once-Daily Formulation of Tacrolimus Versus Standard-of-Care Tacrolimus in *de novo* Kidney Transplant Patients Across Europe.

by Budde, K., et al. Transplant International 2022; 35: 10225.

Aims
This study aimed to compare the posttransplant outcomes of LCP-tacrolimus (LCPT) versus current standard-of-care tacrolimus [immediate-release tacrolimus (IR-Tac) or prolonged-release tacrolimus (PR-Tac), according to centre preference] in *de novo* kidney transplant recipients.

Interventions
Participants were randomly assigned to receive either LCPT or current standard-of-care tacrolimus.

Participants
403 *de novo* kidney transplant recipients (≥18 years).

Outcomes
The primary outcome was the tacrolimus total daily dose (TDD). The secondary clinical outcomes were treatment failure, treatment discontinuation, delayed graft function, local diagnosis of acute rejection requiring treatment, and concomitant immunosuppressive medications.

Follow-Up
6 months.

CET Conclusion
This phase IV multicentre study compared the use of LCP-tacrolimus with standard of care (either standard (SR) or prolonged release (PR) tacrolimus depending on centre preference) in *de novo* kidney transplant recipients. The authors demonstrated that despite a significantly lower total daily dose in the LCP-tacrolimus group, there was no difference in trough levels or short-term clinical outcomes between groups. The study is fairly well-designed, although the decision to allow the control arm to receive SR or PR tacrolimus at centre discretion is slightly odd as the study is left underpowered to show a difference in comparison to either in isolation. It is not really clear if there is any clinical benefit to an overall dose reduction; trough levels are similar so overall exposure is likely to be equivalent. Certainly, the study provides confirmation that the LCP-tacrolimus formulation is safe and equivalent in clinical efficacy to SR and PR formulations.

Jadad Score
3.

Data Analysis
Modified intention to treat.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov—NCT02432833.

Funding Source
Industry funded.

CLINICAL IMPACT SUMMARY

Tacrolimus has become the calcineurin inhibitor (CNI) of choice for maintenance immunosuppression following solid organ transplantation, demonstrating lower risk of acute rejection and improved graft survival compared to cyclosporine (1). It does have some drawbacks, including an increased risk of new-onset diabetes and an unfavourable pharmacokinetic profile with a rapid peak and narrow therapeutic window.

There have been a number of attempts to produce a tacrolimus formulation with a flatter pharmacokinetic profile and less pronounced peak, allowing once-daily dosing. Such a profile may have potential to reduce toxicity by reducing peak levels, and once-daily dosing may have an impact on compliance by reducing pill burden. The most-recent of these formulations is LCP-tacrolimus, which is reported to increase bioavailability and reduce first-pass metabolism compared to earlier formulations (2).

In a recent, phase 4 multicentre study, Budde et al. investigated the role of LCP-tacro in 401 *de novo* kidney transplant recipients across 10 European countries (3). Recipients were randomised to receive LCP-tacro or "standard care," which could be immediate-release (IR) or prolonged-release (PR) tacrolimus alongside basiliximab, mycophenolate and corticosteroids. The authors demonstrated a significantly lower daily tacrolimus dose for the LCP-tacrolimus group to achieve slightly higher trough levels, confirming the improved bioavailability seen in earlier studies. However, there were no significant differences in clinical outcomes including rejection rates, graft survival, graft function or toxicity.

This large study was well-designed and reported, with central block-randomisation stratified by site and use of a modified intent-to-treat analysis. Whilst reflective of real-world variation in practice, the decision to allow either IR or PR tacrolimus as standard of care does limit the conclusions somewhat, as there is insufficient power to compare LCP-tacrolimus to either alternative formulation in isolation.

In reality, this study is unlikely to have much impact on clinical practice. A reduction in daily dose of tacrolimus alone is not sufficient to justify switching to what is presumably a more expensive formulation, although no health economic analysis is presented. Extended follow-up would be required to see if there is any benefit to the flattened pharmacokinetic profile on the risk of CNI toxicity in the longer-term.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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Lung Transplantation From Controlled and Uncontrolled Donation After Circulatory Death (DCD) Donors With Long Ischemic Times Managed by Simple Normothermic Ventilation and *Ex-Vivo* Lung Perfusion Assessment

Alessandro Palleschi[1,2], Alberto Zanella[1,3], Giuseppe Citerio[4,5], Valeria Musso[1,2], Lorenzo Rosso[1,2], Davide Tosi[2], Jacopo Fumagalli[3], Gianluca Bonitta[1], Elena Benazzi[6], Gianluca Lopez[7], Valeria Rossetti[8], Letizia Corinna Morlacchi[8], Clarissa Uslenghi[1,2], Massimo Cardillo[9], Francesco Blasi[1,8], Giacomo Grasselli[1,3], Franco Valenza[1,10] and Mario Nosotti[1,2]*

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Donation after cardiac death (DCD) donors are still subject of studies. In this prospective cohort trial, we compared outcomes after lung transplantation (LT) of subjects receiving lungs from DCD donors with those of subjects receiving lungs from donation after brain death (DBD) donors (ClinicalTrial.gov: NCT02061462). Lungs from DCD donors were preserved *in-vivo* through normothermic ventilation, as per our protocol. We enrolled candidates for bilateral LT ≥14 years. Candidates for multi-organ or re-LT, donors aged ≥65 years, DCD category I or IV donors were excluded. We recorded clinical data on donors and recipients. Primary endpoint was 30-day mortality. Secondary endpoints were: duration of mechanical ventilation (MV), intensive care unit (ICU) length of stay, severe primary graft dysfunction (PGD3) and chronic lung allograft dysfunction (CLAD). 121 patients (110 DBD Group, 11 DCD Group) were enrolled. 30-day mortality and CLAD prevalence were nil in the DCD Group. DCD Group patients required longer MV

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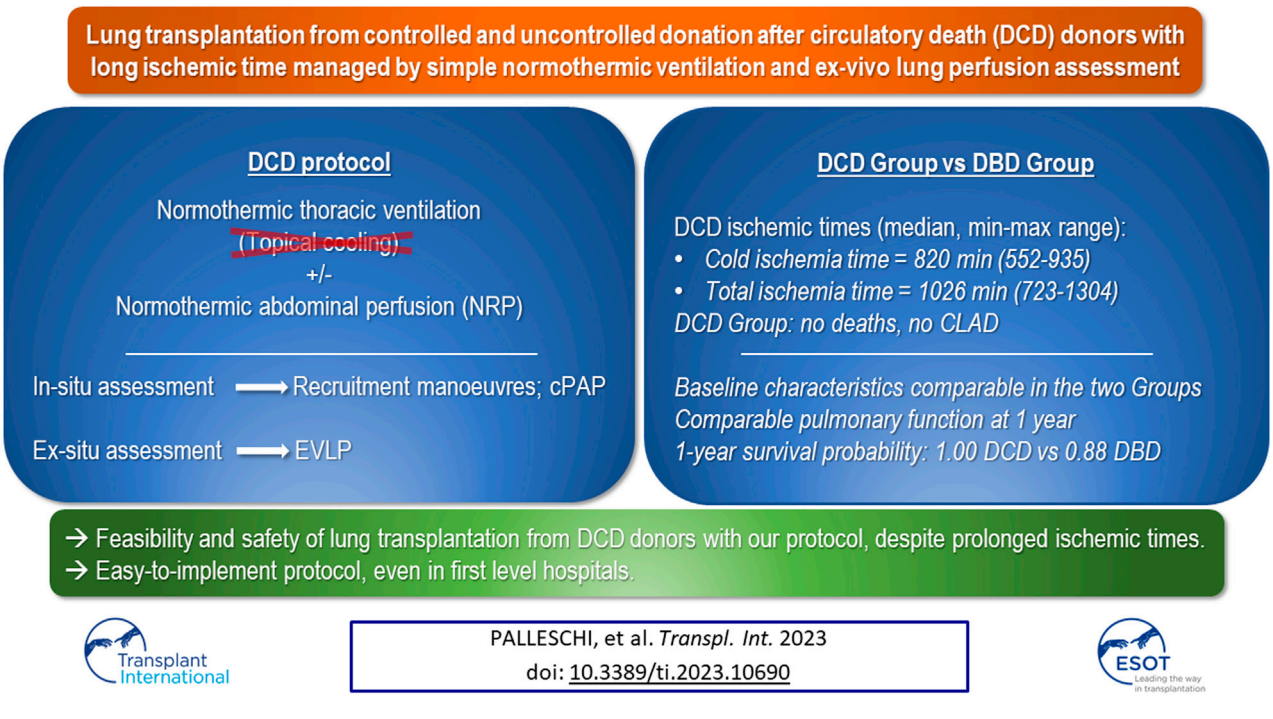
Abbreviations: ALAD, acute lung allograft dysfunction; AR, acute rejection; cDCD, controlled donation after cardiac death; CF, cystic fibrosis; CIT, cold ischemia time; CLAD, chronic lung allograft dysfunction; CPAP, continuous positive airway pressure; CPR, cardio-pulmonary resuscitation; DBD, donation after brain death; DCD, donation after cardiac death; ECMO, extracorporeal membrane oxygenation; EKG, electrocardiogram; EVLP, *ex-vivo* lung perfusion; FEV1, Forced Expiratory Volume in the first second; FVC, forced vital capacity; GEE, generalized estimating equations; ICU, intensive care unit; LAS, Lung Allocation Score; MV, mechanical ventilation; NRP, normothermic regional perfusion; $PaCO_2$, arterial partial pressure of carbon dioxide; PAPm, mean pulmonary artery pressure; PEEP, positive end-expiratory pressure; PGD, primary graft dysfunction; RM, recruitment manoeuvre; TIT, total ischemia time; TPT, total preservation time; uDCD, uncontrolled donation after cardiac death; WIT, warm ischemia time; WLST, withdrawal of life-sustaining treatment.

(DCD Group: 2 days, DBD Group: 1 day, p = 0.011). ICU length of stay and PGD3 rate were higher in DCD Group but did not significantly differ. LT with DCD grafts procured with our protocols appears safe, despite prolonged ischemia times.

Keywords: lung transplantation, chronic lung allograft dysfunction, primary graft dysfunction, donation after circulatory death donors, ischemia time, lung preservation



GRAPHICAL ABSTRACT |

INTRODUCTION

Lung transplantation is a well-established treatment for selected patients with end-stage benign respiratory diseases. Donor's shortage is one of the main factors limiting lung transplantation, hence the great interest in lung procurement from donation after circulatory death (DCD) donors (1,2). Maastricht category III DCD donors are the most widely used and best studied (3). Conversely, the uncontrolled settings of categories I and II are fascinating but challenging for at least three reasons: timing, organ preservation, and assessment. On the other hand, while using category III DCD lungs avoids these issues, it gives rise to ethical concerns about the withdrawal of life-sustaining treatment (WLST). In this scenario, few lung transplantation centres established an uncontrolled DCD (uDCD) program, and even fewer have protocols including both controlled (cDCD) and uncontrolled DCD settings (4).

The impact of the legal and ethical system of the different countries is relevant. In Italy, DCD is legal but suffers from the 20 min of recorded flat electrocardiogram (EKG) required for death declaration. WLST is also allowed by Italian law but has not become common practice yet and has been only recently codified. After a long pre-clinical phase (5,6,7), we refined an original two-steps protocol to manage lungs from DCD donors to overcome the long acirculatory period (8). First, by leveraging the possibility of dissociating ischemia from hypoxia, we adopted an open lung strategy for *in-situ* lung preservation even for prolonged periods, without topical cooling. In the second phase of the protocol, we employed the *ex-vivo* lung perfusion (EVLP) for *ex-situ* graft evaluation and reconditioning. We began our experience with the uncontrolled setting in 2014, and then followed the same principles when dealing with the controlled one (9).

Here we present the results of a clinical trial comparing the outcomes after lung transplantation of subjects receiving lungs from DCD donors with those of subjects who received lungs procured from donation after brain death (DBD) donors.

PATIENTS AND METHODS

Study Design

This study is a single-institution, prospective cohort trial (ClinicalTrial.gov: NCT02061462). We wanted to verify the

safety of lung transplantations performed with organs from DCD donors procured with an original protocol. We compared clinical and functional outcomes of patients undergoing lung transplantation who received grafts from DCD donors (DCD Group) with those of recipients of lungs from DBD donors (DBD Group) in our centre in the same period. We also performed an analysis comparing the outcomes of the DCD Group with those of recipients of lungs from DBD donors requiring machine perfusion (DBD-EVLP Group).

Since November 2014 all subjects provided written informed consent to participate in the trial at the time of enlisting, in accordance with the protocol approved by the local Ethics Committee. Recipients were selected sequentially, based on blood group, size match (total lung capacity) and waiting-list status (i.e., lung allocation score (LAS) or emergency program) (10,11). The type of donor (DBD vs. DCD) did not represent a criterion for donor-recipient matching, thus maintaining randomness of recipients group distribution. Recipients of a DCD lung were asked to renew their consent to receive organs from a DCD donor closely ahead of transplantation. During and after surgery, standard care was provided in both groups, according to our protocol (see **Supplementary Material**). Recipients' and respective donors' variables of interest were recorded in a dedicated electronic database from the date of waiting-list entry to the date of the last follow-up. Institutional board approval for data use was obtained (number 749_2016bis). The follow-up period was concluded on 31st July 2020.

Inclusion and Exclusion Criteria

All patients enlisted for bilateral lung transplantation older than 14 years were deemed eligible. Candidates for multi-organ transplantation or re-transplantation were excluded. Donors aged 65 and older, as well as DCD category I or IV donors, were also excluded.

Lung Allocation Process and Procurement Protocol

The lungs, from both DBD and DCD donors, were offered to our centre by the regional and national organ procurement organizations: the North Italian Transplant program (NITp) and the National Transplantation Centre (CNT), respectively (10,12). Notably, Italian law requires 6 h of observation, or 20 min of flat EKG to declare the patient's death according to neurological or cardiocirculatory criteria, respectively.

Donor Selection Criteria

Both DBD and DCD donor lungs suitability was determined according to standard criteria. Donors with massive lung contusions, history of aspiration of gastric content, pneumonia, or sepsis were excluded. Regarding DCD Maastricht type II donors, subjects with cardiovascular collapse, first treated by an advanced life support crew on the scene, then transferred to the emergency room, were considered as potential donors if declared dead after advanced cardiac life support attempts had failed (8). DCD Maastricht type III were patients admitted to the intensive care unit (ICU), where cardiac arrest occurred after a planned WLST. The following were considered DCD donors refusal criteria: unwitnessed cardiac arrest; no-flow (preceding initiation of cardio-pulmonary resuscitation, CPR) period >15 min and/or low flow >60 min for uDCD; *in-situ* warm ischemia time (WIT) >240 min for both cDCD and uDCD.

DBD Preservation and Procurement Protocol

Our lung procurement procedure consists of a standard bi-pulmonary block retrieval Once organs have been prepared for retrieval and the pulmonary artery (PA) is cannulated, prostaglandin E1 (500 mcg) is injected into the PA, aortic and venae cavae cross-clamp is performed, the left atrial appendage is amputated and/or the posterior aspect of the left atrium is incised, and the anterograde pulmonary flush is performed with 60 mL/kg of cold (4°C–8°C) PerfadexTM. The retrograde pulmonary flush is performed by using 250 mL PerfadexTM per pulmonary vein.

DCD Preservation and Procurement Protocol

Our protocol for lung preservation and retrieval has been previously described in detail (8,9). In short, it consists of a non-rapid normothermic open-lung procurement, namely without pleural topical cooling (i.e., without chest tube placement) before the start of cold flushing. In uDCD donor's management, after heart beating cessation, 5 min of no-touch period are required to clinically confirm the diagnosis of death. A recruitment manoeuvre (RM) is performed by progressively increasing airway pressure over a positive end-expiratory pressure (PEEP) of 5 cmH_2O to obtain a total airway pressure of 35 cmH_2O with 10 bpm of respiratory rate and inspiratory/expiratory [I/E] ratio 1:1. Continuous positive airways pressure (CPAP 10 cmH_2O, 100% FiO_2) is applied until death is confirmed according to circulatory criteria (20 min of flat EKG). Heparin is given (10.000 IU by endovenous push, followed by 3 min of CPR), a new RM is performed, and ventilation is started (respiratory rate 4 breaths/min, tidal volume 6 mL/kg, PEEP 8 cmH_2O, fraction of inspired oxygen [FiO_2] 100%, I/E ratio 1:1). If chest radiographs and bronchoscopic evaluation are normal, the subject is transferred to the operating room, and lung procurement is performed. Lungs are perfused *in situ* with a fibrinolytic agent (15 mg of recombinant tissue plasminogen activator, rTPA), flushed with Perfadex^{TM} (60 mL/kg anterograde) and procured (see **Supplementary Material** for further details).

Similarly, in case of cDCD, after re-intubation of the donor, lung preservation is achieved through protective mechanical ventilation (13). If combined procurement with abdominal organs is proposed, we associate a non-rapid normothermic open-lung strategy with the abdominal normothermic regional perfusion (NRP), as described in the **Supplementary Material** (9).

Ex-Vivo Lung Perfusion

We utilize a custom-made circuit to perform EVLP procedures according to a protocol previously described (14). For the purpose of this trial, EVLP has been used in the following cases:

- lungs from DBD donors with PaO_2/FiO_2 <300 mmHg on a PEEP of 5 cmH_2O and/or with chest X-ray abnormalities after optimization of MV
- lungs from DBD donors on veno-arterial extracorporeal membrane oxygenation (ECMO) for cardio-circulatory support during brain death observation, whose evaluation of gas exchange is suboptimal
- lungs from DCD donors.

At the end of the EVLP protocol, lungs were judged suitable for transplantation according to criteria described elsewhere (15).

Ischemia Times Definition

- Cold ischemia time (CIT): the time between lung cold flushing and beginning of organ implantation (without considering machine perfusion time) for both DBD and DCD grafts.
- Intraoperative WIT: the time from the beginning of organ implantation to reperfusion.
- Total ischemia time (TIT): the time from cross-clamping to reperfusion for the DBD group; from cardiac arrest to reperfusion for category II DCD donors; from the drop of systolic blood pressure <50 mmHg to reperfusion for category III DCD donors. TIT did not include machine perfusion time.
- Total preservation time (TPT): the time from cross-clamping to reperfusion for DBD donors, from the end of CPR to reperfusion for category II DCD donors, and from cardiac arrest to reperfusion for category III DCD donors. TPT included machine perfusion time.

Finally, for category II DCD donors, we also considered a WIT period from cardiac arrest to pulmonary cold flush; for category III DCD donors, we recorded interval 1, 2, 3 and 4 as suggested by the International Society for Heart and Lung Transplantation (ISHLT) (16). For more details on the procurement process see **Supplementary Material**.

Study Endpoints

Primary endpoint was the 30-day mortality after transplantation. The secondary endpoints were the duration of MV, ICU length of stay, the occurrence of primary graft dysfunction (PGD) of grade 3 within the first 72 h after transplantation (17), and the onset of chronic lung allograft dysfunction (CLAD) (18).

Statistical Analysis

Continuous data are presented as median and inter quartile range (IQR). Binary variables are shown as absolute and percentages frequencies. The Mann-Whitney or Chi-square tests were performed, as appropriate. Pulmonary function parameters were measured at 3, 6, and 12 months. The repeated measures for pulmonary function data were analysed using the "mean response profile" method through generalized estimating equations (GEE) by employing time as a categorical variable and logit link function (19). GEE standard errors were calculated with a sandwich estimator. We used the unstructured working correlation matrix selected by correlation information criterion (20). The GEE regression model was adjusted by donor smoking history, donor age, donor-recipient sex mismatch, surgical incision, recipient medical diagnosis, LAS, grade 3 PGD, TIT and WIT for both first and second lung, airway complications.

We chose GEE because it allows a population-averaged interpretation of the regression coefficients. The null hypothesis was that the difference of pulmonary function between the two study groups was constant over time. This was verified using the multivariate Wald test, testing time × group interaction in the GEE regression model. Profile likelihood confidence intervals (CIs) at 95% confidence level were computed. Univariate Wald test for each GEE-estimated parameter was performed. The non-parametric Kaplan-Meier estimator was used to analyse time-to-event data related to overall survival and CLAD onset. Confidence intervals (CIs) were at 95% and 2-sided p-values were calculated. A p-value of <0.05 was considered statistically significant; the inference should be intended for exploratory purposes. All analyses and graphs were carried out using an R software (version 3.2.2) (21).

RESULTS

Study Population

From November 2014 to July 2019, we performed 143 lung transplantations. Out of these, 22 cases were excluded from the study: ten single-lung transplantations, eight donors ≥65 years-old, two re-transplantations, one DCD category IV donor, one recipient <14 years-old (**Figure 1**). The remaining 121 patients were enrolled in the study: 110 in the DBD Group and 11 in the DCD Group (five patients received lungs procured from DCD II donors and six from DCD III donors). The complete results of our DCD program are shown in **Figure 2**.

Recipients' diseases leading to lung transplantation were distributed homogeneously in the two groups; notably, in the DCD and DBD groups, 72.7% and 61.9% of patients suffered from cystic fibrosis (CF), respectively. Recipients were similar in terms of number of urgent transplants, LAS, preoperative mean pulmonary artery pressure (PAPm), and preoperative arterial partial pressure of carbon dioxide ($PaCO_2$). Sex mismatch (female recipient with male donor) occurred more often in the DCD Group (p = 0.009) (**Table 1**). There were no statistically significant differences between the DBD and DCD groups in terms of donors' sex, age, comorbidities, duration of MV, smoking history, percentage of abnormal chest X-ray, PaO_2/FiO_2 ratio, and secretions at bronchoscopy. Donor BMI was significantly higher in the DCD Group (p = 0.022). The percentage of grafts from DBD undergoing machine perfusion was 15.5%; all grafts from DCD donors underwent *ex-vivo* evaluation. With regard to perioperative variables, the two groups were similar in terms of type of incision, need for both intraoperative and post-operative extra-corporeal support, and packed red blood cell, plasma and platelet intraoperative transfusion.

CIT, TIT, and preservation times were significantly higher in the DCD Group (p < 0.001), while intraoperative WIT was similar between the two groups. **Table 2** shows the ischemia

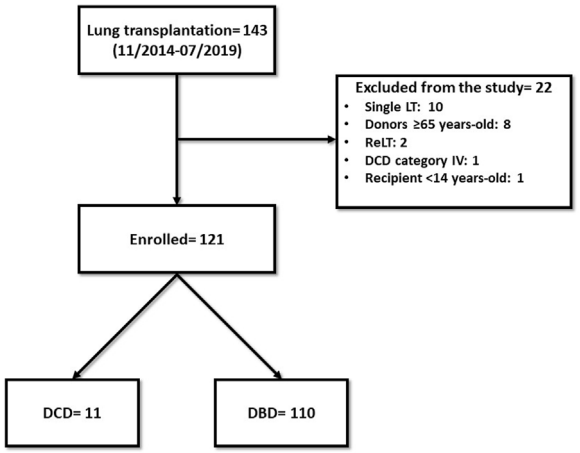


FIGURE 1 | Study population flow-chart. LT: lung transplantation; ReLT: retransplantation.

times of procurement and preservation in the DCD Group, in details.

Post-Operative Course and Outcomes

Post-operative data and outcomes are shown in **Table 3**. No adverse events related to our protocol were recorded in the DCD Group. In the first 30 days 1 patient (0.9%) died in the DBD Group, none in the DCD Group (p = NS). There was a statistically significant difference in median duration of MV (2 days for DCD and 1 day for DBD, p = 0.011). The prevalence of PGD3 within the first 72 h was 27.3% in the DCD Group and 18.2% in the DBD Group (p = 0.742).

Airway complications occurred in two recipients of DCD Group (18.2%) and seven of the DBD Group (6.4%): the difference, however, did not reach statistical significance (p = 0.154). No bronchial anastomotic dehiscence occurred in both groups, but only stenosis. Both cases and the 71.4% of patients required endoscopic treatment in the DCD Group and DBD Group, respectively.

The incidence of both histology-proven acute rejection (AR) and acute lung allograft dysfunction (ALAD) was similar between the two groups (22). The median follow-up period after transplantation was 605 days in the DCD Group and 895 days in the DBD Group. None of the patients receiving lungs from a circulatory death donor experienced CLAD during the period of the study. The probability of CLAD free survival in the DBD Group at 1, 3 and 5 years after transplantation was 0.96, 0.68, and 0.60, respectively (**Figure 3**). There were no deaths in the DCD Group, while overall survival in the DBD Group at 1, 3 and 5 years after surgery was 0.88, 0.75 and 0.70, respectively (**Figure 4**).

Pulmonary Function

At 3 months, pulmonary function values were similar in both groups for Forced Expiratory Volume in the first second (FEV1), forced vital capacity (FVC) and Tiffeneau index. Mean percentage of predicted FEV1 at 3, 6 and 12 months was 76.3%, 78.5%, and 81.7% in the DCD Group and 77%, 83.5%, and 86% in the DBD Group, respectively (**Supplementary Table S1**). The difference in mean FEV1 was statistically significant only at 6 months (p = 0.046), as shown in **Figure 5**. Mean FVC and Tiffeneau index were not significantly different at all time points (**Figure 6**).

The results of the adjusted GEE regression analysis for pulmonary function tests cohort are shown in **Supplementary Table S2**. At the test baseline (3 months), lower FEV1 and FVC were associated with grade 3 PGD and clamshell incision. Moreover, grade 3 PGD, donor age, and airway complications significantly reduced the Tiffeneau index.

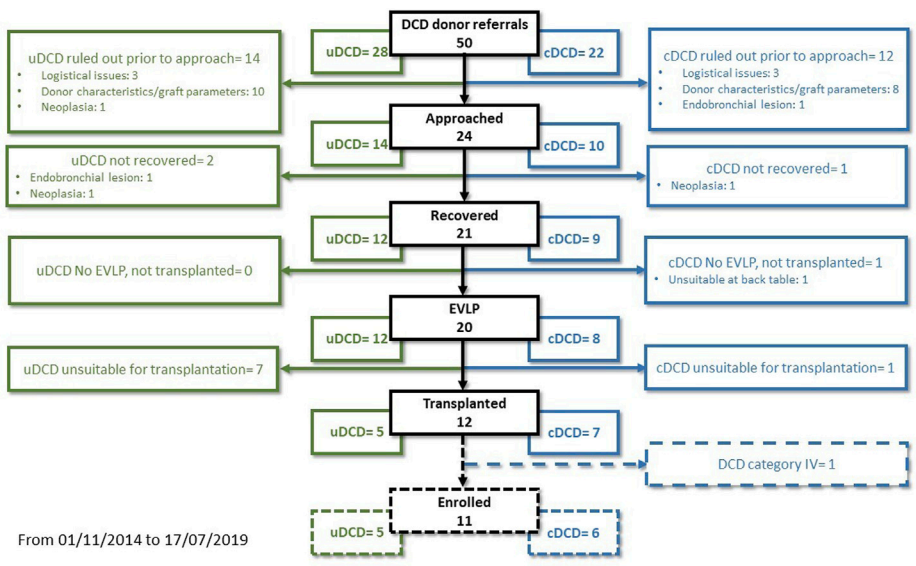


FIGURE 2 | Overall flow-chart of our DCD program.

TABLE 1 | Study population demographic and clinical characteristics.

	DCD Group (*n* = 11)	DBD Group (*n* = 110)	*p*-value
Donor characteristics			
Sex (Female), n (%)	1 (9.1)	41 (37.3)	0.061
Age, median (IQR)	53.0 (8.5)	49.0 (19.0)	0.168
BMI, median (IQR)	27.7 (5.5)	24.8 (4.4)	0.022[a]
Comorbidities (Yes), n (%)	7 (63.6)	48 (43.6)	0.204
Mechanical ventilation (days), median (IQR)	1 (6)	2 (3)	0.611
Smoking history, n (%)			
No	7 (63.6)	73 (66.3)	0.855
Former	1 (9.1)	8 (7.3)	0.827
Yes	3 (27.3)	29 (26.4)	0.948
Chest X-ray, n (%)			
Clear	7 (63.6)	69 (62.7)	0.952
Minor	1 (9.1)	25 (22.7)	0.294
Opacity<1 lobe	3 (27.3)	12 (10.9)	0.116
Opacity ≥1 lobe	0 (0.0)	4 (3.7)	0.520
Donor PaO_2/FiO_2, median (IQR)[b]	372.0 (60.0)	470.0 (137.0)	0.07
Donor secretions, n (%)			
None	5 (45.4)	43 (39.1)	0.681
Minor	3 (27.3)	53 (48.2)	0.185
Moderate	3 (27.3)	13 (11.8)	0.149
Major	0 (0.0)	1 (0.9)	0.751
Sex mismatch, n (%)	5 (45.5)	16 (14.5)	0.009*
Recipient characteristics			
Sex (Female), n (%)	6 (54.5)	52 (47.3)	0.645
Age, median (IQR)	42.0 (17.5)	36.5 (24.8)	0.836
BMI, median (IQR)	20.8 (3.2)	20.5 (4.2)	0.960
Disease, n (%)			
Cystic fibrosis	8 (72.7)	68 (61.9)	0.475
Interstitial lung disease	1 (9.1)	25 (22.7)	0.294
COPD	2 (18.2)	10 (9.1)	0.336
Bronchiectasis	0 (0.0)	3 (2.7)	0.579
Pulmonary vascular disease	0 (0.0)	1 (0.9)	0.751
Other	0 (0.0)	3 (2.7)	0.579
Urgent transplantation, n (%)	0 (0.0)	10 (9.1)	0.297
LAS, median (IQR)	42.9 (9.0)	38.5 (12.6)	0.652
PAPm, median (IQR)	22.0 (2.0)	24.0 (10.0)	0.924
$PaCO_2$, median (IQR)	48 (8)	42 (11)	0.362
ECMO Bridge to transplantation, n (%)	0 (0.0)	10 (9.1)	0.297
Intraoperative			
Incision, n (%)			
Clamshell	9 (81.8)	71 (64.5)	0.249
Bilateral anterior thoracotomy	2 (18.9)	39 (35.5)	0.249
Machine perfusion, n (%)	11 (100)	17 (15.5)	<0.001[a]
CIT, 1st lung (minutes), median (IQR)	595 (159)	338 (157)	<0.001[a]
CIT, 2nd lung (minutes), median (IQR)	820 (155)	557 (181)	<0.001[a]
Intraoperative WIT, 1st lung (minutes), median (IQR)	76 (27)	82 (29)	0.257
Intraoperative WIT, 2nd lung (minutes), median (IQR)	80 (24)	71 (25)	0.539
TIT, 1st lung (minutes), median (IQR)	797 (154)	428 (156)	<0.001[a]
TIT, 2nd lung (minutes), median (IQR)	1,026 (202)	632 (186)	<0.001[a]
TPT, 1st lung (minutes), median (IQR)	1,058 (125)	433 (175)	<0.001[a]
TPT, 2nd lung (minutes), median (IQR)	1,286 (102)	641 (213)	<0.001[a]
ECMO, n (%)	7 (63.6)	51 (46.4)	0.274
Intraoperative Red cells concentrate transfusion (U), median (IQR)	3.0 (4)	3.0 (5)	0.899
Intraoperative Plasma transfusion (U), median (IQR)	1.0 (3)	0.0 (3)	0.778
Intraoperative Platelet transfusion (U), median (IQR)	0.0 (0)	0.0 (0)	0.396

[a]*Statistically significant* p-*value.*

[b]*Median PaO_2/FiO_2 was calculated in 5 and 106 patients in the DCD and DBD Group, respectively.*

BMI, body mass index; IQR, interquartile range; PaO_2/FiO_2, ratio of arterial oxygen partial pressure (PaO_2 in mmHg) to fractional inspired oxygen; COPD, chronic obstructive pulmonary disease; LAS, lung allocation score; PAPm, mean pulmonary artery pressure; $PaCO_2$, arterial partial pressure of carbon dioxide; ECMO, extracorporeal membrane oxygenation; CIT, cold ischemia time; WIT, warm ischemia time; TIT, total ischemia time; TPT, total preservation time; U, units.

Data regarding best FEV1 reached within the first year after transplantation and throughout the follow-up period are shown in **Table 4**. There were no statistically significant differences between the two groups. In both sets of patients, best FEV1 was reached during the first year after surgery: in the DCD Group, the median time to achieve best

TABLE 2 | DCD Group ischemic times (16).

DCD category III (n = 6)	
Interval 1 (minutes), mean (SD)	14.3 (8.5)
Interval 2 (minutes), mean (SD)	26 (8.9)
Interval 3 (minutes), mean (SD)	165.5 (29.9)
Interval 4 (minutes), mean (SD)	151 (37)
DCD category II (n = 5)	
WIT (minutes), mean (DS)	250 (53)

Interval 1: from WLST to BP<50mmHg; Interval 2: WLST to asystole; Interval 3: WLST to pulmonary flushing; Interval 4: BP<50mmHg to pulmonary flushing. SD: standard deviation.

FEV1 was 217 days, whereas in the DBD Group, it was 333 days.

DCD Group vs. DBD-EVLP Group

When comparing the DCD Group with the subset of DBD grafts undergoing EVLP, the difference in terms of duration of MV did not reach statistical significance. The incidence of PGD3 in the DBD-EVLP Group was 23.5% vs. 27.3% in the DCD Group. Also, there was no statistically significant difference regarding airway complications, even though the incidence was higher in the DCD Group (18.2% vs. 5.8% in the DBD-EVLP Group).

The punctual estimates and regression as a function of time of FEV1, FVC, and Tiffeneau index are presented for the DCD Group and DBD-EVLP Group in **Supplementary Table S3** in the **Supplementary material**. No difference was detected between the two groups.

DISCUSSION

We present the results of a prospective trial designed to compare the outcomes of patients receiving grafts from DBD and DCD donors, managed with our original protocol. Our experience suggests that transplantation from both controlled and uncontrolled DCD donors is feasible and safe even after prolonged ischemic times in a non-rapid procurement setting. Notably, the protocol has been activated in first level as well as in secondary hospitals (4,8,9,12,23,24).

In Italy, a mixed "opting-in" and "opting-out" system and, more importantly, 20 min of flat EKG for the declaration of circulatory death were long considered an insurmountable obstacle to the use of DCD donors. Our protocol relied on the possibility for lung tissue to dissociate ischaemia from hypoxia, hence the preservation of lungs for an extended time by using RMs followed by continuous positive airway pressure (*in-situ* preservation phase), and subsequently evaluating them by using EVLP (*ex-situ* preservation phase)(8). Our peculiar strategy allowed a complete expansion of the lungs for optimal perfusion, instead of causing a parenchymal collapse for topical cooling (25). The advantages of this approach were recently confirmed by the work of Healey et al. (26). Also, employing only ventilation allowed us to preserve the lung without the need for chest drains and topical cooling, making it possible to implement our protocol in any situation, also in first level hospitals. Finally, we combined this approach with the NRP for abdominal organ preservation (9,13). Indeed, the ischemia times of our DCD cohort were generally longer than those reported in the literature. Our median TIT was three times greater than that reported by Cambridge or Harefield Center (27,28). The analysis of the mean WIT in DCD category II donors shows that prolonged *in-situ* WIT was not strictly considered a refusal criterion, as we prefer to evaluate the grafts on a case-by-case basis. The acceptance rate in our DCD program was much higher in category III DCD donors than in category II (0.78 versus 0.42) (**Figure 2**).

PGD3 rate was slightly higher in the DCD Group, although the difference did not reach statistical significance; moreover, the duration of MV was significantly longer in the DCD Group, even though the difference with DBD group was only 24 h. Finally, it is interesting to note that the PGD3 rate and pulmonary function at 1 year in the DCD Group were similar to those found in the subgroup of patients belonging to the DBD Group who received lungs treated with EVLP. Overall, DCD lungs seem to have a slower recovery in the early post-operative period.

We did not register any immediate bronchial anastomosis complications that could endanger patients' life; in contrast, two

TABLE 3 | Patients' postoperative data and outcomes.

	DCD Group (n = 11)	DBD Group (n = 110)	p-value
Grade 3 PGD, n (%)	3 (27.3)	20 (18.2)	0.724
MV (days), median (IQR)	2.0 (2.5)	1.0 (2.0)	0.011*
ICU stay (days), median (IQR)	4.0 (5.5)	3.0 (3.0)	0.053
Hospital stay (days), median (IQR)	21 (5)	22 (10)	0.732
Airway complication, n (%)	2 (18.2)	7 (6.4)	0.154
90-days mortality	0 (0.0)	5 (4.6)	0.999
ALAD, n (%)	3 (27.3)	34 (30.9)	0.999
Histologic AR[a], n (%)			
Grade 0	4 (36.3)	28 (28)	0.561
Grade 1	5 (45.5)	56 (56)	0.505
Grade 2	2 (18.2)	14 (14)	0.708
Grade 3	0 (0.0)	2 (2)	0.636

[a]*AR was calculated in 11 DCD and 100 DBD.*

IQR, interquartile range; PGD, primary graft dysfunction; MV, mechanical ventilation; ICU, intensive care unit; ALAD, acute lung allograft dysfunction; AR, acute rejection.

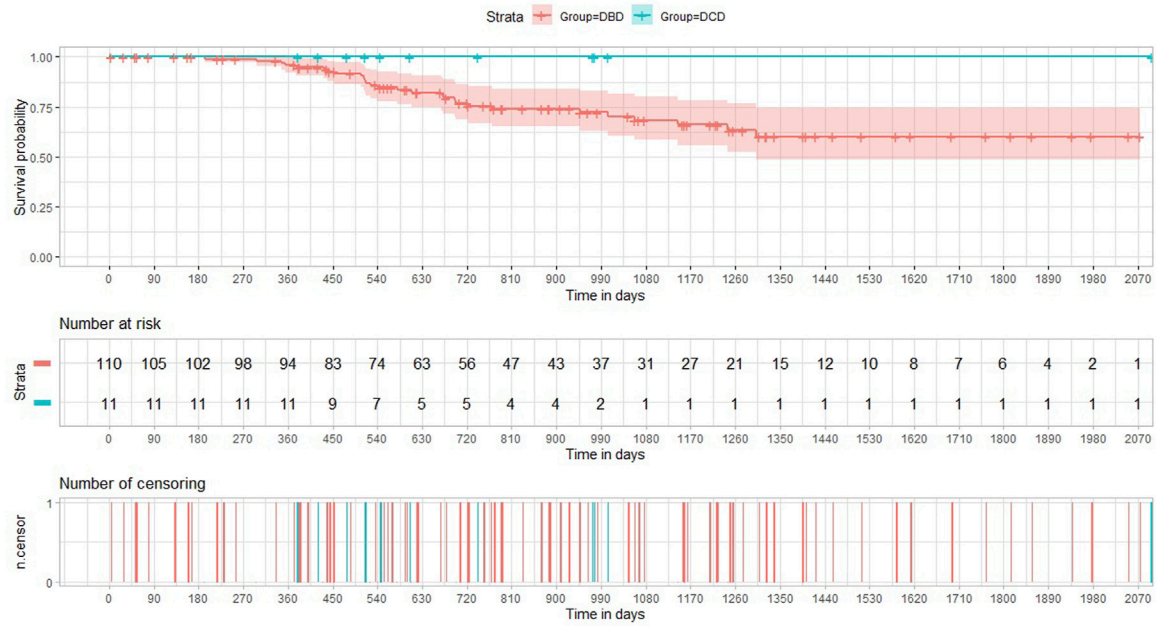


FIGURE 3 | Kaplan-Meier curves illustrating CLAD-free survival in the DCD and DBD Group.

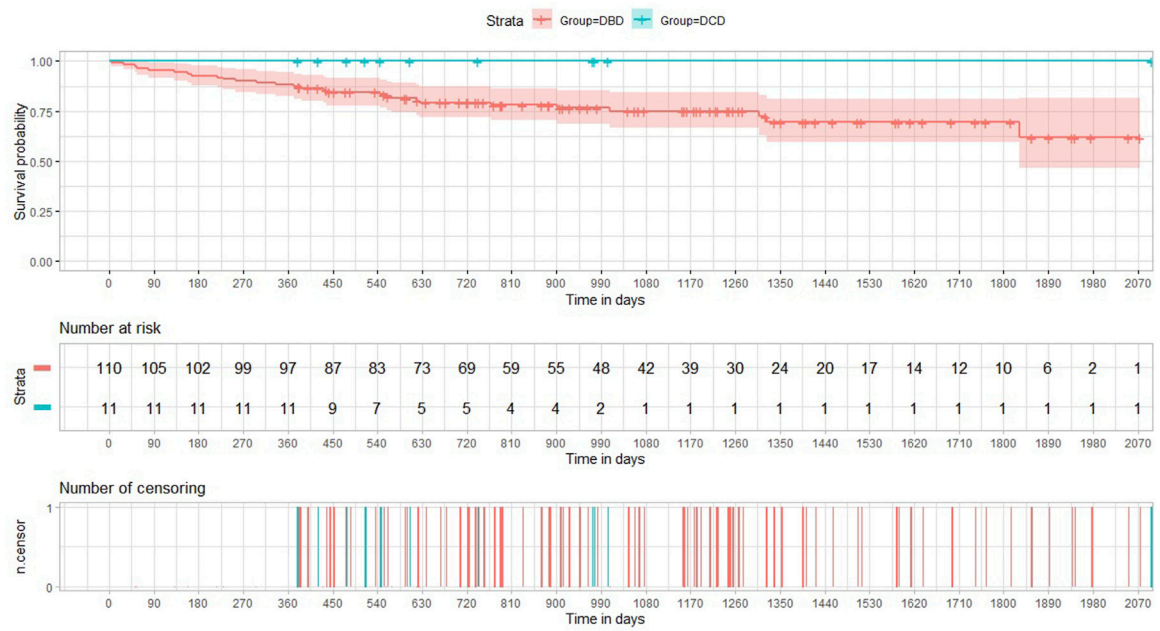


FIGURE 4 | Kaplan-Meier curves illustrating overall survival in the DCD and DBD Group.

patients in the DCD Group developed bronchial stenosis, distally to the anastomosis, in the medium term. Given the small population in the DCD cohort, the complication rate rose to 0.18. This rate is however consistent with the literature (range: 0.05–0.28) and, above all, our patients required only endoscopic treatments. Anyway, we can speculate that the length of ischemia times played a role in this regard, and that our protocol based on ventilation is more protective on pulmonary parenchyma than on large bronchi.

The medium-term outcomes of DCD lungs are encouraging. In the first year we found a homogeneous distribution of acute rejection and ALAD episodes in the two groups. To the best of our knowledge, we are the first to report the prevalence of ALAD in a population of

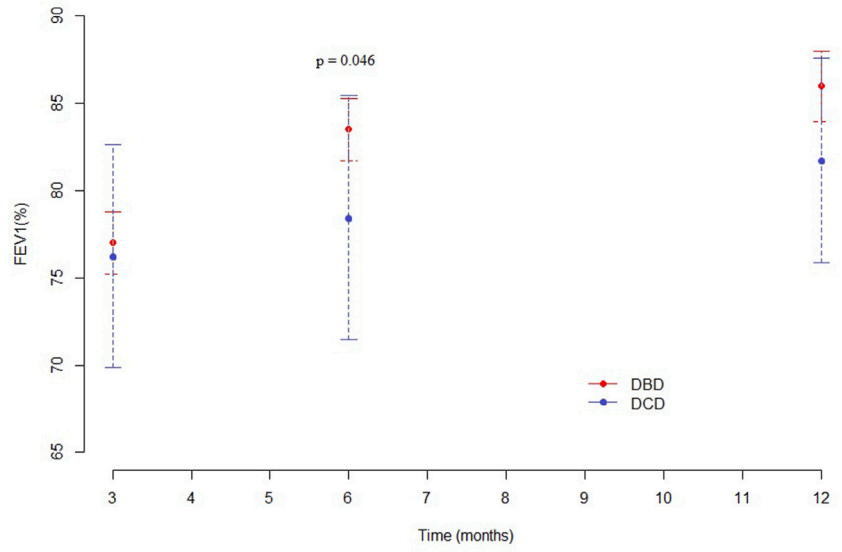


FIGURE 5 | Mean FEV1 (%) at 3, 6, and 12 months after transplantation.

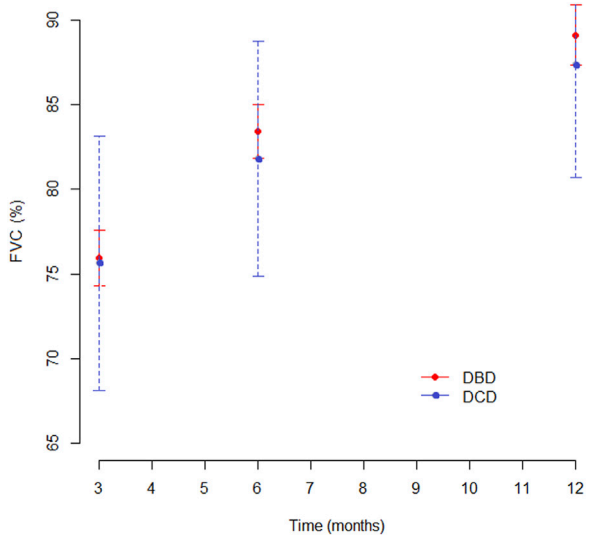


FIGURE 6 | Mean FVC (%) at 3, 6, and 12 months after transplantation.

patients transplanted with lungs by DCD donors. Sabashnikov describes acute rejection rates similar to ours and well balanced between DCD and DBD groups (28). It is possible to speculate that grafts from DCD donors have no particular impact on immunity, and therefore on the onset of rejection. One-year survival probability in the DCD Group was 1.0 versus 0.88 in the DBD Group. This finding is congruent with the result of our recent meta-analysis, where the odds ratio for 1-year overall survival was balanced between the DCD and DBD group (29). Finally, in the DCD Group, no CLAD events were recorded during the study period. Obviously, these two last results should be taken with caution considering the small sample size of our study.

The DCD Group pulmonary function was also adequate. We noted that the FEV1 was similar in the two groups 3 months after transplantation, while the recovery in the following 3 months was faster in the DBD Group: as a consequence, we observed a statistically significant difference at 6 months. One year after transplantation, this difference was no longer detectable. Despite the statistical significance reached at the six-month, it should be pointed out that such a slight difference in percentage

TABLE 4 | Best FEV1 values in the DCD and DBD Groups.

	DCD Group	DBD Group	*p*-value
Best FEV1 within 1st year (%), median (IQR)[a]	97 (38)	87 (23.8)	0.7937
Time to reach best FEV1 within 1st year (days), median (IQR)[a]	196 (149)	234 (184)	0.3297
Best FEV1 (%), median (IQR)	99 (33)	90 (25)	0.7107
Time to reach best FEV1 (days), median (IQR)	217 (486)	333 (455)	0.9129

[a]*Best FEV1 within the first year and days for reaching best FEV1 within the first year were calculated in 11 DCD and 105 DBD.*
FEV1, forced expiratory volume in the first second; IQR, interquartile range.

FEV1 is clinically irrelevant. Data on Best FEV1 did not reveal any statistically significant difference between the two Groups.

This trial has several limitations. Although it is a phase I-II trial, the study population should be larger. Nevertheless, we considered it useful to perform the analyses so as not to dilute cases over time and not to expose the data set to subsequent revisions due to the continuous evolution of knowledge in the lung transplantation field. A practical example is CLAD, of which the definition is constantly evolving. We did not register CLAD cases in the study cohort, but we preferred not to comment this result in light of the absence of a clear and definitive classification (30). Another important limitation is the lack of randomization of recipients; on the other hand, the allocation system makes randomization virtually impossible. It should be noted that our two cohorts of recipients had completely overlapping parameters. Moreover, this study suffers from the possibility of generalization, being based on an original procurement protocol. Despite all this, our study can help to broaden the general knowledge on donation from circulatory arrest, which represents a possible tool to fight the chronic scarcity of lungs. Finally, we have included both category II and category III donors in the DCD group. This could be considered a confusing factor, but the examination of the two subgroups did not reveal differences in outcomes, while their merging gave an overall picture of the results obtained with our procurement protocol.

The results of this trial suggest that lung transplantation with grafts from DCD donors procured with our protocols is safe despite the long ischemia times. The trend towards a higher PGD3 rate, prolonged mechanical post-operative ventilation, and a slower functional recovery after transplantation does not seem to negatively affect clinical outcomes nor pulmonary function at 1 year.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Milano Area 2, number 749_2016bis. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualisation: AP, FV, and MN; Data curation: VM, CU, LM, and EB; Formal analysis: GB; Investigation: GC, VM, and AP; Methodology: AZ, GC, and AP; Project administration; Resources: VM, VR, JF, and MC; Software: GB; Supervision: AP, AZ, and MN; Validation: AP and GC;Visualisation: FB, MN, AP, AZ, and FV; Writing—original draft, and writing—review and editing: All authors.

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CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10690/full#supplementary-material

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REVIEW
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Tolerance Induction in Vascularized Composite Allotransplantation—A Brief Review of Preclinical Models

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Pre-clinical studies are an obligatory tool to develop and translate novel therapeutic strategies into clinical practice. Acute and chronic rejection mediated by the recipient's immune system remains an important limiting factor for the (long-term) survival of vascularized composite allografts (VCA). Furthermore, high intensity immunosuppressive (IS) protocols are needed to mitigate the immediate and long-term effects of rejection. These IS regiments can have significant side-effects such as predisposing transplant recipients to infections, organ dysfunction and malignancies. To overcome these problems, tolerance induction has been proposed as one strategy to reduce the intensity of IS protocols and to thereby mitigate long-term effects of allograft rejection. In this review article, we provide an overview about animal models and strategies that have been used to induce tolerance. The induction of donor-specific tolerance was achieved in preclinical animal models and clinical translation may help improve short and long-term outcomes in VCAs in the future.

Keywords: Vascularized composite allotransplantation, tolerance induction, animal models, swine, rodent

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INTRODUCTION

To date, about 150 vascularized composite allotransplants (VCAs) including over 40 face and 120 extremity transplantations, have been performed worldwide. Approximately 40 VCA programs across five continents have been established to date, and more VCA centers are anticipated to be established in the future (1,2).

In the field of reconstructive surgery, the geometric uniqueness and often resulting functional deficit of a composite tissue defect are major challenges. Vascularized composite allotransplantation (e.g., facial VCA) has revolutionized restoration of form and function of the most complex defects. For example, facial transplantation can now be offered to selected patients at experienced centers with reproducible results (3-5). However, acute and chronic rejection as well as the resulting need for life-long multidrug immunosuppression (IS) limit the more widespread use of this revolutionary biotechnology.

Side-effects related to these IS protocols (e.g., increased susceptibility to infection, malignancy, and organ dysfunction) continue to adversely affect the risk-benefit ratio transplants, particularly in case of VCA which is not a lifesaving but rather a life-giving procedure.

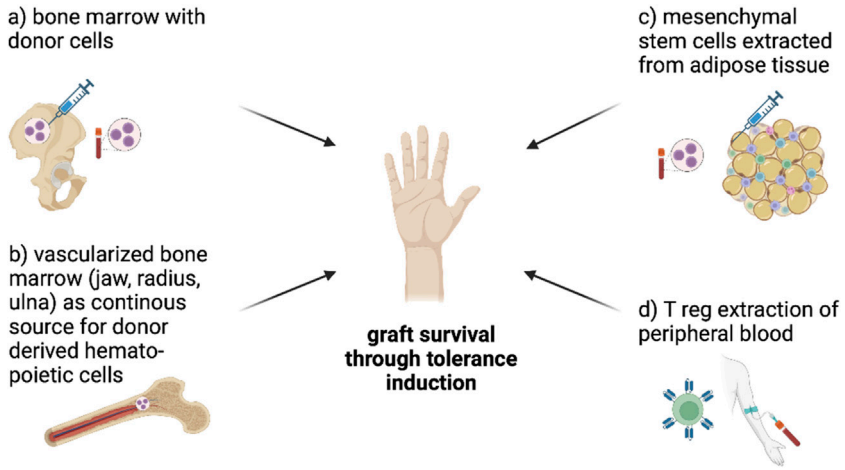

FIGURE 1 | Figure shows cell-based tolerance induction approaches in VCA. Bone marrow transplantation with donor-derived stem cells can induce mixed chimerism and thereby induce tolerance **(A)**, vascularized bone marrow as continuous source for donor derived hematopoietic cells might be able to help minimize immunosuppressants **(B)** and mesenchymal stem cells extracted from adipose tissue **(C)** are already tested in VCA to induce tolerance through mixed chimerism and direct effects on the graft. The extraction of Treg cells **(D)**, modification into CAR-Tcells using CRISPR/C9 and infusion into humans is tested in clinical trials with SOT so far and seems to be a promising approach to induce tolerance in VCA. CAR-Tcells can recognize MHC-I on donor cells and block their interaction with the recipient´s immune system to prevent rejection (Figure created with BioRender.com).

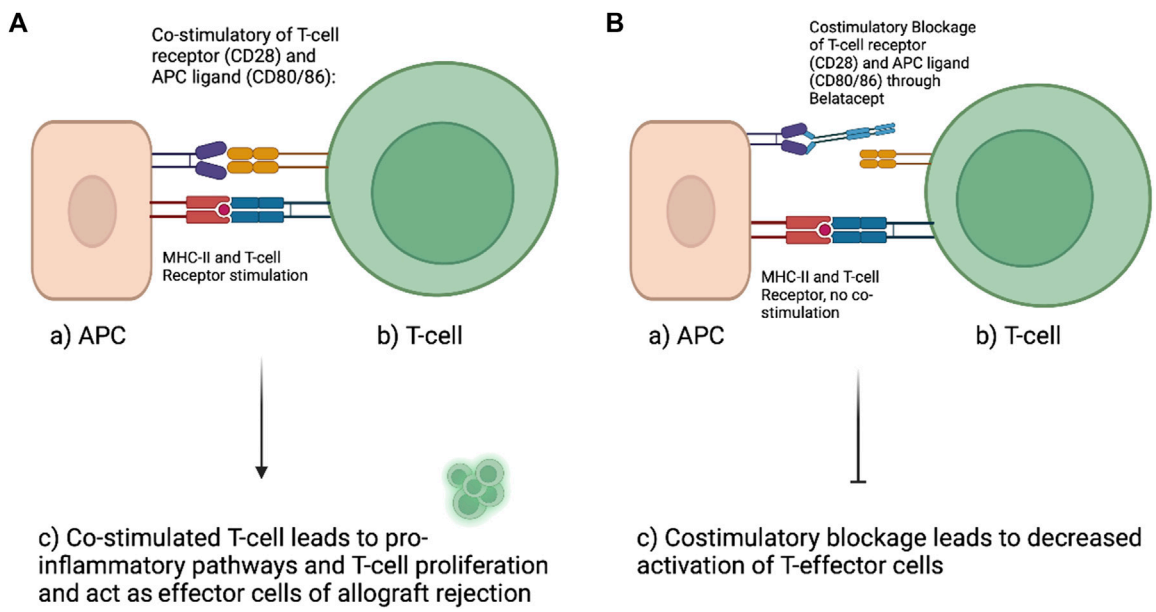

FIGURE 2 | Left **(A)** demonstrates physiologic naïve T-cell activation. Antigen presenting cells (APC) interact with the CD28 surface receptor of naïve T-cells with their ligands CD80/86 while the antigen is presented to the T-cell. This interaction will promote T-cell proliferation and activation of cellular inflammatory pathways. **(B)** With the use of Belatacept (second-generation CTLA4-Ig) the costimulatory interaction between APC and T-cell can be altered. Belatacept blocks the interaction of CD28 with CD80/86 by binding to CD80/86. This interaction limits the activation of T-cells and was shown to reduce alloreactivity and thereby prolong allograft survival (Figure created with BioRender.com).

In this context, tolerance induction, the long-term immunosuppression-free graft acceptance without clinical or histological evidence of rejection, has become the topic of many preclinical and clinical research endeavors. The strategy of tolerance induction is often viewed as the holy grail of achieving improved transplant outcomes for example by preventing or slowing the development of chronic rejection, which is still an important long-term cause of graft loss in solid organ transplantation and presumably also in VCA (6,7).

TABLE 1 | VCA models with detailed therapy regimen in Mice, all models developed tolerance induction.

Mouse: VCA model	Donor	Recipient	Therapy regimen	Days of survival	Results	Ref
Osteomyocutaneous VCA or full thickness skin (FTS) transplantation fully MHC mismatched	DBA/2	C57BL/6	Skin grafting each *n* = 3: I = ALS & Rapa (*n* = 3), II = ALS, Rapa & DN, IV = ALS, Rapa, IL-2 & DN VCA each *n* = 5: I = untreated, II = Rapa, III = Rapa & IL-2, IV = Rapa & DN, V= Rapa, IL-2 & DN, VI = ALS, Rapa & IL-2, VII = ALS, Rapa, IL-2 & DN	10–180	Antigen-induced CD4 derived DN Tregs and a short course of anti-lymphocyte serum, rapamycin and IL-2/Fc fusion protein results in tolerance in VCA but not FTS allografts	(34)
Osteomyocutaneous allografts (OMC)	Balb/c (H2d)	C57BL/6 (H2b)	I = without Treg depletion (*n* = 15), all other groups received anti-CD154, CTLA4-Ig & Rapa: II = POD 0 (*n* = 7), III = POD 30 (*n* = 7), IV = POD 90 (*n* = 7)	50–180	80% of VCA receipients with CoB & Rapa developed tolerance, 20% showed signs of rejection	(31)
Full-thickness trunk skin grafts	Balb/c	C57BL/6	I = untreated control (*n* = 10), II = conditioning therapy only (*n* = 10), III = conditioned with skin transplantation received ASCs (*n* = 6), IV = conditioned with skin transplantation received ASCs & BMCs (*n* = 12) Conditioning: anti-CD4 & anti CD8 monoclonal Anti bodies and non-myeloablative low dose busulfan	17–58	BMCs &ASCs results in skin allograft survival and mixed donor-recipient macrochimerism	(35)
Osteomyocutaneous allografts (alloOMCs) or myocutaneous allografts (alloMC)	Balb/c (H2d)	C57BL/6 (H2b)	I = alloMC with 1.5 × 108 CBMT (*n* = 6), II = alloMC with 3 × 107 CBMT (*n* = 6), alloOMC with VBMT (*n* = 6), IV = syngeneic group OMC without treatment (*n* = 4), V = alloMC without CBMT (*n* = 6) All groups received a costimulated blockage: anti-CD154 & CTLA4Ig plus Rapa short-term	62–120	VBMT with CoB & Rapa led to prolonged graft survival (>120 days), high CBMT also led to prolonged graft survival	(32)
Orthotopic hindlimb transplantation and full thickness skin trans-plantation (third party) fully MHC mismatched	Balb/C (H2d) FVB/N (H2q)	C57BL/6 (H2b)	I = untreated (*n* = 5), II = CTLA4-Ig (*n* = 4), III = CTLA4-Ig & hamster anti-mouse CD154 mAB (*n* = 8), IV = TBI 1 day before surgery, CTLA4-Ig & hamster anti-mouse CD154 mAB (*n* = 6)	8–210	CoB treatment plus TBI 1 day before Surgery increased graft survival (82 days) and showed T cell depletion	(33)
Osteomyocutaneous allografts (alloOMCs) or myocutaneous allografts (alloMC)	Balb/c (H2d)	C57BL/6 (H2b)	I = syngeneic controll (*n* = 2), II = alloOMC untretead (*n* = 5), II = alloMC with anti-CD154, CTLA4Ig & RPM (*n* = 6), IV = alloMC with antiCD154, CTLA4Ig (*n* = 6), V = alloOMC with Rapa (*n* = 6), VI = alloOMC with anti CD154, CTLA4Ig & Rapa (*n* = 15)	12–120	Combined CoB and Rapa (Group VI) led to long-term allograft survival (>120 days, 12 out of 15 animals) and same treatment in alloMC (Group III) without vascularized BM showed reduced allograft survival (53 days + - 11.6)	(30)

DN, double negative Treg based therapy; ALS, anti-lymphocyte serum; Rapa, rapamycin; ASCs, adipose-derived stromal/stem cells; VBMT, Vascularized bone marrow transplantation; CBMT, conventional bone marrow transplantation; CoB, Costimulated Blockade; BMCs, bone marrow cells.

Given the inherent complexity and diversity of VCA procedures, different animal models have been established and evaluated, while additional models are being developed to help answer specific VCA related immunological questions. We present a brief review of the most commonly used preclinical approaches of tolerance induction in the field of VCA.

TOLERANCE INDUCTION IN VCA

Tolerance induction strategies can be broadly categorized into cell- and pharmaceutical based strategies. The goal is to achieve graft acceptance and minimize the need for less specific and highly toxic systemic immunosuppression such as tacrolimus and MMF. The interest in cell-based strategies has been increasing over the past 10 years. Cell-based strategies include the use of stem (e.g.,: bone marrow transplant-derived, mesenchymal stem cells) and/or immune cells (e.g.,: dendritic cells, T reg cells) plus usually a co-administration of a drug therapy regimen (8). **Figure 1** illustrates standard tolerance induction strategies that have been used in preclinical models of VCA. The goal of stem cell-based therapies is the establishment of mixed chimerism which was described in detail in 1988 by Sykes and Sachs as a phenomenon of host and donor bone marrow-derived cell coexistance (9). For example, mixed chimerism can be induced

TABLE 2 | VCA models with detailed therapy regimen in Rats.

Rat: VCA model	Donor	Recipient	Therapy regimen	Days os survival	Results	Ref
Hindlimb, orthotopic	BN, RT1u	LEW, TR1l	Five groups received different circles of treatment *n* = 4 ALS, n = 5 CsA, *n* = 4 CsA + human (h) IL-2/Fc, *n* = 6 ALS + CsA, *n* = 5 ALS + CsA + hIL-2/Fc	8–150	hIL-2/Fc in combination with ALS + CsA enables long-term graft survival and induction of tolerance, furthermore hIL-2/Fc leads to increased Treg proliferation & function but no chimerism were analyzed	(37)
Groin flap fully MHC mismatched	ACI, RT1a for flap LEW for BMC	LEW, RT1l rec. flap ACI received BMC	N = 8 each: I & II: VCA from ACI donors preconditioned at 24 & 72 hrs, prior to VCA transplant, supported with a 7-day IS protocol of s.c. CsA & i.p. anti-ab-TCR monoclonal antibody, III & IV: VCA from ACI donors precond. at 24 & 72 h, prior to transplantation (no IS). V: VCA from noncond. ACI donor under a 7-day IS of CsA & anti-ab-TCR. VI: VCA from noncond. ACI donor, no IS	8–98	The regimen BMC prior 24 h plus anti-ab-TCR/CsA shows the longest graft survival (80+-18 days) and tolerance induction with mixed chimerism	(41)
Osseomusculocutaneous sternum (OMCS, n = 5) or osseomusculocutaneous sternum and thymus (OMCST, n = 5) heterotopic allotransplantation	LEW BN, RT1l	LEW, RT1l	CsA monotherapy (16 mg/kg) tapered to 2 mg/kg and maintained for the duration of the study	150	Study confirms correlation between thymus transplantation and donor-specific chimerism. No signs of rejection in any of the transplants during the 150 days posttransplant (Banff grade 0) but also no tolerance induction	(43)
Heterotopic hindlimb fully MHC mismatched	BN, RT1An	LEW, RT1Al	I = syngeneic control (*n* = 4); II-VI (each *n* = 6) = allogeneic VCA with II = no treatment, III = ALG &CsA, IV = ALG, CsA & ADSC, V = ALG, CsA & TBI, VI = ALG, CsA, ADSC & TBI	10–150	150 days of survival in 4 out of 6 rats in group IV & VI, both received infusion of ADSC, tolerance induction was achieved here but no mixed chimerism were analyzed	(39)
Full-thickness hemiabdominal wall (HAW) or hindlimb osteomyocutaneous (HLOMC) or combined HAW/HLOMC flap fully MHC mismatched	BN, RT1An	LEW, RT1Al	I&II = syngeneic controls; III-V = no IS (III = HAW *n* = 6, IV = HAW/HLOMC *n* = 4, V=HLOMC *n* = 4); VI-VIII ALS, CsA & ADSC (VI = HAW *n* = 8, VII = HAW/HLOMV *n* = 7, VIII = HLOMC *n* = 8)	11–150	150 days of survival only in syngeneic control groups; longest survival in group VIII (HLOMC) with 57 +- 21 days, also showed significantly higher peripheral Chimerism as well as tolerance induction	(36)
Groin flap fully MHC mismatched	ACI RT1A	LEW, RT1Al	*n* = 6 each group: I = untreated, II = 7 days anti-ab-TCR/CsA, III = DRCC, IV = DRCC plus 7 days anti-ab-TCR monoclonal antibody & CsA	8–125	Longest survival in group IV with 125 days after single application of DRCC plus IS regimen for 7 days. DRCC therapy induces tolerance induction plus peripheral blood chimerism in the VCA recipients	(40)
Inferior epigastric flap	BN, RT1An	LEW, RT1Al	*n* = 15 each group: I=VCA, II = VCA & hASCs/GFP, III = VCA & hASCs/CCR7	8–17	Targeted migration of hASCs/CCRP7 infusion to secondary lymphoid organs potently prolong the mean survival time of VCA flaps, no chimerism/tolerance was established	(42)
Hindlimb	BN, RT1An	LEW, RT1Al	I = control (*n* = 5), II = 1 × 106 BM-MSCs (*n* = 6), III = 5 × 106 BM MSCs (*n* = 7),IV = 1 × 106 AD-MSCs (*n* = 8), V = 5 × 106 AD-MSCs (*n* = 9) all recipients: rabbit antirat lymphocyte serum 4 days before & 1 day after surgery, daily Tacrolimus 0–21 days after surgery, single-shot MSC IV on day 1 after surgery	47–120	all cell-treated groups showed prolonged survival, 47% of the MSC-treated exhibited long-term survival of the allograft and tolerance induction (>120 days). Peripheral blood chimerism were only transient	(38)

ADSC, syngeneic adipose-derived stem cells of LEW rat; ALS, Antilymphocyte serum); anti-ab-TCR, anti-ab- T-cell receptor monoclonal antibody; ALG, anti-lymphocyte globulin; DRCC, donor-recipient chimeric cells intraosseous; CCR7, chemokine receptor 7; hASCs, human adipose-derived stem cells.

TABLE 3 | VCA models with detailed therapy regimen in Swine, both induced tolerance.

Swine: VCA model	Donor	Recipient	Therapy regimen	Days of survival	Results	Ref
Fasciocutaneous flap (MGH miniature swine)	SLAacPAA+	SLAadPAA-	T cell depletion with CD3-immunotoxin, 100 cGy TBI prior to HCT, 45-day course of CsA	115–504	Both VCAs (transplanted into stable chimerism $n = 4$ and transplanted at time of hematopoietic cell transplantation (HCT) $n = 2$) showed no signs of rejection following withdrawl of immunosuppression and stable mixed chimerism	(14)
Osteomyocutaneous flap matched for class II SLA, mismatched for class I SLA (MGH miniature swine)	PAA+	PAA-	300cGy total body irradiation +700cGy thymic irradiation, following bone marrow transplantation, co-stimulated blockade using CTLA4-Ig + FK506	400	3 out of 5 animals achieved long-term survival without Immunosuppression, 2 recipients developed idiopathic pneumonia-like syndrome (euthanized 36–39 POD), tolerance induction and mixed chimerism was achieved	(44)

CsA, Cyclosporine A.

TABLE 4 | VCA model with detailed therapy regimen in Non-Human Primate (NHP); vascularized bone marrow (VBM), bone marrow cells (BMC) infusion.

NHP: VCA model	Donor	Recipient	Therapy regimen	Days of survival	Results	Ref
Heterotopic partial face transplantation with ($n = 4$)/without vascularized bone ($n = 3$) (mandible segment) MHC class I-mismatched	Cynomolgus macaques		Tacrolimus IV/IM plus MMF OV/orally and all daily	7–430	VCA with vascularized bone led to prolonged rejection-free graft survival and showed sporadic macrochimerism; no tolerance induction as graft was rejected after withdrawal but low dose maintenance of immunosuppression could be achieved	(15)

by transfer of donor hematopoietic stem cells (HSCs) from bone marrow or cytokine-mobilized peripheral stem cells. Typically, the recipient is conditioned in order to control alloreactivity and generate space for donor HSCs engraftment. The co-existence of donor and recipients HSC is thought to promote donor-specific tolerance while maintaining immunocompetence (10,11).

Mixed chimerism alone was shown to not consistently achieve tolerance induction and thus Leonard et al. described different approaches achieving mixed chimerism combined with adjuvant cellular therapies leading to tolerance induction in pigs (3,10). The longest tolerance induction through establishment of mixed chimerism was demonstrated in a pig model by Leonard et al. (12,13) in 2015 (14) after performing VCA in stable mixed chimerism or concurrent with induction of mixed chimerism following hematopoietic stem cell transplantation. Both groups showed no signs of rejection up to 504 days after transplantation.

To achieve a continuous supply of donor derived bone marrow elements, Barth et al. further analyzed the role of vascularized bone in a heterotopic partial face transplantat model in NHPs (15). VCA containing vascularized bone not only led to prolonged, but rejection free graft survival (430 days) compared to VCA without vascularized bone (7 days). Schneeberger et al. developed the "Pittsburg Protocol" as an approach in humans following upper-extremity transplantation. Bone marrow cell-based treatment allowed a maintenance low-dose immunosuppression protocol compared to conventional protocols (16). However, long-term results have not been published and further studies are needed to evaluate the safety and reliability of this approach.

Conditioning of the recipient entails toxic side effects, thus approaches of bone marrow infusion-mediated immunomodulation without the necessity of conditioning are studied at the time (17). In this context other immune cells have been studied. Mesenchymal stem cells (MSCs) are multipotent stem cells that can differentiate into mesenchymal cell lineages. MSCs are derived from the bone marrow but can also be extracted from for example adipose tissue (= adipose derived MSCs) and have shown promising immunomodulatory effects by regulation of T-cell proliferation and inhibition of dendritic cell differentiation as they interact with the innate and adaptive immune system (18). Not only can MSCs induce mixed chimerism in the recipient, but they can also modulate cytokine expression in VCAs and hereby may be able to prolong graft survival(19). Promising results have been shown in small and large animal models, and clinical trials using mesenchymal stem cells have been established in solid organ transplants with promising results (8).

Ongoing clinical trials in humans evaluate the potential of dendritic cells as major regulator of the human immune system. An injection of immature dendritic cells was shown to have an immunosuppressant effect and to achieve inhibition of memory T cells (20). Another promising approach is the use regulatory T-cells. Tregs help maintain and regulate self-tolerance, antimicrobial resistance, tumor immunity and transplant rejection. Tregs exert their immunosuppressive effects *via* cell-

to-cell interaction with target immune cells, *via* removal of IL-2 (potent factor in T cell survival and growth), *via* anti-inflammatory molecules (TGF-beta) or through costimulatory pathways such as binding to cytotoxic T-lymphocyte-associated protein 4. Nevertheless, irregularity in Tregs can lead to autoimmune disorders, reduced disease tolerance or higher risk for cancer(21). Recent studies have investigated the potential therapeutic value of genetically modified patient-derived Tregs, such as antigen-specific Chimeric Antigen Receptor (CAR)-Tregs, targeting for example MHC-class I expressed by donor cells. Tregs can be isolated from patient´s blood samples, cultured and expanded to produce for example CAR-Treg cells. Those conditioned T cells can be infused into the patient. Clinical trials did show the safety, efficacy, and proof of concept of Treg therapy in kidney and liver transplantation (22). The engineering of Tcells (e.g.: CAR-Tregs) might be a promising supplementary treatment of rejection in the future. CAR-T cells are typically designed to recognize donor MHC molecules, thus localizing to donor tissues and exerting their regulatory effects in a precise and targeted manner (21).

Pharmaceutical therapy regimens are the current gold standard in VCA and are mostly modifications of regimens that have already been established in SOT (23). Usually, an induction therapy (e.g.,: Anti-Thymoglobulin, Alemtuzumab) is administered first and a maintenance immunosuppressive regimen (Tacrolimus, Mycophenolate Mofetil (MMF), Prednisone) is given (8). Anti-Thymocyte globulin (ATG) is used as induction agent in most cases and leads to decreased T-cell mediated rejection (10). The maintaining therapy of the calcineurin inhibitor (CNI) Tacrolimus, Corticosteroids (Prednisone) and MMF (inhibits proliferation of lymphocytes) often involves side effects such as myopathy, diabetes mellitus, impaired kidney function, abdominal pain or diarrhea. Aside from the standard immunosuppressive regiments, other pharmaceutical approaches have been tested, for example tolerance inducing medications.

Cell mediated graft rejection in VCA is fueled by the CD28 and CD80/86 co-stimulatory pathway of T-cells (**Figure 2**). Preventing or interfering with co-stimulation of T cells is an approach for improved allograft survival and a more specific pharmaceutical approach that may cause less severe side effects compared to the conventional approaches (11). CD28 surface T-cell receptors communicate with antigen-presenting cells through CD80 and CD86 ligands and evokes both T-cell proliferation and pro-inflammatory pathways. Drugs like Belatacept or Abatacept are already established anti-rejection medications used in humans to interfere with co-stimulatory pathways. Both inhibit the costimulatory pathway through CTLA4-Ig or CD 125 (Fusion protein of cytotoxic T-lymphocyte-associated antigen 4 Immunoglobulin and IgG1 Fc) by inhibiting the linking of CD28 (T-cell surface) and CD80/86 (APC surface) (15,17). Belatacept can serve as replacement for CNI after kidney transplantation as side effects appear to be less than with CNI. Belatacept is already in clinical application following hand and/or face transplantation along with low dose of CNI/MMF/prednisone. Subsidiary, topical administration of immunosuppression might also reduce the systematically dosage of immunosuppressants. Tacrolimus and steroids are available as creams or mouthwashes as "transplant-targeted therapy" and show promising results in first approaches (8).

LARGE VERSUS SMALL ANIMAL MODEL

Rodent models are of great value especially in VCA research and are most popular for preclinical studies as they allow precise genetic breeding, are easy to reproduce and handle, produce low costs in housing and breeding and enable therefore larger study groups. The majority of the described animal models in this article used rat models and most commonly the hindlimb model was used. Mice allow more precise genetic manipulation which makes analysis of immunological mechanisms easier when compared to other small animal models. However, disadvantages of rat and mice models are related to their smaller size in terms of anatomic structures and the lack of similarity to the human immune system. Furthermore, the results of rodent models have often failed to be successfully transferred to large animal models or human trials (8). In addition, their short lifespan can make it difficult to truly assess long-term graft survival. Most importantly, the development of a naive immune system in largely germ-free laboratory environments and the lack of class II antigen expression on rodent vascular endothelium suggests a fundamentally different immune system than that of humans (11, 24-27).

Large animals are suited as preclinical models as they allow evaluation of a hypothesis in a complex system that has strong similarities to humans in terms of anatomical structures, size, and immune responses, making data potentially more reliable for translation to humans (28). Swine models also allow genetic manipulation to control for example the degree of MHC mismatch in transplantation and share great similarity with humans in structure, cellular and antigenic attributes in skin (8). NHP models are also of great value as they are even more similar in human anatomy than pigs and showed similar transplant rejection to humans. Hand transplantation is feasible and did show good motor function in NHPs (29). Disadvantages are cost and for example with primates' ethical concerns, slower reproductive cycles. Especially the skin of humans and pigs show a great similarity which is even more important with skin being a crucial part of VCA and complicating factor on the way to long-term graft survival and tolerance induction. Having a great similarity with pig skin makes pig animal model most valuable for research. Finally, the high costs of livestock breeding and research in large animals, the ethical status in NHP models on the one hand and the simplicity of reproducibility, handling of rodents and low costs on the other hand may explain why most research groups are using rodents.

Mouse Models

Costimulatory Blockade (CoB) takes advantage of the mechanism that naïve helper and cytotoxic Tcells must be activated through different pathways to become effector T-cells that can ultimately promote graft rejection. Antibodies, antigens, and

immunosuppressive drugs (e.g.: Belatacept) can inhibit T-cell costimulatory pathways and, combined with adjuvant strategies such as total body irradiation (TBI) or stem cell transplantation, may induce mixed chimerism and thereby induce graft tolerance in VCA (10). Tolerance induction in all mice models described here (**Table 1**) was achieved *via* drug-based strategies utilizing costimulatory blockade in combination with adjuvant stem cell and/or TBI therapy regimen. Tolerance has been successfully induced up to 120–210 days in all mouse models described in this article. Five out of six models used an osteomyocutaneous hindlimb allograft transplant model while one used a full-thickness skin graft model without inclusion of bone or muscles. Lin et al. (2016) and Anggelia et al. (2021) showed successful induction of donor-specific tolerance (survival >120 days) by application of CoB (anti-CD154, CTLA4-Ig) with a short treatment of rapamycin (30,31). Both studies achieved graft survival up to 120 days. Lin et al. (2021) added high-dose bone marrow transplantation cells to the regimen of CoB (anti-CD154, CTLA4-Ig) and rapamycin and induced tolerance by mixed chimerism for >120 days in vascular bone marrow transplantation (32). Oh *et al.* (2020) combined the CoB (anti-CD154, CTLA4-Ig) with TBI and demonstrated tolerance induction and a depletion of alloreactive T cells for >210 days. Depletion of alloreactive T cells appears to be a promising mechanism for long-term graft feasibility and a key to long-term viability in this approach (33). Lin et al. (2013) used a different approach *in vitro*; the group used a donor antigen-specific $CD4^{-}CD8^{-}$ double negative Treg-based therapy plus anti-lymphocyte serum (ALS) plus rapamycin plus Il-2/Fc fusion proteins and showed tolerance induction in VCA but interestingly not in full thickness grafts (34). Davis et al. (2014) applied adipose-derived stromal cells (ASC) with non-myeloablative low-dose busulfan plus anti-CD4/CD8 and induced tolerance for >180 days as well as mixed macrochimerism (35). The longest graft survival plus depletion of alloreactive Tcells for over 210 days could be shown *via* CoB plus TBI 1 day before surgery by Oh et al (2020). This approach is furthermore relinquishing immunosuppressants which could be a promising in terms of that patients are mostly young and healthy when receiving VCA and therefore suffer a lot from long-term toxic side effects.

Rat Models

Tolerance induction was achieved in five out of eight described rat models (**Table 2**), and stem cells were used in most approaches to obtain longer-term graft survival. In total, four studies used a hindlimb, two a skin flap, one abdominal wall with/without hindlimb and one group an osseomusculuocutanous sternum with/without thymus model. Ramirez et al. (2013) showed a successfully induced tolerance and peripheral chimerism using a regimen of cyclosporine A (CsA), ALS, and adipocyte-derived stem cells (36). Jindal et al. (2015) used human IL-2 fusion protein and combined this regimen with ALS plus CsA (37). This regimen showed the longest graft survival with >150 days plus tolerance induction but no mixed chimerism were analyzed.

Plock et al. (2015) changed the regimen to only a short course of FK-506 plus either adipose- or bone marrow-derived mesenchymal stem cells and induced tolerance as well as long-term graft survival and transient mixed chimerism (>120 days) (38). Cheng et al. (2013) used adipocyte-derived stem cells, CsA and ALS. Tolerance induction and long-term graft survival could be achieved up to 150 days but no mixed chimerism were analyzed (39). A single donor-recipient chimeric cells (DRCC) therapy by *ex vivo* cell fusion (of bone marrow cells) was applied by Cwykiel et al. (2021) in combination with CsA and antibody therapy and resulted in graft survival of >79 days, tolerance induction plus peripheral chimerism (40). In 2016, Sieminow et al. showed establishment of mixed chimerism by preconditioning recipient bone marrow cells (BMC) followed by anti-α-β-T cell receptor (TCR) monoclonal antibody plus CsA for 7 days post-transplant and achieved a median graft survival of 90 days plus tolerance induction (41). Ma et al. (2019) demonstrated a potential attenuation of rejection by infusion of human adipose-derived stem cells (hASC) plus chemokine receptor 7 (CCR7). CCR7 was able to guide hASC to secondary lymphoid organs to have immunomodulatory effects on Tcells. Chimerism or tolerance induction have not been established (42). Zor et al. (2020) analyzed the role of a simultaneous thymus transplantation. Chimerism were detectable >150 days posttransplant under continuous CsA therapy in an osseomusculoutaneous sternum and thymus allotransplantation, but without induction of tolerance (43). The longest survival with simultaneously induction of tolerance here was shown by Cheng et al. (2013) with a regimen of adipose-derived stem cells, CsA and ALS with or without TBI. Unfortunately, the establishment of mixed chimerism has not been analyzed. Most approaches here show that using stem cell therapies can induce tolerance and graft survival.

Swine Models

In swine (**Table 3**), tolerance induction was attempted by establishing mixed chimerism with and without co-stimulatory blockage. One group used a full-skin flap, the second group used an osteomyocutaneos flap. Leonard et al. (2014) successfully induced tolerance in their large animal model. The recipient was preconditioned by T-cell depletion plus TBI, followed by hematopoietic cell transplantation. VCA was either performed into stable mixed chimerism or after establishing of mixed chimerism 85–105 days later. After transplantation, a 45-day course of CsA was given. No signs of rejection were seen between 115–504 days posttransplant (14). Lellouch et al. (2022) showed tolerance induction by mixed chimerism across MHC class-I-mismatch up to 400 days posttransplant. Treatment was TBI and thymic irradiation 2 days before surgery. Bone marrow transplantation was performed on the day of surgery, followed by co-stimulatory blockade (CTLA4-Ig + FK405) for 30 days and treatment of anti-IL6R monoclonal antibody (mAb) plus methylprednisolone (44). Both approaches show promising results in terms of long-term graft survival, similarity to the human immune system and absence of long-term immunosuppression and serve as proof of concept in a preclinical large animal model.

NHP Model

As described above, to have a continuous source for donor stem cells, vascularized bone marrow transplantation might be an approach to induce tolerance through establishment of mixed chimerism (continuous source of donor-derived hematopoietic stem cells). This approach was tested by Barth et al. (**Table 4**) as he further analyzed the role of vascularized bone marrow transplantation (VBM) in a VCA model with a heterotopic partial face transplantation in NHPs. VCA containing vascularized bone not only led to prolonged, but also rejection free graft survival (430 days) compared to VCA without vascularized bone (7 days). However, withdrawal of immunosuppressants led to graft loss and tolerance induction was not achieved but VBM enabled a low dose maintenance immunosuppressants regimen and sporadic macrochimerism were detected (15). Although this approach was unable to achieve tolerance induction through mixed chimerism, these results serve as proof of concept. However, stable macro or micro chimerism has not been documented in human VCA. Further studies are necessary, for example on how to enhance the ability of vascularized bone marrow to induce chimerism, e.g., *via* Treg modulative strategies to increase acceptance of donor derived hematopoietic stem cells (21).

CONCLUSION

Both small and large animal models are relevant for preclinical VCA research. Small animal models are advantageous because of low costs in breeding and housing and especially mice are well suited for analysis of immunological mechanisms. Large animal models offer greater similarity to human immune system and human anatomy, but models tend to be more complex and cost intensive. Tolerance induction in preclinical models was achieved using both cellular based and pharmaceutical based strategies. Clinical translation of such strategies to human trials has yet to be done successfully, however, the induction of donor-specific tolerance may ultimately help improve the risk benefit ratio of VCA transplantation.

AUTHOR CONTRIBUTIONS

LH and MK-N contributed to conducting and writing this review as shared first co-authors. VS and JF contributed in data analysis and literature research. MK-N, AD, SR, MK and TH contributed in research design and input on the manuscript. MK and AD supervised the manuscript process as shared last authors. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

LH is a recipient of a scholarship (Walter-Benjamin) from the German Research Foundation (DFG).

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

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ORIGINAL RESEARCH
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Effects of T3 Administration on *Ex Vivo* Rat Hearts Subjected to Normothermic Perfusion: Therapeutic Implications in Donor Heart Preservation and Repair

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The present study investigated the effects of triiodothyronine (T3) administration in *ex vivo* model of rat heart normothermic perfusion. T3 is cardioprotective and has the potential to repair the injured myocardium. Isolated hearts were subjected to normothermic perfusion (NP) with Krebs-Henseleit for 4 h with vehicle (NP) or 60 nM T3 in the perfusate (NP + T3). Left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), perfusion pressure (PP) and percentage of change of these parameters from the baseline values were measured. Activation of stress induced kinase signaling was assessed in tissue samples. Baseline parameters were similar between groups. LVEDP was increased from the baseline by 13% (70) for NP + T3 vs. 139% (160) for NP group, $p = 0.048$. LVDP was reduced by 18.2% (5) for NP + T3 vs. 25.3% (19) for NP group, $p = 0.01$. PP was increased by 41% (19) for NP + T3 vs.91% (56) for NP group, $p = 0.024$. T3 increased activation of pro-survival Akt by 1.85 fold ($p = 0.047$) and AMPK by 2.25 fold ($p = 0.01$) and reduced activation of pro-apoptotic p38 MAPK by 3fold ($p = 0.04$) and p54 JNK by 4.0 fold ($p = 0.04$). Administration of T3 in normothermic perfusion had favorable effects on cardiac function and perfusion pressure and switched death to pro-survival kinase signaling.

Keywords: heart, normothermic perfusion, thyroid hormone, cold cardioplegia, kinase signalling

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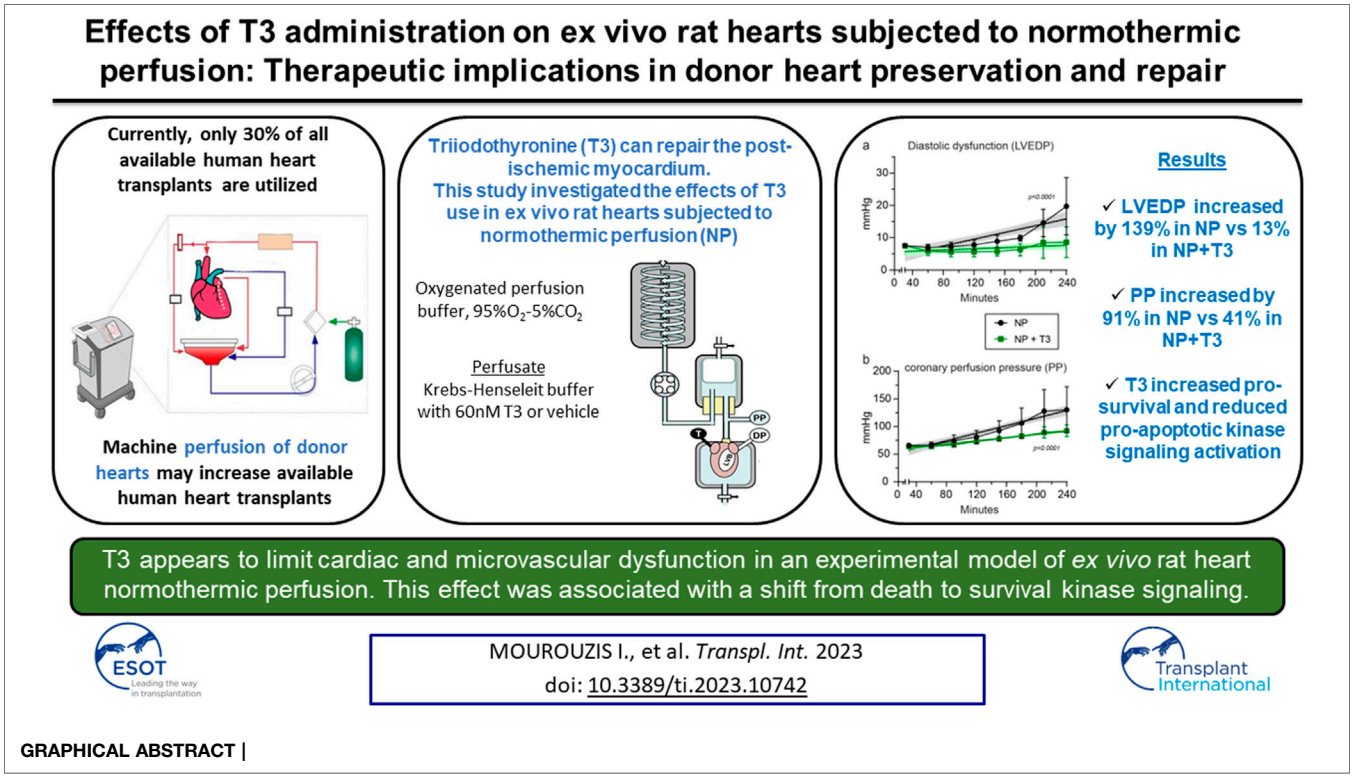

GRAPHICAL ABSTRACT |

INTRODUCTION

Heart transplantation remains the cornerstone therapy for end stage heart failure with an overall median survival of 12.5 years (1). Preservation solutions and techniques are crucial for donor organ quality which affects morbidity and survival after transplantation (2). Currently, cold storage is the standard method for organ preservation. However, prolonged cold storage may increase the risk of early graft dysfunction due to residual ischemia, reperfusion and rewarming injury (3). Furthermore, the demand for the use of marginal donor organs requires methods for organ assessment and repair. Machine normothermic or hypothermic perfusion has been recently attempted as a promising preservation technique (4). Machine perfusion may enable the use of cardioprotective agents to enhance tolerance of the donor heart to ischemia and prevent cardiac remodeling (5, 6). Thus, this new therapeutic challenge may increase both donor pool and post transplantation survival (7).

Thyroid hormone has traditionally been used in heart transplantation, but its mode of action is not fully understood (8–11). It is now recognized that thyroid hormone, beyond its classical actions on metabolism, has cardioprotective and reparative actions due to its differential effects on healthy and injured myocardium. Thus, thyroid hormone pretreatment can precondition the heart against ischemia-reperfusion (12) and triiodothyronine (T3) administration at reperfusion can limit reperfusion injury and cardiac remodeling (13, 14). T3 action is mediated by a delicate balance between pro-apoptotic and pro-survival kinase signaling pathways (15). This kinase signaling balance seems to be critical for cardiac injury and remodeling after an ischemic insult (16). More recently, the cardioprotective and reparative effects of T3 have also been demonstrated in humans (17, 18).

Based on this evidence, the aim of the present study was to compare normothermic perfusion to cold standard cardioplegia technique and investigate the potential of T3 administration to optimize normothermic perfusion in an *ex vivo* rat heart experimental model. This issue is of therapeutic importance and has not previously been studied.

MATERIALS AND METHODS

Animals

Wistar male rats, 380–500 g, were used for this study. The rats were handed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Pub. No. 8323, Revised 1996). The protocol of the study was approved by the Animal Care and Use Committee of Department of Pharmacology, Medical School, National and Kapodistrian University of Athens (license 842/20-02-2017, EL 25BIOexp 10).

Experimental Protocol

In order to assess the effects of cold cardioplegic arrest versus normothermic perfusion on cardiac function and perfusion pressure, the following experiments were performed (**Figure 1A**).

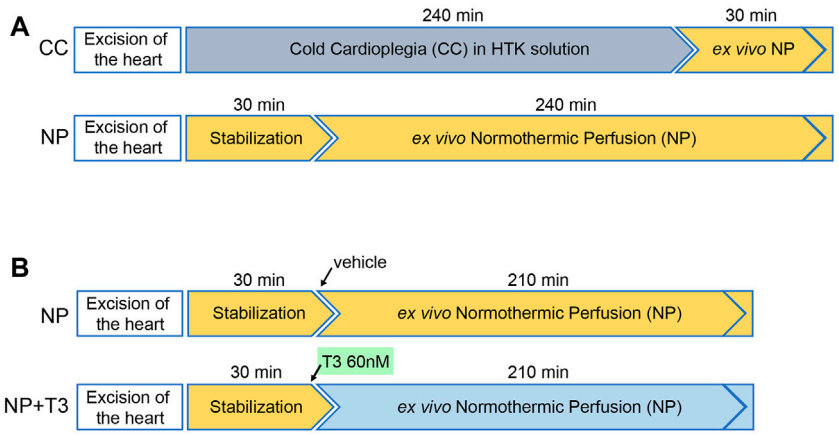

FIGURE 1 | Schematic diagram showing the experimental design of the study. **(A)** Experiments performed to compare Cold Cardioplegia (CC) to normothermic perfusion (NP), **(B)** Experiments performed to investigate the effects of triiodothyronine (T3) in rat hearts with NP.

a. Hearts excised and subjected to cold cardioplegic (CC) arrest for 240 min and then perfused in Langendorff apparatus allowing 30 min recovery (group CC, n = 10),
b. Hearts excised and subjected to normothermic perfusion (NP) in Langendorff apparatus for 210 min after an initial period of 30 min perfusion (stabilization period), (group NP, n = 10).

In order to assess the effects of T3 administration on cardiac function and perfusion pressure during *ex vivo* normothermic perfusion, the following experiments were performed (**Figure 1B**).

a. Hearts excised and subjected to normothermic perfusion in Langendorff apparatus for 210 min after an initial period of 30 min perfusion (stabilization period). At the end of stabilization, KH buffer was supplemented with vehicle (group NP, n = 10),
b. Hearts excised and subjected to normothermic perfusion in Langendorff apparatus for 210 min after an initial period of 30 min perfusion (stabilization period). At the end of stabilization, KH buffer was supplemented with 60nM T3 (group NP + T3, n = 8),

During stabilization both groups were treated the same. At the end of the perfusion, LV was isolated, frozen in liquid nitrogen and kept at −80°C for molecular analysis.

Anesthesia and Recovery After Cold Static Storage

Rats were anaesthetized with ketamine HCl and heparin 1000 IU was given intravenously. Following anesthesia, thoracotomy was performed to expose the heart and major vessels. The heart and large vessels were excised and immersed into ice cold histidine-tryptophan-ketoglutarate (HTK) cardioplegia solution identical to the solution used for cardiopreservation during heart transplantation (composition in mmol/L NaCl 15, KCl 9, MgCl 2 4, Adenosine 5, a-ketoglutarate 1, Tryptophan 2, Histidine 180, Histidine-HCl, 8, Mannitol 30). Changes in cold HTK solution were performed to achieve removal of blood (within 90 s) and then the heart was transferred to 100 mL of new cold HTK cardioplegia solution and stored at 4°C for 240 min. After this period, hearts were cannulated *via* the ascending aorta and perfused in the Langendorff apparatus with KH buffer allowing 30 min recovery. An intraventricular balloon was inserted into the left ventricle *via* the left atrium and allowed measurement of left ventricular pressure under isovolumic conditions. In order preload to be similar in both groups and equal to normal preload of the isolated rat heart, balloon volume was set at 250 μL. Constant flow was adjusted at 15 mL/min. The perfusion apparatus was heated to ensure a temperature of 37°C throughout the course of the experiment. Hearts were paced at 320 beats/min with a Harvard pacemaker. Pacing started 5–7 min after perfusion was achieved.

Anesthesia and Recovery After Normothermic Perfusion

Rats were anaesthetized with ketamine HCl and heparin 1000 IU was given intravenously. The hearts were rapidly excised, placed in ice-cold Krebs-Henseleit (KH) buffer (composition in mmol/L: sodium chloride 118, potassium chloride 4.7, potassium phosphate monobasic 1.2, magnesium sulfate 1.2, calcium chloride 1.4, sodium bicarbonate 25, and glucose 11) and mounted on the aortic cannula of the Langendorff perfusion system. Perfusion with oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer was established within 60 s after thoracotomy. The heart perfused to a non-working isolated rat heart preparation at a constant flow according to the Langendorff technique as previously described (12, 14, 19, 20). A water filled balloon, connected to a pressure transducer, was advanced into the left ventricle through an incision in the left atrium. Pressure signal was transferred to a computer using a data analysis software (IOX, Emka Technologies) which allowed

continuous monitoring and recording (21). The intraventricular balloon allowed measurement of left ventricular pressure under isovolumic conditions. Left ventricular balloon volume was adjusted to produce an average initial left ventricular end-diastolic pressure of 6–8 mmHg in all groups and was held constant thereafter throughout the experiment. Since the balloon was not compressible, left ventricular contraction was isovolumic. As intraventricular volume was maintained at a constant value, preload, did not change. Thus, the left ventricular peak systolic pressure and the left ventricular developed pressure (LVDP), defined as the difference between left ventricular peak systolic pressure and left ventricular end-diastolic pressure, represented indexes of systolic function obtained under isometric conditions.

The perfusion apparatus was heated to ensure a temperature of 37°C throughout the course of the experiment. Constant flow was adjusted at 15 mL/min. Hearts were paced at 320 beats/min with a Harvard pacemaker. Pacing started 5–7 min after perfusion was achieved.

Administration of Triiodothyronine

3,5,3′-triiodothyronine (T3) was purchased from Sigma Chemicals (St Louis MO, USA). T3 was dissolved in ethanol by adding a small volume of 25% NaOH and diluted in 0.9% normal saline to obtain a stock solution. T3 concentration in stock solution was 1 mg/mL. Stock solutions were kept at −20°C and before each experiment a quantity of this solution containing T3 was added in Krebs-Henseleit (KH) buffer to a final concentration of 60 nM. This dose has previously been shown to be cardioprotective against ischemia-reperfusion injury in an isolated rat heart experimental model (14). T3 administration initiated after the first 30 min of perfusion (stabilization period) in the isolated heart apparatus. Vehicle (T3 diluent) was injected in hearts subjected to normothermic perfusion without T3.

Measurement of Mechanical Function

Left ventricular function was assessed by recording the left ventricular developed pressure (LVDP, mmHg) and the positive and negative first derivative of LVDP (+dp/dt and −dp/dt). Diastolic function was assessed by monitoring isovolumic left ventricular end-diastolic pressure (LVEDP) as a measure of diastolic chamber distensibility. Perfusion pressure under constant flow conditions was used to assess coronary vessel resistance (PP, mmHg). All parameters were also expressed as percentage of change from the baseline values.

Molecular Analysis

In order to investigate potential molecular mechanisms underlying the effects of T3 in normothermic perfusion, the pattern of stress induced kinase signaling activation was assessed in NP and NP + T3 hearts at the end of perfusion. Molecular analysis was performed as previously described (21,22). A sample from the left ventricular tissue (200–220 mg) was homogenized in ice-cold buffer containing 10 mM Hepes (pH: 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mM DTT and 10 μg/mL leupeptin. 200 μL of 10% Igepal was added and samples were left in ice for 30 min. Homogenization was repeated and the homogenate was centrifuged at 1000 g for 5 min, 4°C. The supernatant representing the cytosol-membrane fraction was kept at −80°C for further processing. Protein concentrations were determined by the bicinchoninic acid (BCA) method, using bovine serum albumin as a standard. Samples were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by boiling for 5 min in Laemmli sample buffer containing 5% 2-mercaptoethanol. Aliquots (40 μg) were loaded onto 9% (w/v) acrylamide gels and subjected to SDS-PAGE in a Bio-Rad Mini Protean gel apparatus. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond ECL) at 100 V and 4°C, for 1.5 h using Towbin buffer for Western blotting analysis. Subsequently, filters were probed with specific antibodies against total p38 MAPK and dual phospho-p38 MAPK, total c-jun NH2-terminal kinases (JNKs) and dual phospho-JNKs, total Akt and dual phospho-Akt (Cell Signaling Technology, dilution 1:1000), total AMPK and phospho (Thr172)-AMPK (Cell Signaling Technology, dilution 1:1000) overnight at 4°C. Filters were incubated with appropriate anti-mouse (Amersham) or anti-rabbit (Cell Signalling) HRP secondary antibodies and immunoreactivity was detected by enhanced chemiluminescence using Lumiglo reagents (New England Biolabs). Chemiluminescence was detected by the image analysis system FluorChem HD2 (AlphaInnotech Corporation, 14743, Catalina Street, San Leandro, CA) equipped with a CCD camera and analysis software. Five samples from each group were loaded on the same gel for comparisons between groups. Data were expressed as the ratio of phosphorylated to total protein expression.

Statistics

Values are presented as mean (Standard Deviation). Normal distribution of variables was estimated with Shapiro-Wilk test of normality. Normally distributed data were compared using an independent t-test. Skewed data were analysed non-parametrically using Mann-Whitney U test. Serial measurements of LVEDP and PP were compared by mixed, repeated measures analysis of variance (mixed ANOVA) to test for the effect of treatment, time and the interaction (tests for "within-subjects" factor and "between-subjects" factor); the respective non-linear fit-curves with the 95% CI error were produced with non-linear fit analysis. When significant, differences within and between each group were tested by a *post hoc* analysis using the Bonferroni correction for multiple comparisons if needed. A two-tailed test with a p value less than 0.05 was considered significant. Analysis was performed using the GraphPad 8 software.

RESULTS

Comparison of Normothermic Perfusion to Cold Cardioplegic Arrest

Cardiac function and perfusion pressure were assessed at the end of the recovery period in CC group and the end of normothermic perfusion in NP group. After cold cardioplegia, LVEDP was 33.6 (11.7) mmHg in CC group vs.18.5 (13.3) in NP group, $p = 0.02$.

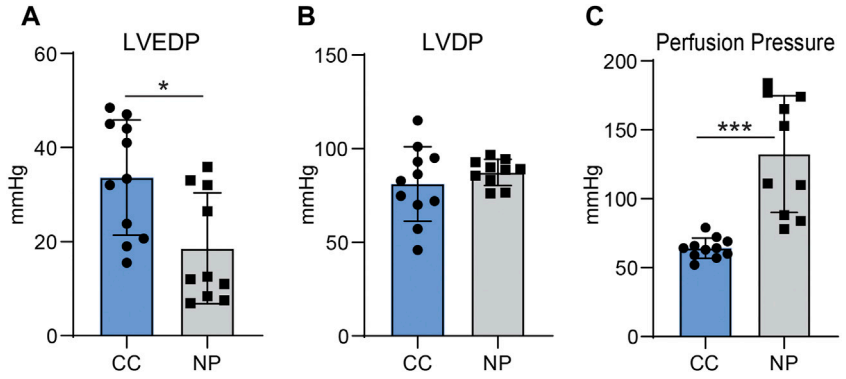

FIGURE 2 | Left ventricular end-diastolic pressure (LVEDP, **(A)**, left ventricular developed pressure [LVDP, **(B)**] and perfusion pressure [PP, **(C)**] in hearts preserved with cold cardiop legia (CC, n = 10) and hearts with normothermic perfusion (NP, n = 10). Values represent Means, bars stand for standard deviation. *p < 0.05, ***p; 0.001.

TABLE 1 | Cardiac function and perfusion pressure at the end of stabilization (baseline parameters) and the end of normothermic perfusion. Data are presented as Mean (SD).

	NP (n = 10)			NP + T3 (n = 8)		
	End of stabilization	**End of perfusion**	**% Change from baseline**	**End of stabilization**	**End of perfusion**	**% Change from baseline**
LVDP (mmHg)	116.7 (9.0)	87.3 (6.9)	−25 (5)%	112.3 (8.2)	90.0 (7.3)	−18 (5)%*
LVEDP (mmHg)	7.6 (0.42)	19.7 (12)	139 (160)%	7.5 (0.43)	8.5 (5.6)*	13 (70)%*
+dp/dt (mmHg/sec)	3740 (826)	3038 (612)	−18 (10)%	3593 (803)	3032 (850)	−15 (6)%
−dp/dt (mmHg/sec)	2189 (268)	1490 (216)	−32 (6)%	2045 (227)	1515 (268)	−25 (9)%#
PP (mmHg)	67 (4.4)	130 (41)	91 (56)%	66 (2.4)	92 (10)*	41 (19)%*

LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dp/dt, rate of increase of LVDP; −dp/dt, rate of decrease of LVDP; PP, perfusion pressure.
*p < *0.05 vs. NP;* #p = *0.08 vs. NP.*

LVDP was similar between the groups; 81 (18.9) mmHg for CC and 87.3 (7.0) for NP, p = n.s. Perfusion Pressure was 64.2 (7.0) mmHg in CC group vs. 132 (51) mmHg in NP group, p = 0.001 (**Figure 2**).

Effects of T3 Administration in Normothermic Perfusion

Cardiac function and perfusion pressure were measured at the end of stabilization period (baseline parameters) and the end of normothermic perfusion. Baseline parameters were similar between NP and NP + T3 groups (**Table 1**). LVEDP was 19.7 (12) mmHg in NP group and 8.6 (5.8) mmHg in T3 treated group p = 0.026 (**Figure 3A**). LVDP was 87 (20) mmHg in NP group and 90 (21) mmHg in T3 treated group, p = 0.49. PP was 130 (39.1) mmHg in NP and 92 (10.6) mmHg in T3 treated group, p = 0.019 (**Table 1**).

Functional parameters and perfusion pressure were also expressed as percentage of change from the baseline values. LVEDP gradually increased by 139% from baseline in NP group, and only by 13% in NP + T3 group, p = 0.048 (**Table 1**). The decline in LVDP was found to be less after T3 treatment; 18.2% in NP + T3 vs. 25.3% in NP, p = 0.01 (**Table 1**). PP increased by 91% from baseline in NP group and by 41% in NP + T3 group, p = 0.024 (**Table 1**).

Measurements of cardiac function and perfusion pressure were also performed every 30 min from the end of stabilization period to the end of normothermic perfusion (**Figure 3**). Mixed Repeated Measures ANOVA analysis for LVEDP (dependent variable) at different time points between the groups showed that the main effect of treatment (T3, "between-subjects" factor) on LVEDP was statistically significant (F = 4.0, p = 0.04). Thus, T3 treatment induced a sustained improvement of LVEDP over time. Furthermore, there was a statistically significant effect (F = 9.1, p = 0.002) regarding the main effect of time ("within-subjects" factor). There was also a statistically significant interaction effect between time and treatment (p = 0.044).

Mixed Repeated Measures ANOVA analysis for PP (dependent variable) at different time points between the groups showed that the main effect of treatment (T3, "between-subjects" factor) on PP was statistically significant (F = 5.5, p = 0.03). Thus, T3 treatment induced a sustained improvement of PP over time. Furthermore, there was a statistically significant effect (F = 28.8, p = 0.000002) regarding the main effect of time ("within-subjects" factor) on deterioration

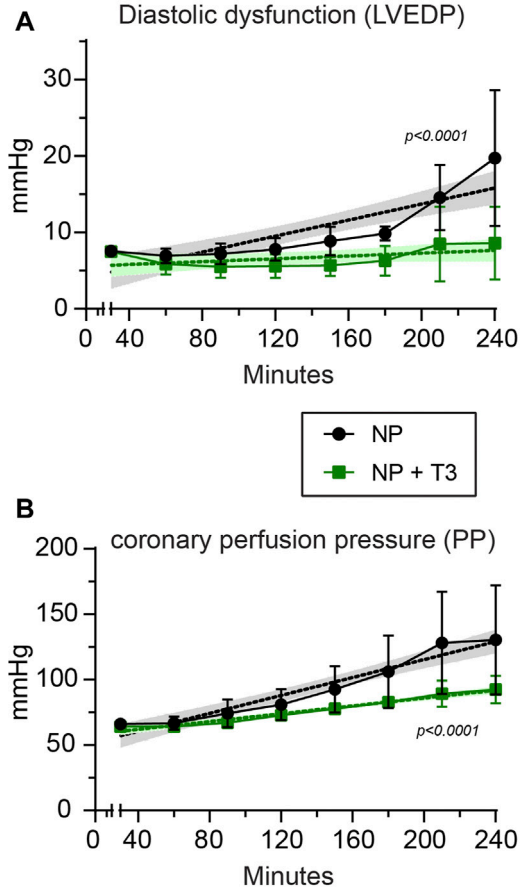

FIGURE 3 | Left ventricular end-diastolic pressure [LVEDP, **(A)**], and perfusion pressure [PP, **(B)**] in hearts subjected to normothermic perfusion in Langendorff apparatus for 210 min after an initial period of 30 min perfusion (stabilization period). At the end of stabilization, KH buffer was supplemented either with vehicle (NP, $n = 10$) or with 60 nM T3 (NP + T3, $n = 8$). Values represent Means, bars stand for standard deviation. The respective non-linear fit-curves with the 95% Confidence Intervals produced with non-linear fit analysis are shown with dotted lines.

of PP. A statistically significant interaction effect between time and treatment was found ($p = 0.024$).

At the end of normothermic perfusion, the ratio of LV weight to body weight was 2.35 (0.3) for the NP hearts and 2.39 (0.26) for NP + T3 hearts, p = n.s.

Effects of T3 Administration on the Activation of Stress Induced Kinases

Stress induced kinase signaling activation was assessed in NP and NP + T3 hearts at the end of normothermic perfusion. T3 administration during normothermic perfusion resulted in suppression of proapoptotic signalling and upregulation of the pro-survival signalling pathways.

Phosphorylated to total Akt and phosphorylated to total AMPK levels were both significantly increased by 1.85 ($p = 0.047$) and 2.25-fold ($p = 0.01$) in T3 treated hearts (NP + T3) as compared to NP hearts, $p < 0.05$ (**Figures 4A,B**).

Phosphorylated to total p38 MAPK levels were lower by 3 fold in NP + T3 as compared to NP hearts, $p = 0.04$. Phosphorylated to total p54 and p46 JNKs levels were lower by 4.0 ($p = 0.04$) and 3.7-fold ($p = 0.03$) respectively in NP + T3 as compared to NP hearts (**Figures 4C,D**).

DISCUSSION

Normothermic machine perfusion is an emerging preservation modality which maintains organ metabolism and thus, prevents the harmful effects of cold ischemia. Machine perfusion is non inferior to cold cardioplegia and is particularly beneficial for high risk donors and donation of hearts after circulatory death (23). However, optimal microcirculatory support is crucial for successful normothermic perfusion. After removal of the organ, the disconnection from all autonomic and endocrine systems may cause deregulation of vascular tone which leads to impairment in microcirculation and tissue hypoxia which in turn results in tissue injury, edema and remodeling (viscous circle). Several pharmacological interventions aiming to induce vasodilation, reduce edema and enhance metabolism have been investigated as potential donor organ preservation modalities (24). Triiodothyronine has long been used for hemodynamic support and as replacement therapy along with other hormones for donor organ preservation and resuscitation (8–11). Beyond these classical actions, T3 at doses several folds above normal is shown to reduce myocardial ischemia-reperfusion injury, prevent cardiac remodeling and promote regeneration (12, 14, 25). More importantly, this cardioprotective and reparative action has recently been shown in humans (17). T3 regulates vascular function, induces angiogenesis and mitochondrial biogenesis (26–28). Based on this evidence, we hypothesized that high dose T3 could potentially optimize cardiac preservation in the setting of normothermic perfusion.

In the present study, an *ex vivo* rat heart normothermic perfusion model was established to identify differences between normothermic perfusion and cold cardioplegia and to investigate the potential of high dose T3 to optimize normothermic perfusion. A decline of 5%–10% of left ventricular developed pressure per hour perfusion has been reported in this experimental model (29). In this setting, coronary perfusion pressure (an indirect index of microcirculation) was found to be higher in hearts subjected to normothermic perfusion compared to cold cardioplegia, probably indicating impaired microcirculation after normothermic perfusion. It is of note that microcirculation is impaired after reperfusion in by-pass surgery (30), acute myocardial infarction (31) and heart transplantation (32, 33) despite vascular blood flow restoration in all these conditions. Furthermore, the presence of microvascular dysfunction has been associated with postischemic cardiac remodeling and impaired healing (31, 34). The optimization of microcirculation after reperfusion remains a therapeutic challenge. In this context, according to our data, T3 administration in normothermic

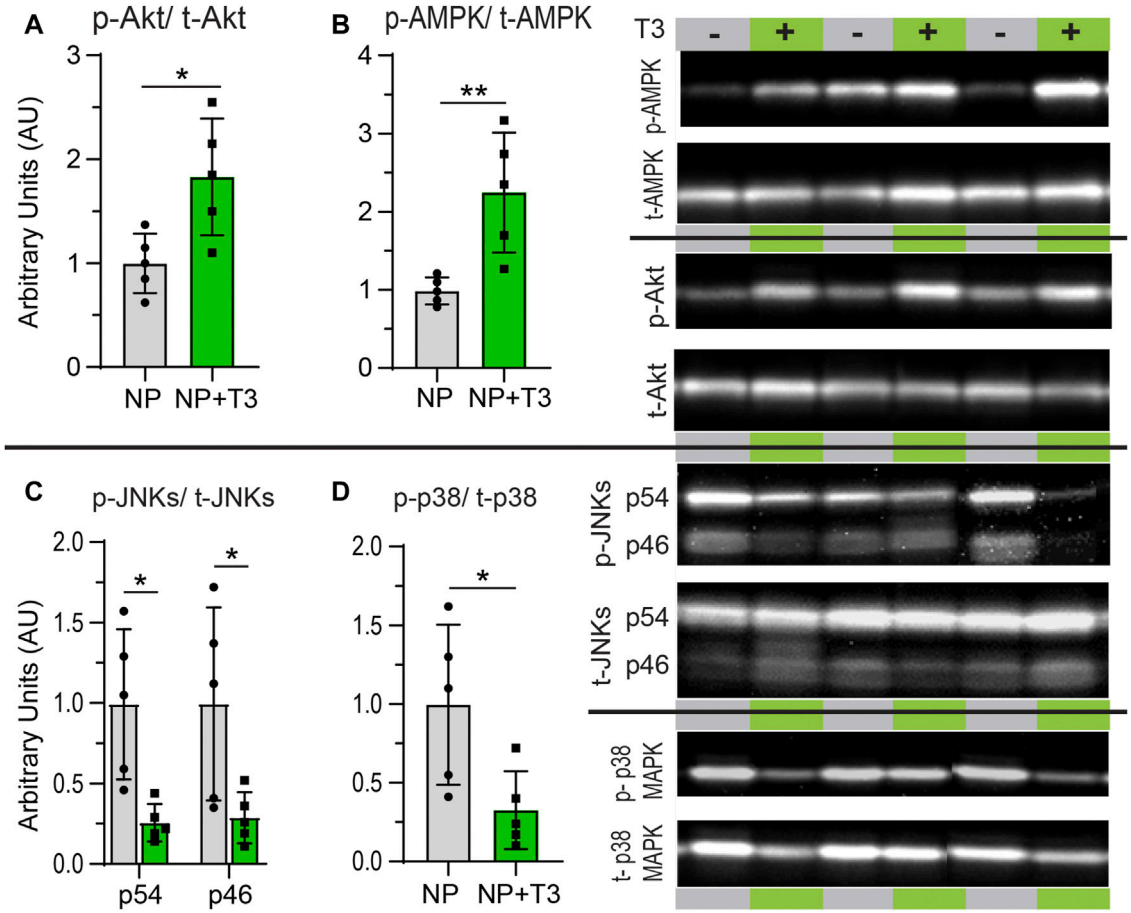

FIGURE 4 | Representative western blots and densitometric assessment in arbitrary units of the ratio of phosphorylated Akt to total Akt expression **(A)**, phosphorylated AMPK to total AMPK expression **(B)**, phosphorylated p54 and p46 JNKs to total JNKs expression **(C)** and phosphorylated p38 MAPK to total p38 MAPK expression **(D)** in hearts at the end of normothermic perfusion supplemented either with vehicle (NP) or with 60 nM T3 (NP + T3). Data represent Means from $n = 5$ hearts per group. Bars stand for standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$.

perfusion resulted in significant lower coronary perfusion pressure as compared to untreated hearts. Furthermore, the decline in LVDP was also significantly less in treated hearts and LVEDP was increased by only 13% of the initial value after normothermic perfusion in treated hearts as compared to 139% increase in the untreated group. Heart weight at the end of perfusion was similar between the groups probably indicating that T3 had no effect on tissue edema. Collectively, these data suggest that T3 may have favorable effects on microcirculation and prevent tissue hypoxia induced functional changes after normothermic perfusion. These observations are consistent with previous reports showing that T3 treatment improves tissue hypoxia in experimental settings of impaired microcirculation such as ischemia–reperfusion and sepsis. Thus, T3 administration early at reperfusion improved post-ischemic recovery of function and limited apoptosis particularly in the mid layer of the myocardium in which extensive microvascular network exists (14). Furthermore, T3 administration prevented cardiac and liver tissue hypoxia and microvascular dysfunction in experimental sepsis (35). Interestingly, the potential of T3 to prevent tissue hypoxia has also been demonstrated in patients with sepsis in a preliminary study (36).

The mechanisms underlying the cardioprotective effect of T3 have been under intense investigation. Thyroid hormone is shown to protect the heart against ischemic/reperfusion injury *via* regulation of intracellular kinase signaling pathways (15). Cellular kinase signaling pathways play an important pathophysiologic role in the ischemic injury, post-ischemic cardiac remodeling and heart failure (16). It is now recognized that a critical balance between pro-apoptotic and pro-survival pathways determines myocardial injury or survival after ischemia. In this context, activation of p38 MAPK results in apoptosis, vascular permeability, depression of cardiac function and immune cell activation and cytokine upregulation (37). Accordingly, inhibition of p38 MAPK activation lowered systematic levels of pro-inflammatory cytokines in a brain dead donor model (38). Furthermore, addition of a p38 MAPK inhibitor to the Euro Collins and University of Wisconsin solutions mitigated ischemia/reperfusion injury in lung, liver and kidney grafts

(39). p38 MAPK is critical for organ repair after stress and inhibition of p38 MAPK enables proliferation of adult mammalian cardiomyocytes and heart regeneration (40). JNK activation also leads to apoptosis and inhibition of this kinase reduced histological rejection and improved graft survival in a rat model of transplantation (41).

Activation of pro-survival Akt signaling pathway prevents apoptosis, induces angiogenesis and regulates calcium handling (42). Interestingly, crosstalk between Akt and p38 MAPK signaling exists and seems to regulate the levels of cryoprotection vs. apoptosis in endothelial cells (43). Upon stress, AMPK appears to serve a critical role in regulating cardiac metabolism. Activation of AMPK improved left ventricular function and survival in experimental myocardial infarction (44).

On the basis of these data, the present study investigated potential changes in the ratio of phosphorylated to total kinase protein expression (an index of kinase activation) after T3 administration in normothermic perfusion. T3 significantly reduced the activation of the pro-apoptotic p38 MAPK and JNK signaling while enhanced pro-survival Akt signaling and AMPK. This molecular signature indicates that T3 administration can switch intracellular death signaling to survival with potential implications in postischemic remodeling and late heart failure.

Clinical Implications -Limitations of the Study

Ex vivo perfusion is a new strategy for organ preservation and reconditioning prior to transplantation (4, 45). Extensive research in this field is underway to identify optimal perfusate composition and duration. The perfusate commonly used in clinical practice is cell based consisted of leukocyte depleted, packed red blood cells supplemented with anticoagulants and vasodilators. However, the use of red cells may not be suitable for prolonged periods of perfusion. There is now evidence that non cell solutions as Ringer's lactate or Steen solution supplemented with nutrient and metabolic substrates may optimize normothermic perfusion (24). Along this line, the present study provides evidence that addition of high dose of T3 in K-H can preserve *ex vivo* rat hearts subjected to normothermic perfusion. Furthermore, for the first time, this therapeutic modality is shown to induce the activation of intracellular repair signaling. Until now, thyroid hormone has been added to solutions at replacement doses and along with other hormones to resuscitate and preserve donor organs (11). Thus, the cardioprotective effect of this hormone has not been evaluated.

There are limitations of this study which have to be taken into consideration. The study was performed in small animals and its findings are needed to be validated in large animals and humans. However, the rat species is shown to be of high translational validity for pharmacological studies of ischemia/reperfusion (17). The potential effects of T3 in extended periods of normothermic perfusion have not been investigated. Cardiac damage was assessed by functional indices and not histological examination. The benefit of T3 in cell-based solutions has not been investigated. However, it has recently been shown that T3 may have favorable effects on erythrocyte aggregation and hemorheology.

In conclusion, T3 appears to limit cardiac and microvascular dysfunction in an experimental model of *ex vivo* rat heart normothermic perfusion. This effect was associated with a switch of death to survival kinase signaling.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Department of Pharmacology, Medical School, National and Kapodistrian University of Athens (license 842/20-02-2017, EL 25BIOexp 10).

AUTHOR CONTRIBUTIONS

IM and DK equally contributed in experiments, methodology, collection of data, formal analysis, and review manuscript. IM: supervision, writing the original draft. VB and DA: contributed in isolated heart experiments. AK: contributed in molecular analysis experiments. AL: statistical analysis and review manuscript. CP: conceptualization, funding acquisition, project administration, supervision, writing the original draft and review manuscript.

CONFLICT OF INTEREST

The following patent is relevant to the work in this manuscript: PCT/4972/2021. Pharmaceutical composition comprising L-triiodothyronine (T3) for use in the treatment of tissue hypoxia and sepsis. CP and IM are the inventors and hold royalties in relation to this patent.

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

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Heparin Thromboprophylaxis in Simultaneous Pancreas-Kidney Transplantation: A Systematic Review and Meta-Analysis of Observational Studies

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Thrombosis is a leading causes of pancreas graft loss after simultaneous pancreas kidney (SPK), pancreas after kidney (PAK), and pancreas transplant alone (PTA). There remains no standardized thromboprophylaxis protocol. The aim of this systematic review and meta-analysis is to evaluate the impact of heparin thromboprophylaxis on the incidence of pancreas thrombosis, pancreas graft loss, bleeding, and secondary outcomes in SPK, PAK, and PTA. Following PRISMA guidelines, we systematically searched BIOSIS®, PubMed®, Cochrane Library®, EMBASE®, MEDLINE®, and Web of Science® on April 21, 2021. Primary peer-reviewed studies that met inclusion criteria were included. Two methods of quantitative synthesis were performed to account for comparative and non-comparative studies. We included 11 studies, comprising of 1,122 patients in the heparin group and 236 patients in the no-heparin group. When compared to the no-heparin control, prophylactic heparinization significantly decreased the risk of early pancreas thrombosis and pancreas loss for SPK, PAK and PTA without increasing the incidence of bleeding or acute return to the operating room. Heparin thromboprophylaxis yields an approximate two-fold reduction in both pancreas thrombosis and pancreas loss for SPK, PAK and PTA. We report the dosage, frequency, and duration of heparin administration to consolidate the available evidence.

Keywords: pancreas transplantation, meta-analysis, heparin, simultaneous pancreas-kidney transplantation, systematic review, thrombosis, thromboprophylaxis

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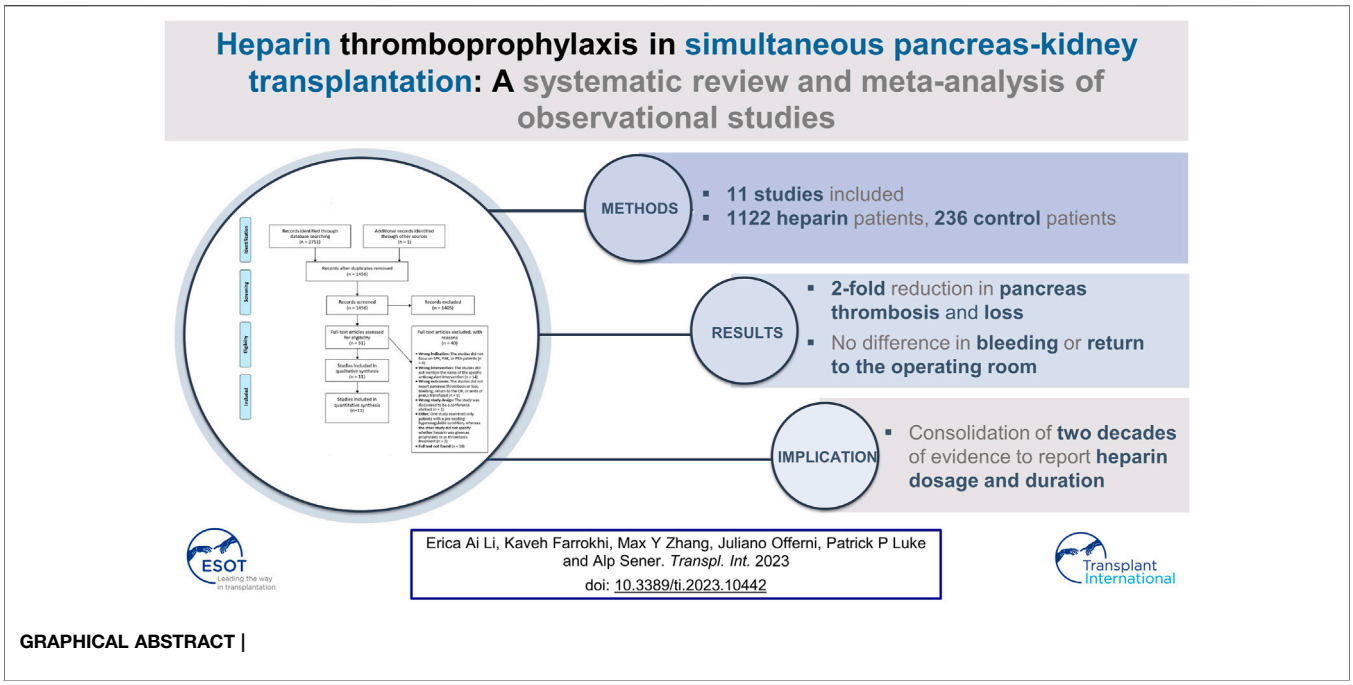
Abbreviations: IU, International unit; PAK, Pancreas after kidney transplant; PTA, Pancreas transplant alone; SPK, Simultaneous pancreas-kidney transplant.



GRAPHICAL ABSTRACT |

INTRODUCTION

Pancreas transplantation remains the only curative procedure for type 1 diabetes mellitus (T1DM) patients, resulting in long-term control of HbA1c without the risk of serious hypoglycemic events. (1) The first pancreas transplant was performed in 1966 at the University of Minnesota, and since then advancements in immunosuppression, surgical techniques, and surgeon experience have resulted in good overall outcomes for patients (2).

Pancreas transplantation most often occurs simultaneously with kidney transplantation in uremic patients. Simultaneous pancreas-kidney (SPK) transplantation comprises the vast majority of all pancreas transplants, with pancreas after kidney (PAK) being the second most common and pancreas transplant alone (PTA) being the least common. (3, 4) Although T1DM remains the most important indication for pancreas transplantation, there have been a growing number of transplants done in T2DM patients. (5) Other, less frequent indications include transplantation for chronic pancreatitis and after pancreatomy due to malignancy (6).

A common but important complication in pancreas transplantation is thrombosis, which has been reported to have an incidence from 4%–20% (7–9). Thrombosis has been reported as one of the leading causes of pancreas graft loss and technical failure. (10) Despite this, the role of prophylactic anticoagulation remains controversial, with some groups reporting favorable outcomes following anticoagulation and other groups reporting no benefit. (11, 12) There remains no standardized thromboprophylaxis protocol in pancreas transplantation, and transplant centers have different internal protocols and practices. The purpose of this systematic review and meta-analysis is to evaluate the available literature to explore the impact of thromboprophylaxis on the incidence of thrombosis, graft loss, bleeding, and secondary outcomes in SPK, PAK, and PTA.

METHODS

Data Sources and Search Strategy

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guideline was followed to construct this review. We searched the following six databases: BIOSIS®, PubMed®, Cochrane Library®, EMBASE®, MEDLINE®, and Web of Science®. The search strategy is provided in **Supplementary Appendix SA1** (Supplemental Digital Content). The search end date was April 21st, 2021. This systematic review and meta-analysis was registered on PROSPERO (CRD42021260585) and may be accessed at: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=260585.

Inclusion and Exclusion Criteria

The objective of this review was to assess the outcomes related to prophylactic heparin in SPK, PAK and PTA. We therefore included prospective and retrospective studies written in English and published in peer-reviewed journals in this study. Studies that explored heparin thromboprophylaxis in the intraoperative and/or post-operative period after SPK, PAK or PTA where included. Studies that reported outcomes including incidence of pancreas thrombosis in the early post-transplant period, pancreas graft loss, bleeding episodes, acute return to the operating room (OR), and units of packed red blood cells (pRBC)

TABLE 1 | Characteristics of included studies.

Author, year	Study design	Transplant type	Intervention	Intervention regimen	Intervention group (N =)	Control group (N =)	Intervention group: Mean age (SD)	Control group: Mean age (SD)	Immuno-suppression	MINORS score (%)	Notes
Aboalsamh, 2016	Retrospective	SPK	IV Heparin	IV postoperative heparin 500 IU/h decreased by 100 IH/day over 5 days, then ASA 81 mg/day	29	33	44.0 (1.8)	41.5 (1.5)	ATG, tacrolimus, MMF, methylprednisolone, prednisone	75.0	
Arjona-Sanchez, 2017	Retrospective	SPK	IV Heparin	IV intraoperative sodium heparin, followed by postoperative LMW heparin 40 mg/day for 3 days, then ASA 100 mg/day	127	51	39.1 (7.5)	37.9 (6.4)	NR	75.0	
Fertmann, 2006	Retrospective	SPK	IV Heparin	IV postoperative heparin 2–4 IU/kg	29	—	42.9 (1.6)	—	ATG/ALG, tacrolimus, MMF, methylprednisolone, rapamycin	75.0	Only the cohort treated with heparin alone was used for analysis
Fertmann, 2011	Retrospective	PAK + PTA	IV Heparin	IV postoperative heparin 2–4 IU/kg according to Hb, partial thromboplastin time	13	—	35.7 (2.2)	—	ATG/ALG, tacrolimus, MMF, cyclosporine, methylprednisolone, "steroids"	75.0	Only the cohort treated with heparin alone was used for analysis
Humar, 2001	Retrospective	SPK	IV Heparin	IV postoperative heparin 300–500 U/h for 5 days, then ASA for 3 months	193	—	37.3 (NR)	—	ALG/daclizumab, tacrolimus, MMF, prednisone	75.0	
		PAK	IV Heparin	IV postoperative heparin 300–500 U/h for 5 days, then ASA for 3 months	205	—	40.2 (NR)	—	ALG/daclizumab, tacrolimus, MMF, prednisone		
Kim, 2012	Retrospective	SPK PAK PTA	SC Heparin SC Heparin SC Heparin	IV intraoperative heparin 70 U/kg, followed by SC heparin 3,000–5000 U with intention to aPTT prolongation, followed by ASA during admission	67 10 42	— — —	— — —	— — —	— — —	75.0	
Raveh, 2019	Retrospective	SPK + PAK + PTA	IV Heparin	IV intraoperative heparin infusion targeting an aPTT of 45–50s	32	10	—	—	ATG, basiliximab, methylprednisolone	79.2	
Scheffert, 2014	Retrospective	SPK + PAK + PTA	IV Heparin	IV postoperative heparin 200–400 U/h for 33–68h, followed by ASA 300 mg *per*	52	100	41 (7.5)	40.0 (8.0)	ALG/IL-2 agonist, tacrolimus, MMF, cyclosporine, prednisone	79.2	90% of patients were given heparin within the first 24 h postoperative period. The median (IQR)

(Continued on following page)

TABLE 1 | (*Continued*) Characteristics of included studies.

Author, year	Study design	Transplant type	Intervention	Intervention regimen	Intervention group (N =)	Control group (N =)	Intervention group: Mean age (SD)	Control group: Mean age (SD)	Immuno-suppression	MINORS score (%)	Notes
				rectum/day, then oral ASA 325 mg/day							heparin dose was 300 (200–400) IU/h, or 5 (3.4–6) IU/kg/h, without titrating to a goal activated partial thromboplastin time. The median (IQR) duration was 48 (33–69) hours
Schenker, 2009	Retrospective	SPK + PAK + PTA	SC or IV Heparin	Either postoperative SC LMW heparin 3,000–3800 IU/day or postoperative IV heparin 400–600 U/h for 9 ± 4.9 days, followed by SC LMW heparin	188	—	41.6 (8.2)	—	ATG, tacrolimus, MMF, prednisolone, sirolimus	75.0	
Shin, 2014	Retrospective	SPK + PAK + PTA	SC or IV Heparin	Either SC postoperative heparin 3,000–5000 U every 8 h or IV postoperative heparin 400–1000 U/h, followed by oral warfarin for 3 months	135	—	37.0 (9.0)	—	ATG, basiliximab, tacrolimus, MMF, prednisolone, "steroids	75.0	
Stratta, 2014	Retrospective	PTA	IV heparin	IV intraoperative heparin 2000–3000 U (30–50 U/kg), followed by IV postoperative heparin 300U/h for 1 day, then 400U/h for 1 day, then 500U/h until post-op day 5	40	—	42.2 (8.7)	—	ATG, tacrolimus, MMF, "corticosteroids"	75.0	The SPK group was excluded from analysis because only 37 out of 162 SPK patients received heparin

ALG, anti-lymphocyte globulin; ASA, acetylsalicylic acid; ATG, anti-thymocyte globulin; IV, intravenous; MMF, mycophenolate mofetil; NR, not reported; SC, subcutaneous.

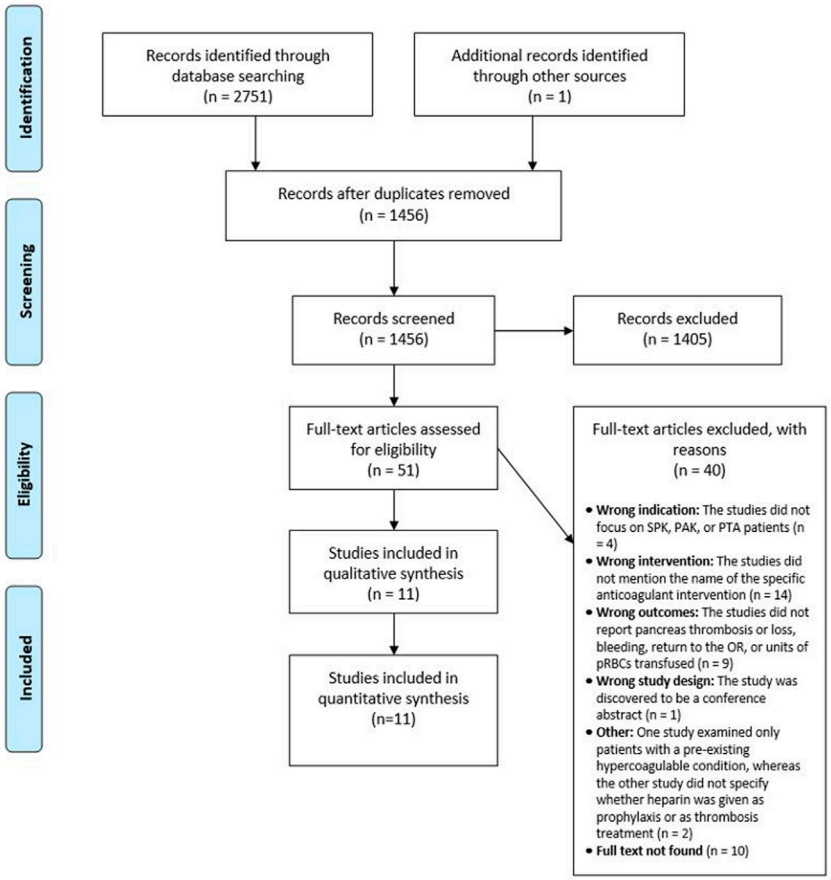


FIGURE 1 | PRISMA Flow diagram.

transfused were included. Given that the inclusion criteria involved heparin as the intervention and thrombosis as the primary outcome, all the included papers reported thromboses that were relevant to the intervention. Early pancreas thrombosis was defined as mention of "early" thrombosis or thrombosis that occurred within 30 days post-transplant. Reviews, editorials, case-reports, conference proceedings, and animal studies were excluded. Studies involving other types of solid organ transplants, where they focused on an intervention other than prophylactic anticoagulation, or where anticoagulation was used to treat diagnosed thrombotic events, were also excluded.

Study Selection

Studies underwent screening of study titles and abstracts by two reviewers (E.A.L and K.F.) using Rayyan (Rayyan Systems, Boston, United States). The included studies then underwent full-text screening by the same two reviewers. Screening conflicts were reviewed and resolved during meetings with all the reviewers.

Data Extraction and Quality Assessment

The following data were extracted from the selected articles: title, author(s), journal, study type, transplant type(s), number of patients, mean age, mean BMI, and sex proportion. Recipient specific factors included: mean time of diabetes diagnosis, and mean time on dialysis. Operative factors included: type of anticoagulation, timing of administration (intra/postoperative), dose, and frequency. Donor specific factors included: warm and cold ischemia time. Outcomes included: pancreas thrombosis in the early post-transplant period, pancreas graft loss due to thrombosis, postoperative bleeding incidence, acute return to the operating room, and units of pRBC used. Pancreas thrombosis was defined as any instance where "pancreas thrombosis" was mentioned. Pancreas loss was defined as mention of "pancreas loss" or "pancreatectomy." Bleeding was defined as mention of "bleed," "hemorrhage," or "hematoma". Acute return to the OR was defined as mention of "re-exploration," "relaparotomy," or "thrombectomy." Data were extracted by two reviewers (E.A.L and K.F) and verified for accuracy and completeness by a different reviewer (M.Y.Z). The Methodological Index for Non-Randomized Studies (MINORS) was used to assess risk of bias of manuscripts (**Table 1**). (13) The MINORS score examines 12 methodological parameters for non-randomized studies and an additional four parameters for comparative studies.

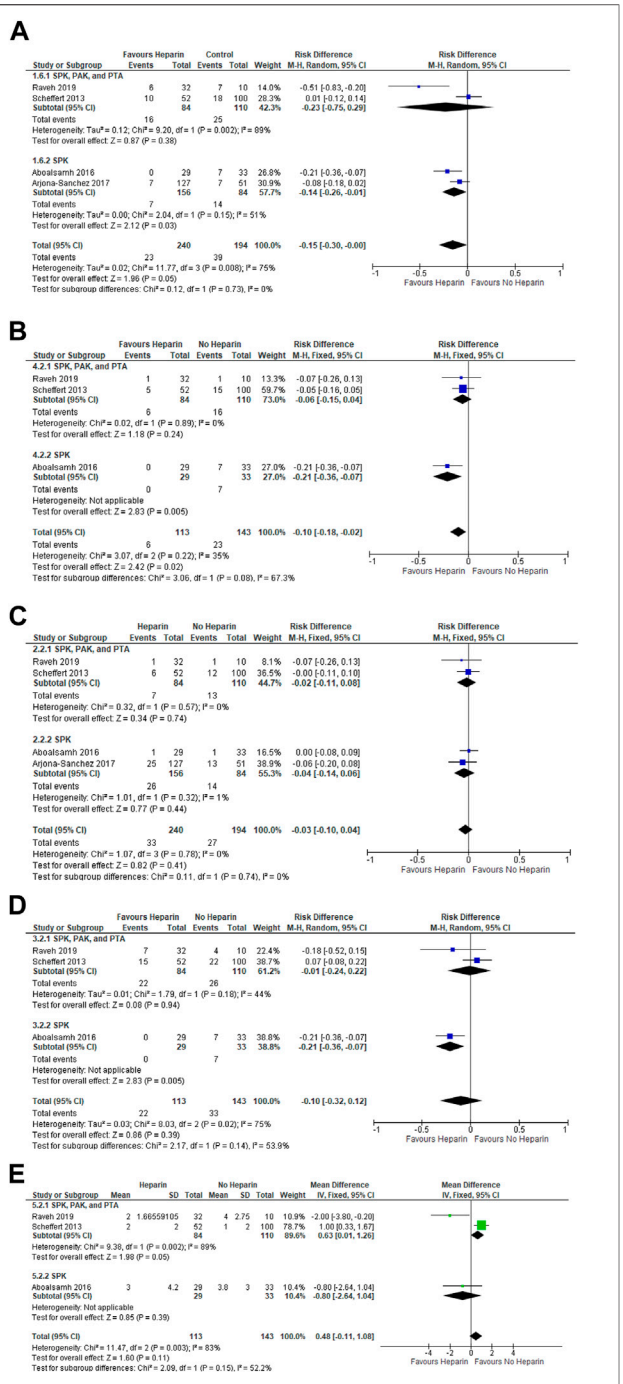


FIGURE 2 | Forest plots examining the impact of heparin on the **(A)** incidence of pancreas thrombosis, **(B)** incidence of pancreas loss due to thrombosis, **(C)** incidence of postoperative bleeding, **(D)** incidence of acute return to the OR, **(E)** mean units of pRBCs transfused.

Studies that scored 60% or higher were considered high quality and included.

Statistical Analysis

Two methods of data synthesis were performed to account for differences in study methodology. First, we conducted meta-analysis of the comparative studies involving a no-heparin control using Review Manager (RevMan, Version 5.4, The Nordic142 Cochrane Center, Copenhagen, Denmark). Study heterogeneity was examined using the I^2 statistic. An $I^2 < 50\%$ suggested low study heterogeneity and a fixed-effect model was used, whereas an $I^2 > 50\%$ suggested high study heterogeneity and a random-effects model was used. Results were visualized as forest plots. Publication bias was assessed using funnel plots for each outcome.

Second, we pooled the populations from the comparative and non-comparative studies to allow for comparison of demographic, intraoperative, and postoperative characteristics between the heparin and no-heparin groups. Statistical analysis was performed using R (Version 4.1.2., Boston, United States) and GraphPad QuickCalcs (GraphPad Software, Inc., California, United States). Two-tailed Fisher's exact tests were conducted for the categorical variables and results were reported as incidence (percentage of total). Unpaired t-tests were conducted for the continuous variables and results were reported as mean (SD). Cohen's kappa coefficient was obtained to assess inter-rater reliability. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Search Results

The study inclusion process is summarized in the PRISMA flow diagram (**Figure 1**). After removal of duplicates, 1,456 studies underwent Level 1 abstract and title screening, of which 51 studies were eligible for Level 2 full-text screening. Eleven studies were ultimately included for quantitative synthesis (11, 12–22). All 11 studies were retrospective. Level 1 screening had moderate agreement with a Cohen's kappa of 0.56 and Level 2 screening had substantial agreement with a Cohen's kappa of 0.67.

Study Characteristics

Characteristics of the included studies are summarized in **Table 1**. These included studies comprised of a total of 1,358 patients, with 1,122 patients in the intervention group and 236 patients in the control group. Mean recipient age was 40.4 (7.85) for the heparin group and 39.7 (6.90) for the control group, with no significant difference detected ($p = 0.96$). There were three studies involving only SPK transplant patients, (11, 12, 17) one study with both PAK and PTA patients; (16) one study with both SPK and PAK patients; (22) and five studies combining SPK, PAK, and PAK patients; (14, 18–21). There was also one study involving both SPK and PTA patients. However, only the PTA data was suitable for quantitative synthesis (**Table 1**) (15).

Study Quality and Publication Bias

Methodological quality of the included studies is summarized in **Table 1** and in **Supplementary Appendix SA2** (see Supplemental Digital Content). All studies lacked prospective data collection and prospective calculation of the study size. However, all studies possessed a clearly stated aim, with appropriate study endpoints. There were five comparative studies involving a no-heparin

control, and these studies had baseline equivalence between groups and adequate statistical analysis.

Funnel plots were assessed for publication bias, with one funnel plot for each outcome of interest. We did not observe any overt asymmetry or pattern in the funnel plots for the incidence of thrombosis, graft loss, bleeding, acute return to the OR, or mean units of pRBCs transfused (see Supplemental Digital Content, **Supplementary Appendix SA3**).

Analysis of Comparative Studies

Heparin Reduced the Incidence of Pancreas Thrombosis

There were four studies available for analysis, comprising of 240 patients in the heparin treatment group and 194 patients in the control group. (11, 12, 18, 20) Overall, there was a significantly lower incidence of pancreas graft thrombosis in the heparin group compared to the no-heparin group, with a risk difference of −0.15 (95% CI = −0.30, −0.00; p = 0.05) (**Figure 2A**). Subgroup analysis revealed no significant difference in pancreas thrombosis between treatment and control groups when looking at the studies that mixed their SPK, PAK, and PTA patients together (Risk difference = −0.23; 95% CI = −0.75, 0.29; p = 0.38). However, there was a significantly lower incidence of early pancreas thrombosis in the heparin group compared to the control group when looking at the studies that only included SPK patients (Risk difference = −0.14; 95% CI = −0.26, −0.01; p = 0.03). In fact, the total incidence of pancreas thrombosis in the heparin group was less than half of that in the no-heparin group.

Heparin Reduced the Incidence of Pancreas Loss

Three studies were available for analysis, consisting of 113 patients in the heparin group and 143 patients in the no-heparin control group. (12, 18, 20) When assessing the overall effect, there was a significantly lower incidence of pancreas loss due to graft thrombosis in the heparin group compared to the control group (Risk difference = −0.10; 95% CI = −0.18, −0.02; p = 0.02) (**Figure 2B**). When examining the subgroup consisting of SPK, PAK, and PTA patients, there was no significant difference between groups in incidence of pancreas loss (Risk difference = −0.06; 95% CI = −0.15, 0.04; p = 0.24). For the SPK subgroup, there was a significantly lower incidence of pancreas loss in the heparin group (Risk difference = −0.21; 95% CI = −0.36, −0.07; p = 0.005).

Heparin did Not Impact Postoperative Incidence of Bleeds, Incidence of Acute Return to the OR, or Units of pRBC Used

Four studies were available for analysis of the incidence of bleeding, consisting of 240 patients in the heparin group and 194 patients in the no-heparin control group. (11, 12, 18, 20) Overall, there was no significant difference in the incidence of bleeding between groups (Risk difference = −0.03; 95% CI = −0.10, 0.04; p = 0.41) (**Figure 2C**). Subgroup analysis also revealed no differences in the incidence of bleeds in neither the combined transplant subgroup (Risk difference = −0.02; 95% CI = −0.11, 0.08; p = 0.74), nor the SPK subgroup (Risk difference = −0.04; 95% CI = −0.14, 0.06; p = 0.44).

Three studies were available for analysis of the incidence of acute return to the OR and the mean number of packed RBCs used. (12, 18, 20) There were 113 patients in the heparin group and 143 patients in the control group (**Figures 2D,E**). Overall, there was no significant difference in the incidence of acute return to the OR between the heparin and no-heparin groups (Risk difference = −0.10; 95% CI = −0.32, 0.12; p = 0.39). There were no significant differences when examining the combined SPK, PAK, PTA subgroup (Risk difference = −0.01; 95% CI = −0.24, 0.22; p = 0.90). There was a significantly lower risk of acute return to the OR in the SPK subgroup (Risk difference = −0.21; 95% CI = −0.36, −0.07; p = 0.005).

Furthermore, there was overall no significant difference in units of pRBCs used between the heparin and no-heparin groups (Mean difference = 0.48; 95% CI = -0.11,1.08; p = 0.11). Subgroup analysis also revealed no significant differences in the combined SPK, PAK, PTA subgroup (Mean difference = 0.63; 95% CI = 0.01, 1.26; p = 0.05) and the SPK subgroup (Mean difference = −0.80; 95% CI = −2.64, 1.04; p = 0.39).

Analysis of Both Comparative and Non-Comparative Studies

SPK Only

There were studies that met inclusion criteria that lacked a no-heparin control. To incorporate these studies, we grouped their patient cohorts with the heparin cohorts from the comparative studies. The no-heparin group derived from the comparative studies was used as the control. When examining the SPK subgroup, there were five studies available for analysis, (11, 12, 17, 19, 22) with two of these studies being comparative studies. (11, 12) For the study by Fertmann et. al. 2006, only the cohort treated with heparin alone was used for analysis (**Table 1**). Donor mean age, donor mean BMI, mean cold ischemia time, and mean warm ischemia time were significantly higher in the heparin group compared to the control group (**Table 2**). The rate of thrombosis and pancreas loss due to thrombosis were significantly lower in the heparin group compared to the no-heparin control group. There was no significant difference in the incidence of bleeding, acute return to the OR, or units of pRBCs transfused between groups.

Analysis of all Included Studies: SPK, PAK and PTA

Eleven studies were available for analysis (11, 12, 14–22), of which four were comparative studies. (11, 12, 18, 20) For the study by Fertmann et. al. 2011, only the cohort treated with heparin alone was used for analysis (**Table 1**). Both Fertmann studies were included because two different patient cohorts were used for each study. Fertmann 2006 included only SPK patients, whereas Fertmann 2011 included only PAK and PTA patients. The combined cohort consisted of SPK, PAK, and PTA patients. There were significantly more male patients in the no-heparin group compared to the heparin group (**Table 3**). The rate of thrombosis and pancreas loss due to thrombosis were significantly

TABLE 2 | Analysis of SPK studies.

	Total N	Heparin		Total N	No Heparin		*p*
		Mean (SD) or incidence (%)			Mean (SD) or incidence (%)		
Demographic Characteristics							
Recipient Mean Age	209	40.9	(6.33)	84	39.3	(5.32)	0.045
Recipient Mean BMI	156	23.0	(2.62)	84	23.3	(2.18)	0.32
Recipient Incidence Male	349	231 (66.2%)	(9.00)	84	57 (67.9%)	(7.75)	0.80
Mean Time Diabetes (Years)[a]	127	24.0	(6.00)	51	24.0	(4.8)	1.00
Mean Time Dialysis (Months)[a]	29	33.2		33	33.9		0.61
Donor Mean Age[a]	82	32.6	(2.24)	33	31.1	(2.20)	0.0015
Donor Mean BMI[a]	29	25.9	(0.90)	33	24.6	(0.60)	<0.001
Donor Proportion Male[a]	222	134 (60.4%)		33	16 (48.5%)		0.26
Intraoperative Characteristics							
Mean Cold Ischemia Time (h)	156	10.8	(2.72)	84	9.4	(2.49)	<0.001
Mean Warm Ischemia Time (h)[a]	29	0.5	(0.03)	33	0.4	(0.00)	<0.001
Postoperative Complications							
Thrombosis	469	43 (9.2%)		83	14 (16.9%)		0.048
Pancreas loss due to thrombosis[a]	96	1 (1.0%)		33	7 (21.2%)		0.0003
Bleeding	349	92 (26.4%)		84	14 (16.7%)		0.07
Return to the OR[a]	222	50 (22.5%)		33	14 (16.9%)		1.00
Units of pRBC[a]	29	3.0	(4.20)	33	3.8	(3.00)	0.39

[a]*Only one study available.*

TABLE 3 | Analysis of all included studies.

	Total N	Heparin		Total N	No Heparin		*p*
		Mean (SD) or incidence (%)			Mean (SD) or incidence (%)		
Demographic Characteristics							
Recipient Mean Age	655	40.4	(7.85)	184	39.7	(6.90)	0.96
Recipient Mean BMI	343	22.3	(2.77)	184	23.7	(2.67)	0.74
Recipient Incidence Male	860	531 (61.7%)	(8.6)	184	114 (62.0%)	(7.75)	1.00
Mean Time Diabetes (Years)	489	25.4	(24.75)	51	24.0	(4.80)	0.96
Mean Time Dialysis (Months)[a]	217	28.2	(7.85)	33	33.9		0.93
Donor Mean Age	497	30.6	(11.00)	133	25.0	(8.62)	0.80
Donor Mean BMI	444	22.8	(3.71)	133	24.9	(8.67)	0.80
Donor Proportion Male	802	453 (56.5%)		133	91 (68.4%)		0.010
Intraoperative Characteristics							
Mean Cold Ischemia Time (h)	436	11.9	(3.48)	184	11.2	(3.53)	0.91
Mean Warm Ischemia Time (h)	81	0.55	(0.18)	133	0.5	(0.14)	0.73
Postoperative Complications							
Thrombosis	1,204	163 (13.5%)		194	39 (20.1%)		0.0205
Pancreas loss due to thrombosis	586	43 (7.3%)		143	23 (16.1%)		0.0029
Bleeding	788	148 (18.8%)		194	27 (13.9%)		0.12
Return to the OR	661	142 (21.5%)		143	33 (23.1%)		0.66
Units of pRBC	144	2.5	(2.53)	143	1.9	(2.65)	0.044

[a]*Only one study available.*

lower in the heparin group. Furthermore, there was no significant difference in the incidence of bleeding or return to the OR but there was a significantly higher mean number of units of pRBC transfused in the heparin group compared to the no-heparin group.

Analysis of Contemporary Studies: SPK, PAK and PTA

Lastly, we explored the effect of heparin when including only contemporary works published within the past 10 years. The analysis of comparative studies conducted in the previous section involved only contemporary studies from 2013 and onward. When combining both the comparative and non-comparative studies, seven papers were available for analysis, which included studies from 2012 and onward (11, 12, 14–20). Four of these studies were comparative studies. Analysis of these contemporary works revealed similar trends to that of the analysis of all included studies (**Table 4**).

DISCUSSION

Graft thrombosis remains a leading cause of pancreas graft loss after SPK, PAK, and PTA. Despite this, the evidence for

TABLE 4 | Analysis of contemporary studies from the past 10 years.

	Total N	Heparin		Total N	No Heparin		*p*
		Mean (SD) or incidence (%)			Mean (SD) or incidence (%)		
Demographic Characteristics							
Recipient Mean Age	502	37.9	(8.92)	(8.92)	39.7	(6.90)	0.91
Recipient Mean BMI	343	22.3	(2.77)	(2.77)	23.7	(2.67)	0.74
Recipient Incidence Male	502	287 (57.2%)	(9.2)	(9.2)	114 (62.0%)	(7.75)	0.29
Mean Time Diabetes (Years)	421	20.5	(6.0)	(6.0)	24.0	(4.80)	0.90
Mean Time Dialysis (Months)[a]	29	33.2	(8.92)	(8.92)	33.9		0.61
Donor Mean Age	375	28.5	(10.52)	133	25.0	(8.62)	0.85
Donor Mean BMI	375	22.1	(3.87)	133	24.9	(8.67)	0.74
Donor Proportion Male	335	184 (54.9%)		133	91 (68.4%)		0.0091
Intraoperative Characteristics							
Mean Cold Ischemia Time (h)	367	10.3	(4.19)	184	11.2	(3.53)	0.89
Mean Warm Ischemia Time (h)	81	0.55	(0.18)	133	0.5	(0.14)	0.83
Postoperative Complications							
Thrombosis	534	85 (15.9%)		194	39 (20.1%)		0.18
Pancreas loss due to thrombosis	367	10 (2.7%)		143	23 (16.1%)		<0.0001
Bleeding	359	54 (15.0%)		194	27 (13.9%)		0.80
Return to the OR	232	47 (20.3%)		143	33 (23.1%)		0.52
Units of pRBC	113	2.3	(2.68)	143	1.9	(2.65)	0.23

[a]*Only one study available.*

heparin thromboprophylaxis is mixed and there is great variability in the regimens used. We systematically reviewed the available literature to investigate the effect of prophylactic heparin on pancreas graft thrombosis and loss, as well as on other postoperative complications. We conducted two methods of quantitative synthesis: the first involving analysis of the included comparative studies, and the second involving analysis of both comparative and non-comparative studies. The first method of analysis revealed that heparin significantly reduced the overall incidence of early pancreas thrombosis and pancreas loss, without impacting the incidence of acute return to the OR, bleeding, or units of pRBC transfused. The second method of analysis revealed similar findings for SPK, PAK, and PTA, with an increase in the mean units of pRBCs transfused in the heparin group compared to the no-heparin group.

Analysis of comparative studies revealed greater overall effects and lower study heterogeneity in the SPK subgroup compared to the combined SPK, PAK, and PTA subgroup. This was evident for the pancreas thrombosis outcome. Previous studies report differential rates of pancreas thrombosis and pancreas loss for SPK, PAK and PTA, (16, 23) which when combined as a subgroup, may offset any true trends. Even so, we found that the overall incidence of pancreas thrombosis was over two times lower in the heparin group, while the overall incidence of pancreas loss was over three times lower in the heparin group. Because SPK requires greater time and technical involvement, the impact of heparin may be especially evident in this subgroup. With pancreas thrombosis being a common cause of pancreas loss, (8) heparin has an appreciable effect on improving graft survival, particularly after SPK transplantation.

Analysis of both comparative and non-comparative SPK studies showed a significantly higher mean donor age, mean donor BMI, mean cold ischemia time, and mean warm ischemia time in the heparin group. These variables have been previously shown to be associated with graft thrombosis. (24) Heparin remained effective in reducing the incidence of thrombosis and pancreas loss despite this group possessing factors associated with pancreas thrombosis. For this review, caution is warranted when interpreting these demographic differences because for three variables, there was only one study in the no-heparin group. The observed statistical significance may then be a by-product of the heterogeneity in the reporting of demographic data between the intervention group and control group. Furthermore, some pancreas transplant centers have avoided the use of systemic anticoagulation to prevent bleeding complications. (25, 26) However, a number of transplant groups note that graft thrombosis and loss are more detrimental than bleeds that can be controlled by transfusion or laparotomy. (27, 28) Importantly, our analysis shows that in SPK transplants, the beneficial effects of heparin did not increase the risk of acute bleeding requiring laparotomy or use of blood transfusions.

When examining all the included comparative and non-comparative studies, we confirm that heparin is associated with a significant reduction in thrombosis and pancreas loss for SPK, PAK, and PTA. In this combined analysis, patients treated with heparin were transfused with a mean of 0.62 units of additional pRBC compared to the control—which although is statistically significant—is not clinically significant. We may attribute this statistical difference to the large sample sizes and relatively small standard deviations for both groups. By incorporating all the included studies, we also balance the demographic differences between the heparin group and no-heparin group, without observing any changes in the efficacy of heparin in mitigating early post-transplant thrombosis and pancreas loss.

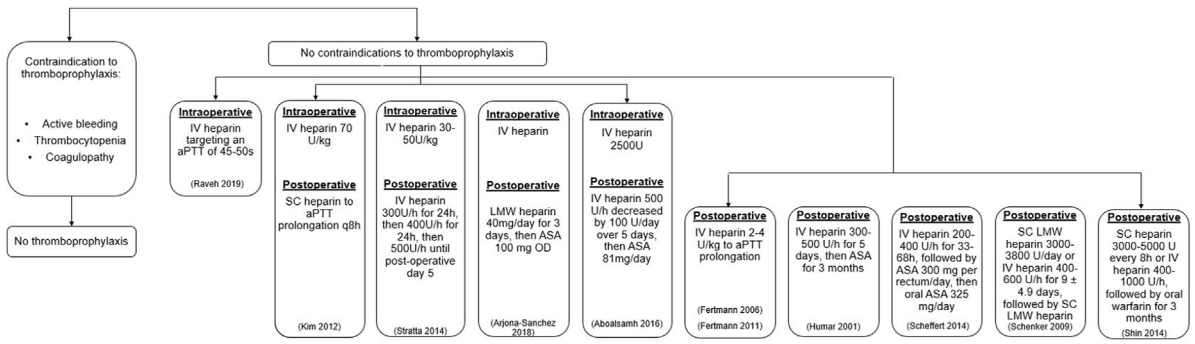


FIGURE 3 | Flow diagram of the prophylactic heparin regimens presented in this review. ASA, acetylsalicylic acid; IU, international unit; IV, intravenous; SC, subcutaneous.

This analysis is limited by the lack of prospective, randomized controlled studies, as well as by the inclusion of only English studies. The available literature presents with higher degrees of confounding and greater variability in reporting, which may limit the conclusions drawn. A limitation with retrospective data is that we lack insight on the decision-making process behind which patients were heparinized. Even in comparative studies, there exists patient-specific factors—such as a history of a clotting disorder or a prolonged cold ischemia time—which may influence the decision for heparin thromboprophylaxis. (15) Changes in personnel, surgical technique, and postoperative management in institutions over time may also impact patient outcomes. Of the included studies, there is also great variability in the timing and dosage of heparin administered. This may be the result of evolving institutional practices and a lack of consistent evidence on prophylactic heparin usage. Additionally, there are technical factors that may influence the risk of thrombosis, such as the type of exocrine drainage. Well-established evidence indicates that bladder drainage may confer long-term urologic and metabolic complications, with contemporary practice utilizing enteric drainage, or occasionally portal-enteric drainage. (29) We controlled for these institutional and technical factors by analyzing only the included contemporary studies. Given that the impact of heparin on post-operative outcomes remained the same even with this contemporary analysis, the effects of heparin may be robust enough to withstand evolving institutional and surgical practices.

This review highlights the gaps in the literature, while providing a synthesis of the data that is available to us at this current time. To our knowledge, this is the first systematic review in existence that explores the impact of heparin thromboprophylaxis in SPK, PAK, and PTA. The pooled sample sizes for the heparin and no-heparin groups are sufficiently large such that the assumption of normal sampling distribution may be fulfilled by the Central Limit Theorem. (30) The large sample sizes help account for the probability of error from the above limitations. Future research that prospectively compares the impact of heparin to that of a no-heparin control is warranted. Given that all the comparative studies in this review were published within the last 10 years, this suggests an influx of higher quality studies exploring this subject in recent times. We foresee more high-quality studies will become published in the near future, which will warrant additional meta-analyses.

Based on the findings of this study, we present a flow diagram outlining the available treatment regimens (**Figure 3**). In the absence of contraindications to heparin thromboprophylaxis, intraoperative intravenous heparin 30–70 IU/kg was used. (15, 19) During the postoperative period, either subcutaneous heparin 3,000–5000 IU 1–2 times per day (14, 19, 21) or intravenous heparin infusion 200–1000 IU/h for 1–14 days (12, 14–17, 20–22) were reported. Subsequent maintenance with aspirin 81–325 mg daily was then used (11, 12, 20).

CONCLUSION

We demonstrate that prophylactic heparinization produces over a two-fold reduction in early pancreas thrombosis and pancreas loss for SPK, PAK and PTA, without increasing the incidence of bleeding or acute return to the OR. With early postoperative complications, such as pancreas graft thrombosis, persisting as a leading cause for graft loss, heparin thromboprophylaxis holds promise for enhancing graft survival without imposing additional postoperative complications. This meta-analysis culminates 2 decades of available evidence to highlight the efficacy of heparin thromboprophylaxis for improving graft survival for SPK, PAK, and PTA patients.

AUTHOR CONTRIBUTIONS

EL: Participated in research design, search strategy formation, literature screening, data extraction, statistical analysis, manuscript writing. KF: Participated in research design, search strategy formation, literature screening, data extraction, manuscript writing. MZ: Participated in research design, data extraction, manuscript writing. JO: Participated in research design, literature screening, PL: Participated in research design, manuscript writing. AS: Participated in research design, search strategy formation, literature screening, data extraction, statistical analysis, manuscript writing.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10442/full#supplementary-material

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ORIGINAL RESEARCH
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"Train the Trainers" Program to Improve Knowledge, Attitudes and Perceptions About Organ Donation in the European Union and Neighbouring Countries: Pre- and Post- Data Analysis of the EUDONORGAN Project

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EUDONORGAN, a European Union-funded project to improve organ and tissue donation, included a blended-based "Train the Trainers" program, which was implemented with the support of an international consortium from Croatia, Italy, Slovenia, and Spain. The web-based training included seven modules for which medical aspects, educational tips, and practical activities were scored using a 5-point Likert scale. The overall mean scores of satisfaction were higher than 4 for each module, without significant differences between HCPs and OKPs. In the face-to-face training survey similar scores above 4 were obtained for most items. Knowledge acquisition improved significantly in both HCPs and OKPs, as well as in transplant/donor coordinators, medical doctors, registered nurses, anesthesiologists/intensivists, and intensive care nurses. Improvements in attitudes and perceptions regarding organ donation were also observed, particularly among HCPs. In the accomplishment of the learning process, a successful pass mark of 95% was obtained. The "Train the Trainers" program was associated with an improvement in learning and attitudes of healthcare and non-healthcare professionals for the benefit of organ and tissue donation.

Keywords: transplantation, organ donation, training, blended-learning, knowledge

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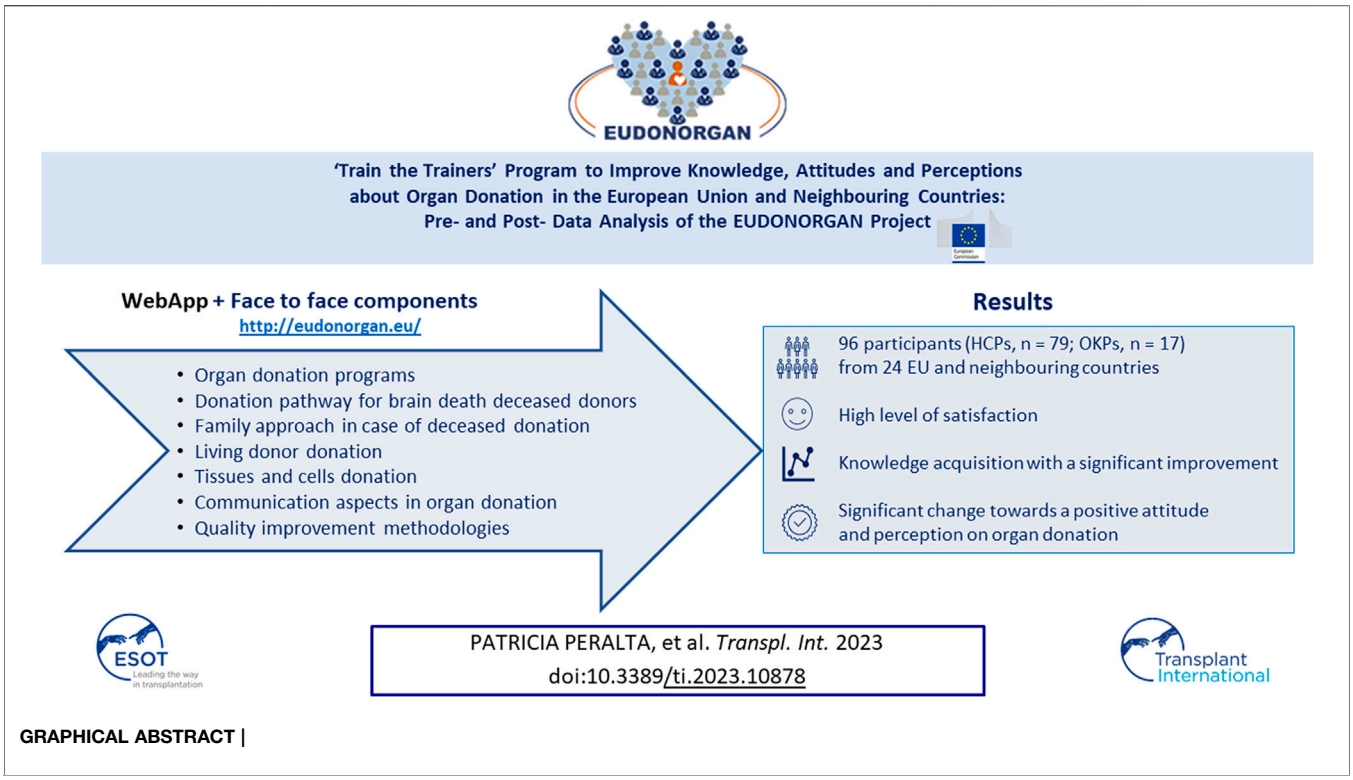
Abbreviations: EC, European Commission; EU, European Union; HCPs, healthcare professionals; ICU, intensive care unit; MD, medical doctor; OKPs, other key players; RN, registered nurse; SD, standard deviation.



GRAPHICAL ABSTRACT |

INTRODUCTION

Over the past 50 years, organ transplantation has become an established practice worldwide, bringing immense benefits to hundreds of thousands of patients with end-stage failure of organs for most of whom organ transplantation is the only available treatment (1). The shortage of organs, listed as a major priority, and the supply-demand gap are two limiting factors for organ procurement. In response to these major challenges, the European Commission (EC) issued a communication on organ donation and transplantation (2) that proposed the Action Plan on Organ Donation and Transplantation that complemented the organ specific legislation (3). After a first half-period of completion of the Action Plan, the EC undertook the ACTOR Study, which emphasized the importance of implementing educational activities and improving as there were many opportunities for countries to share experiences and to learn from each other (3). As the study indicated, several EU-funded projects were proposed with the aim of providing training, sharing of knowledge, implementation of programs, development of tools, and to identify the best organizational models (3). In a final assessment of the impact of the Action Plan, a final report (4) provided an overview of the efforts made showing the benefits of the EU-funded resulting in guidelines, trainings, and manuals to exchange knowledge and best practices among countries.

The EU-funded pilot project EUDONORGAN was a pioneer EU-funded project that contributed to the Action Plan as an initiative for increasing organ and tissue donation in the EU and neighbouring countries. To this purpose, two types of core activities focused on training and social awareness were developed and implemented at EU level. The "Train the Trainers" program was based on active learning and adult learning principles and employed a blended learning methodology by means of e-learning (*via* WebApp) and face-to-face training. The course was addressed to healthcare professionals (HCPs) and other relevant key players (OKPs). The objective of this study was to present the results of pre- and post-data analysis of the "Train the Trainers" activities.

MATERIALS AND METHODS

EUDONORGAN Project

EUDONORGAN project was a service contract awarded by the EC from the EU budget, on the initiative of the European Parliament. It was developed by an international consortium, made up of institutions from four European countries, --Croatia, Italy, Slovenia and Spain--, that provided similar organ donation models and successful transplantation rates. The consortium partners were the Institute for Transplantation and Biomedicine-Ministry of Health of Republic of Croatia (Croatia); the Italian National Transplant Centre-Italian National Institute of Health (Italy); the Institute of the Republic of Slovenia for the Transplantation of Organs and Tissues (Slovenia); and the University of Barcelona, Fundació Bosch i Gimpera, the Donation and Transplantation Institute (DTI), and

Dinamia, with the support of the Spanish National Transplant Organization (Spain).

The aim of the project was to contribute actively to increase organ donation rates in Europe focusing on two main actions: the implementation of a "Train the Trainers" program on organ and tissue donation, and organizing six social awareness events on organ donation with the support of the trained professionals. Both activities were oriented to HCPs and relevant OKPs, such as patients and patient support groups, representatives of public and governmental agencies, representatives of health institutions, opinion leaders, and the media. EUDONORGAN was launched in September 2016 and lasted 36 months, with the implementation of the "Train the Trainers" program in 2017, and the social awareness events between 2018 and 2019.

The whole timeframe of the project was proposed to be implemented considering the policies established for EU Member States in the field of transplantation and it required to consult and involve the Competent Authorities to establish a European network, following the indications of the Directive 2010/53/EU (1).

Educational Methodology

Training Design, Contents and Participants

The objective of the "Train the Trainers" program was to assist and provided HCPs and relevant OKPs with knowledge, educational strategies and communication techniques to monitor and improve overall performance in the management of donated and transplanted organs. The training included the implementation of a curriculum to support capacity-building efforts and train professionals who will, in turn, be able to conduct future training actions. The design of the program started by establishing a training methodology, the educational contents, and the selection of participants according to the criteria agreed upon by the consortium partners.

The methodology followed analysis of trends in education and literature research to ensure effective educational strategies to engage participants through the "Train the Trainers" program. Based on blended-learning methods that share the common element of engaging participants in doing things and thinking about what they are doing (5), the training offered the advantages of both online (WebApp) and face-to-face components in terms of flexibility of time and place (6,7), accessibility to the best of the educational elements (6), and autonomy with a gradual development of independent learning (7). From a competence-based perspective, blended-learning methods allowed participants to further fine-tune their skills and capabilities, which optimize direct application of experience and knowledge in their own professional environment (8) and promote efficiency, motivation, cognitive effectiveness, and flexibility of learning style (9).

The WebApp (http://eudonorgan.eu) provided a learner-centered platform. Educational modules on organ donation, educational tips and quizzes were delivered through microcapsules of curated content (microlearning) with fine-grained and inter-connected learning activity (10). The storytelling was the narrative learning method used to create a link between lived experience and curricular content (11). Specifically, it showed a family of characters and scenarios through a wide range of game elements in a gradual, entertaining and easy to understand way to keep participants interested and motivated (12).

The face-to-face component employed learning strategies: process mapping exercises, case studies, buzz sessions, collaborative activities and on-ground simulations, that boosted hands-on learning, networking and promoted great interactivity. The methodology followed six adult learning principles (13–16) adapted to the training. This included self-directed experiences; performance-based training to establish a relation between participants' previous knowledge and their training expectations; experiential learning; critical thinking; learning based on real-world situations; and value learning to further apply the acquired competencies when organizing future training actions on organ donation.

The educational contents were proposed in compliance with the EU legislation (1,17). According to the high-quality standards required (18), these contents should ensure that healthcare personnel directly involved in the chain from donation to transplantation or disposal are suitably qualified or trained and competent, and shall develop specific training programs for such personnel (1) and, consequently, needed to cover the most relevant information on organ and tissue donation. Seven educational modules were designed and adapted to each group of HCPs and OKPs, with the support of international experts, and finally agreed by the members of the consortium. The which included the following content: organ donation programs, donation pathway for brain death deceased donors, family approach in case of deceased donation, living donor donation, tissues and cells donation, communication aspects in organ donation, and quality improvement methodologies. The topics and learning objectives of these modules are described in the **Supplementary Table S1**.

International experts and participants selection was performed in parallel with the design of the training methodology. Participants from EU Member States and neighbouring countries were invited to join in the training program. The selection of participants followed the recommended criteria agreed by the competent authorities described in the **Supplementary Table S2**. The objective was to create a heterogeneous pool of trained and dedicated professionals on organ donation that will continue improving in the working environment. Participants were trained on how to best identify donors, how to best organize donation activities (taken into account national specificities) and how to pass on the main positive aspects of donation within the hospitals and to the rest of society (18). The criteria for the selection of HCPs included professionals that were able to demonstrate medical expertise in the field of organ/tissue and cell donation and transplantation. Eligible candidates could be medical doctors (MD) and registered nurses (RN) with different specialties, such as transplant/donor coordinators, anesthesiologists, intensivists, nephrologists, internal medicine physicians, general nurses, or intensive care nurses. The selection of OKPs was focused on actors with proven capacity and motivation to learn and to transfer the knowledge acquired in organ and tissue donation and transplantation *via* the

training course, such as active members of patient support groups, communication officers of national and regional authorities, journalists in the field of care, healthcare establishments, and key opinion leaders.

Training Implementation

The "Train the Trainers" program started in June 2017 with a series of informative webinars to get all participants familiar with the main topics of the program, the training objectives, and the characteristics of the methodology. Before beginning the training, participants were requested to complete an 18-item test of knowledge and a survey on attitudes and perceptions towards organ and tissue donation. The content of knowledge questionnaires was based on information included in the educational modules. Knowledge questionnaires were different for HCPs and OKPs, whereas the survey on attitudes and perceptions remained the same. Once completed the questionnaires, participants were ready to access to the training program. They were direct responsible for pacing their own self-learning.

The program continued with face-to-face sessions. A total of 9 guests and 11 international experts from six EU countries (Croatia, France, Italy, Slovenia, Spain, and Netherlands) joined the on-site training. The on-site sessions were designed to put into practice the knowledge acquired previously during the online part and to facilitate the switch from the theoretical knowledge to hands-on practice. A learning culture was created with in-class time dedicated to exploring organ and tissue donation topics in greater depth and creating enriching experiences. Apart from the educational contents, an educational kit was provided to participants with essential knowledge on adult learning in medical education and tips on teaching methodologies and strategies.

The training course finished in September 2017. Certificates of achievement were issued and delivered to participants who had completed the program successfully.

Evaluation

Continuous evaluation of the participant's performance was carried out to allow assessing the extent to which the objectives were achieved. The Kirkpatrick impact evaluation model (19) was proposed to measure the educational intervention. The evaluation framework outlined by this author defined four levels of evaluation based on outcomes of satisfaction, learning, change in learner behaviors (20), and organizational change/patient outcome (9). In EUDONORGAN project, this evaluation model was partially used adapted to the design the tailored "Train the Trainers" program and only satisfaction and learning levels based on knowledge, attitudes and perceptions were considered.

The satisfaction level referred to the degree to which learners find the training favorable, engaging, and scientifically relevant (19). After completion of the training, the overall satisfaction of the program was evaluated. For the web-based training, three categories for each educational model, including medical aspects, educational tips, and practical activities were assessed using a 5-point Likert scale (1 = poor, 2 = fair, 3 = good, 4 = very good, 5 = excellent), with a final score as the sum of the scores corresponding to the three categories. For the face-to-face training, 18 items related to different aspects of training methodology and experience, quality of workshops and presentation, specific debates, practical exercises, etc., were defined, and three categories --contents, presentation, and questions and answers--, were assessed for 15 items, whereas other categories were defined for the remaining three items (organization, course information provided, and global evaluation). All items, however, were evaluated using a 5-point Likert scale (1 = poor, 5 = excellent).

The learning level contained three components (knowledge, attitudes and perception (10, 20).

In relation to knowledge acquisition, pre- and post-test tailored-made questions by HCPs and OKPs were designed by three experts. The pre-test multiple-choice questionnaire included 18 items related to the topics given in the seven educational modules, with four possible options, one of which was correct. Only one attempt was allowed. Each item was scored "1" if the answer was correct or "0" if it was incorrect. The post-test multiple-choice questionnaire included 39 items (18 of which were the same questions as those provided in the pre-test). Again, each item was scored "1" if the answer was correct or "0" if it was incorrect. The 18 items that were same in the pre- and post-test were used to assess differences in knowledge acquisition, whereas results of the post-test questionnaires of 39 items were used to establish the accomplishment of training, with a pass mark of 70% of correct responses. The pre-test and post-test multiple-choice questionnaires are reported in the **Supplementary Tables S3, S4**.

To measure participants' attitudes pre- and post-surveys were also designed regarding organ and tissue donation. These surveys included a total of seven questions, five of which with three different answer choices and the remaining 2, with different close-ended answers.

Finally, pre- and post-surveys measuring participants' perceptions of the process of donation after brain death consisted of a set of 20 terms (solidarity, stressful, organized, complicated, positive, painful, opportunity, awkward, correct, strange, dignified, mistrustful, respectful, barbaric, encouraging, dubious, clear, chaotic, easy and discreditable) that from their perspective best describe the process of donation after brain death), five of which should be chosen.

Statistical Analysis

Categorical data are expressed as frequencies and percentages, and continuous data as mean and standard deviation (SD). In the bivariate analysis, the chi-square test or the Fisher's exact test were used for the comparison of categorical variables, and the Student's t-test, the Wilcoxon signed-rank test or the Kruskal-Wallis test for the comparison of pre- and post-test quantitative data according to conditions of application. Data for HCPs and OKPs were also stratified by gender, age decades, profession, specialty, and position. Statistical significance was set at $p \leq 0.05$. All data was analyzed by using the Statistical Package for Social Sciences (SPSS), version 10.0 for Windows.

TABLE 1 | Demographic data and characteristics of healthcare professionals.

Variables	N (%)
Total participants	79 (100)
Gender	
Men	32 (40.5)
Women	47 (59.5)
Age, years, mean (SD)	40.1 (8.4)
Profession	
Medical doctor	49 (62.0)
Registered nurse	27 (34.2)
Medical student	2 (2.5)
Healthcare manager	1 (1.3)
Specialty	
Anesthesiology/intensive care	41 (51.1)
General nurse	20 (25.3)
Intensive care nurse	5 (6.3)
Transplant/donor coordinator	3 (3.8)
Nephrology	2 (2.5)
Internal medicine	2 (2.5)
Other	6 (3.8)
Position	
Transplant/donor coordinator	37 (46.8)
Anesthesiologist/intensive care	26 (32.9)
Medical doctor	3 (3.8)
Other	13 (16.5)
Participants per country	
6, France, Italy	12
5, Belgium, Poland	10
4, Estonia, Greece, Lithuania, Spain	16
3, Bosnia & Herzegovina, Bulgaria, Croatia, Cyprus, Hungary, Ireland, Latvia, Malta, Sweden	27
2, Finland, Netherlands, Portugal, Romania, Serbia, Slovenia	12
1, Turkey, Germany	2

Data expressed as frequencies and percentages in parenthesis unless otherwise stated.

TABLE 2 | Demographic data and characteristics of other relevant key players (non-healthcare professionals).

Variables	N (%)
Total participants	17 (100)
Gender	
Men	4 (23.5)
Women	13 (76.5)
Age, years, mean (SD)	40.8 (11.4)
Profession	
Patients' group representative	7 (41.1)
Communication expert	5 (29.4)
Journalist	3 (17.6)
Documentalist	1 (5.9)
Other	1 (5.9)
Participants per country	
2, Bulgaria, Ireland, Spain	6
1, Croatia, Cyprus, France, Hungary, Lithuania, Portugal, Romania, Serbia, Slovenia, Slovakia, Sweden	11

Data expressed as frequencies and percentages in parenthesis unless otherwise stated.

RESULTS

Participants

A total of 96 participants (HCPs, $n = 79$; OKPs, $n = 17$) from 24 EU and neighbouring countries completed the training program. In the group of HCPs, there were 32 men and 47 women, with a mean (SD) age of 40.1 (8.4) years, whereas in the group of OKPs, there were 4 men and 13 women, with a mean age of 40.8 (11.4) years. In the group of HCPs, 51.1% of participants were anesthesiologists or intensivists and 25.3% were RN. Thirty-seven (46.8%) were transplant/donor coordinators. In the group of OKPs, patients' group representatives accounted for 41.2% of participants followed by communication experts (29.4%). Profession-related characteristics and countries of origin of participants are shown in **Tables 1, 2**.

Satisfaction With the Program

For the web-based training considering medical aspects, educational tips, and practical activities of the seven modules, the overall mean (SD) scores of satisfaction were higher than 4 for each module, with 4.4 (0.6) for module 1, 4.5 (0.5) for module 2, 4.5 (0.5) for module 3, 4.5 (0.6) for module 4, 4.4 (0.6) for module 5, 4.4 (0.6) for module 6, and 4.3 (0.7) for module 7, without significant differences between HCPs and OKPs. In the group of HCPs (**Table 3**), women scored significantly higher than men in modules 3, 5, and 7, but significant differences by age, profession, specialty or position were not found. In the group of OKPs (**Table 4**), mean scores were also higher than 4 for all modules, but significant differences by gender, age, and profession were not observed.

Regarding the face-to-face training survey, data from HCPs and OKPs were gathered, with more than 80 participants who completed the survey in most of the items, and a highest response rate at 85 participants (88.5%). Results of the face-to-face training also showed high scores (above 4) for all items evaluated, except for communication workshop with scores above 3. In the global evaluation, mean (SD) scores of 4.4 (0.8) were obtained for both categories of "applicability to my job" and "overall course assessment" (**Table 5**).

Knowledge Acquisition

Knowledge acquisition after training showed a statistically significant improvement in both HCPs and OKPs, with mean (SD) percentages of correct responses increasing from 72% (13.4) to 96.2% (5.6) and from 64% (18.3) to 92.8% (7.3), respectively (**Table 6**). In the group of HCPs, improvement in knowledge acquisition was significant in all age categories, professions, and specialties. Pre- and post-test comparisons were particularly significant for RN vs. MD and intensive care unit nurses vs. general nurses and other specialties (**Table 6**). Transplant/donor coordinators showed a meaningful improvement (pre-test 71.5% [13.8] vs. post-test 96.7% [5.6], $p < 0.0001$) as well as anesthesiologists and intensivists. In the group of OKPs, statistically significant improvements in knowledge acquisition were observed in women, age segments 25–34 and 45–54 years, patients' group representatives and communication experts (**Table 6**). However, between-group differences either in pre-test or post-test results in HCPs or OKPs were not observed.

Finally, in the 39-item questionnaire to assess the accomplishment of the learning process, a successful pass mark of 95% was obtained.

TABLE 3 | Satisfaction with the web-based training program among 79 healthcare professionals.

Categories	Participants	Module 1	Module 2	Module 3	Module 4	Module 5	Module 6	Module 7
Gender								
Men	32	4.2 (0.6)	4.3 (0.6)	4.3 (0.6)	4.3 (0.6)	4.0 (0.7)	4.0 (0.7)	4.0 (0.7)
Women	47	4.5 (0.6)	4.6 (0.5)	4.6 (0.5)	4.6 (0.5)	4.6 (0.6)	4.4 (0.6)	4.5 (0.7)
p-value		0.098	0.102	**0.017**	0.071	**0.003**	0.221	**0.007**
Age, years								
25–34	16	4.4 (0.5)	4.6 (0.4)	4.5 (0.5)	4.6 (0.4)	4.5 (0.5)	4.4 (0.6)	4.4 (0.6)
35–44	37	4.3 (0.7)	4.5 (0.6)	4.5 (0.5)	4.5 (0.7)	4.4 (0.7)	4.4 (0.7)	4.3 (0.8)
45–54	20	4.5 (0.7)	4.5 (0.6)	4.6 (0.6)	4.5 (0.6)	4.5 (0.7)	4.3 (0.7)	4.4 (0.6)
55–64	6	4.3 (0.6)	4.5 (0.5)	4.6 (0.4)	4.6 (0.3)	4.3 (0.5)	4.4 (0.7)	4.2 (0.7)
p-value		0.882	0.258	1.083	0.668	1.324	0.177	0.464
Profession								
Medical doctor	49	4.4 (0.6)	4.5 (0.6)	4.6 (0.5)	4.5 (0.6)	4.5 (0.6)	4.3 (0.7)	4.4 (0.7)
Registered nurse	27	4.3 (0.3)	4.6 (0.6)	4.5 (0.6)	4.5 (0.6)	4.4 (0.7)	4.4 (0.6)	4.3 (0.7)
Medical student	2	4.3 (0.0)	4.7 (0.5)	4.3 (0.0)	4.5 (0.2)	4.3 (0.0)	4.0 (0.5)	4.3 (0.9)
Healthcare manager	1	5.0	5.0	5.0	5.0	5.0	5.0	5.0
p-value		0.846	0.644	0.887	0.97	0.672	0.561	0.726
Specialty								
Anesthesiology/intensive care	41	4.4 (0.6)	4.4 (0.6)	4.5 (0.5)	4.5 (0.6)	0.5 (0.7)	4.3 (0.7)	4.4 (0.7)
General nurse	20	4.2 (0.7)	4.5 (0.6)	4.5 (0.5)	4.4 (0.6)	4.3 (0.7)	4.4 (0.6)	4.2 (0.7)
Intensive care nurse	5	4.3 (0.8)	4.5 (0.7)	4.3 (0.7)	4.5 (0.7)	4.7 (0.5)	4.3 (0.9)	3.9 (1.6)
Transplant/donor coordinator	3	4.4 (0.7)	4.7 (0.6)	5.0 (0.0)	4.7 (0.6)	4.9 (0.2)	4.7 (0.6)	4.9 (0.2)
Nephrology	2	4.7 (0.5)	4.7 (0.5)	5.0	5.0	5.0	4.5 (0.7)	4.5 (0.7)
Internal Medicine	2	4.3 (0.9)	4.2 (1.2)	4.7 (0.5)	4.5 (0.7)	4.7 (0.5)	4.3 (0.9)	3.9 (1.6)
Other	6	4.3 (0.5)	4.5 (0.5)	4.5 (0.4)	4.5 (0.4)	4.5 (0.5)	4.3 (0.5)	4.3 (0.5)
p-value		0.898	0.937	0.483	0.885	0.498	0.989	0.726
Position								
Transplant/donor coordinator	37	4.5 (0.5)	4.6 (0.5)	4.6 (0.5)	4.6 (0.4)	4.5 (0.6)	4.4 (0.6)	4.4 (0.7)
Anesthesiologist/intensive care	26	4.2 (0.7)	4.4 (0.7)	4.0 (0.6)	4.4 (0.7)	4.4 (0.7)	4.2 (0.7)	4.2 (0.7)
Medical doctor	3	4.5 (0.7)	4.6 (0.6)	4.6 (0.5)	4.5 (0.7)	4.5 (0.7)	4.5 (0.7)	4.4 (0.7)
Other	13	4.2 (0.9)	4.6 (0.4)	4.3 (0.7)	4.6 (0.5)	4.4 (0.5)	4.1 (1.02)	4.8 (0.4)
p-value		0.49	0.401	0.447	0.651	0.97	0.756	0.491
Total	79	4.4 (0.6)	4.5 (0.6)	4.5 (0.5)	4.5 (0.5)	4.4 (0.6)	4.3 (0.6)	4.3 (0.7)

Data as mean and standard deviation in parenthesis. Values in bold mean statistical significance.

TABLE 4 | Satisfaction with the web-based training program among 17 other key players (non-healthcare professionals).

Categories	Participants	Module 1	Module 2	Module 3	Module 4	Module 5	Module 6	Module 7
Gender								
Men	4	4.3 (1.0)	4.6 (1.0)	4.3 (1.0)	4.3 (1.0)	4.3 (1.0)	4.3 (1.0)	4.3 (1.0)
Women	13	4.6 (0.4)	4.8 (0.3)	4.6 (1.0)	4.6 (0.5)	4.7 (0.3)	4.5 (1.0)	4.3 (0.6)
p-value		0.589	0.469	0.631	0.589	0.469	0.221	0.772
Age, years								
25–34	6	4.6 (0.3)	4.7 (0.3)	4.7 (0.7)	4.7 (0.4)	4.7 (0.3)	4.4 (0.7)	4.4 (0.7)
35–44	3	4.7 (0.6)	4.8 (0.4)	4.6 (0.5)	4.8 (0.4)	4.8 (0.4)	4.7 (0.3)	4.6 (0.5)
45–54	7	4.3 (0.7)	4.5 (0.8)	4.3 (0.7)	4.1 (0.8)	4.4 (0.7)	4.3 (0.7)	4.1 (0.8)
55–64	1	5.0	5.0	5.0	5.0	5.0	5.0	4.0
p-value		0.585	0.742	0.491	0.351	0.723	0.592	0.582
Profession								
Patients' group representative	7	4.6 (0.5)	4.6 (0.4)	4.4 (0.6)	4.6 (0.5)	4.6 (0.4)	4.5 (0.5)	4.3 (0.5)
Communication expert	5	4.3 (0.8)	4.5 (0.9)	4.4 (0.9)	4.2 (1.0)	4.5 (0.8)	4.3 (0.8)	3.9 (0.9)
Journalist	3	4.8 (0.4)	4.9 (0.2)	4.7 (0.6)	4.8 (0.4)	4.8 (0.4)	4.8 (0.4)	4.9 (0.2)
Documentalist	1	4.3	4.3	5.0	4.7	4.3	3.3	3.3
Other	1	5.0	5.0	4.7	5.0	5.0	4.6	4.7
p-value		0.609	0.55	0.847	0.765	0.7	0.486	0.207
Total	17	4.5 (0.5)	4.6 (0.5)	4.5 (0.6)	4.5 (0.6)	4.6 (0.5)	4.4 (0.6)	4.3 (0.7)

Data as mean and standard deviation in parenthesis.

TABLE 5 | Satisfaction with the face-to-face training program in all participants.

Items	Participants	Mean (SD)
1. Welcome session		
Contents	81	4.2 (0.9)
Presentation	81	4.2 (0.9)
Questions and answers	81	4.3 (0.9)
2. Project overview and training methodology		
Contents	82	4.4 (0.9)
Presentation	82	4.4 (0.9)
Questions and answers	82	4.4 (0.9)
3. Online training experience		
Contents	82	4.5 (0.8)
Presentation	82	4.6 (0.9)
Questions and answers	81	4.5 (0.9)
4. Living donation		
Contents	84	4.4 (0.9)
Presentation	83	4.4 (0.8)
Questions and answers	84	4.5 (0.8)
5. Deceased donation		
Contents	84	4.6 (0.8)
Presentation	83	4.7 (0.7)
Questions and answers	84	4.7 (0.7)
6. Quality management presentation		
Contents	82	4.3 (0.9)
Presentation	81	4.4 (0.8)
Questions and answers	83	4.3 (0.9)
7. Quality management workshop		
Contents	84	4.2 (0.9)
Presentation	82	4.2 (0.9)
Questions and answers	83	4.3 (0.9)
8. Teaching and learning strategies		
Contents	83	4.1 (0.9)
Presentation	83	4.1 (0.8)
Questions and answers	83	4.3 (0.9)
9. Communication workshop		
Contents	83	3.7 (1.2)
Presentation	84	3.7 (1.1)
Questions and answers	83	3.9 (1.2)
10. Subject specific debates		
Contents	74	4.2 (0.9)
Presentation	74	4.2 (0.9)
Questions and answers	75	4.2 (1.0)
11. Megacase practical exercise		
Contents	84	4.7 (0.8)
Presentation	84	4.7 (0.8)
Questions and answers	84	4.7 (0.8)
12. Communication exercise		
Contents	83	4.0 (1.1)
Presentation	83	4.1 (1.1)
Questions and answers	83	4.1 (1.0)
13. Group work		
Contents	77	4.5 (0.7)
Presentation	75	4.6 (0.7)
Questions and answers	76	4.5 (0.7)
14. Group work presentation		
Contents	59	4.4 (0.7)
Presentation	58	4.5 (0.7)
Questions and answers	59	4.5 (0.7)
15. Wrap up and next steps		
Contents	52	4.6 (0.7)
Presentation	52	4.5 (0.9)
Questions and answers	52	4.6 (0.7)
16. Organization		
Level of organization	85	4.4 (0.9)
Level of teaching	85	4.4 (0.8)
Technical direction	84	4.8 (4.4)
Secretariat	85	4.5 (0.8)
Educational material	85	4.5 (0.7)
Audiovisual support	85	4.3 (0.7)
17. Course information provided		
Before registration	85	4.2 (1-0)
Alter registration	85	4.4 (0.8)
During the course	85	4.5 (0.8)
18. Global evaluation		
Applicability to my job	85	4.4 (0.8)
Overall course assessment	84	4.4 (0.8)

Attitudes and Perceptions

Attitudes regarding organ and tissue donation in HCPs and OKPs are shown in **Table 7**.

Answers recorded in the post-test survey showed a statistically significant change towards a positive attitude when referring to the willing to donate organs of their relatives both in HCPs and OKPs. Also, 100% of HCPs and OKPs answered "yes" regarding donation of their own organs after death. An improvement in the percentage of participants that considered that organ and tissue donation should be part of the end of life care, both in HCPs and OKPs was also found.

Results of the perception survey showed that both HCPs and OKPs selected more positive than negative terms that better described the process of donation after brain death as compared with pre-test assessment (**Figure 1**). HCPs significantly improved the selection of solidarity, opportunity, and dignified concepts, and significantly reduced the selection of negative items such as stressful and painful ($p < 0.05$). Positive perceptions were also recorded among OKPs, but differences between pre- and post-test analysis were not statistically significant.

DISCUSSION

The EUDONORGAN project (21) was proposed within the framework of EU Action Plan on Organ donation and the legislation established in the Directive 2010/53/EU, as one of the initiatives aimed to increase organ availability, to enhance efficiency and accessibility of transplant systems, and to improve quality and safety. The Action Plan advocated appointing of transplant donor coordinators and promoting quality improvement programs in hospitals hence optimizing deceased organ donation, exchanging best practice on donation from living donors, and strengthening communication skills of professionals and patient support groups. Other EU-funded projects focused on improving outcomes from deceased organ donation included to improve collaboration with ICUs (ACCORD) (22), to compare and improve deceased organ donation programs (MODE) (4), to assess protocols and critical steps (COORENOR) (23), and to develop quality system indicators (ODEQUS) (24).

TABLE 6 | Learning (knowledge acquisition) scores in all participants.

Variables	Healthcare professionals (HCPs) (*n* = 79)				Other relevant key players (OKPs) (*n* = 17)			
	Participants	Correct answers, %		*p*-value[a]	Participants	Correct answers, %		*p*-value[a]
		Pre-test	Post-test			Pre-test	Post-test	
Gender								
Men	32	71.4 (12)	94.8 (6.9)	<0.001	4	54.1 (32.5)	87.5 (8.3)	0.109
Women	47	72 (14.3)	97 (4.5)	<0.001	13	67 (11.8)	94.4 (6.4)	0.002
p-value[b]		0.693	0.67			0.281	0.096	
Age, years								
25–34	16	72.2 (14.8)	95.8 (6.6)	0.001	6	70.4 (5.7)	96.3 (2.9)	0.026
35–44	37	70.7 (14.1)	97.3 (4.6)	<0.001	3	57.4 (12.8)	88.8 (11.1)	0.102
45–54	20	75.4 (12.9)	96.8 (5.6)	<0.001	7	75 (19.5)	95.8 (5.3)	0.042
55–64	6	70.4 (8.5)	92.6 (6.4)	0.027	1	55.5	94.4	
p-value[b]		0.688	0.479			0.281		
Profession (HCPs)								
Medical doctor	49	74.1 (12.4)	95.4 (6.5)	<0.0001				
Registered nurse	27	69.8 (14.7)	97.3 (4.2)	<0.0001				
Medical student	2	55.5 (7.9)	100					
Healthcare manager	1	66.6	100					
p-value[b]		0.93	0.173					
Profession (OKPs)								
Patients' group representative					7	69 (15)	94.4 (3.2)	0.027
Communication expert					5	67.7 (17.7)	92.2 (6.3)	0.068
Journalist					3	42.6 (23.1)	85.1 (12.8)	0.109
Documentalist					1	66.6	100	
Other					1	72.2	100	
p-value[b]						0.297	0.232	
Specialty								
Anesthesiology/intensive care	41	75 (12.5)	94.8 (6.6)	<0.0001				
General nurse	20	70.8 (15.8)	97.2 (4.2)	<0.0001				
Intensive care nurse	5	62.2 (10.7)	94.4 (5.5)	0.042				
Transplant/donor coordinator	3	77.7	100					
Nephrology	2	55.5	100					
Internal medicine	2	75.0 (11.8)	97.2 (3.9)					
Other	6	65.3 (13.8)	100	0.043				
p-value[b]		0.243						
Position								
Transplant/donor coordinator	37	71.5 (13.8)	96.7 (5.6)	<0.0001				
Anesthesiologist/intensive care	26	75 (12.5)	95.3 (6.1)	<0.0001				
Medical doctor	3	72.2 (11.)	96.3 (6.4)					
Other	13	69.4 (13.9)	96.3 (5.5)	0.001				
p-value[b]		0.349	0.852					
Total	79	72 (13.4)	96.2 (5.6)	<0.0001	17	64 (18.3)	92.8 (7.3)	<0.0001

[a]*Within-group comparison.*
[b]*Between-group comparison.*

EUDONORGAN was an educational project addressed to HCPs. However, and for the first time in an EU project, OKPs who turned be able to advocate for organ donation and train colleagues in their countries, regions and/or hospitals were also considered to extend the capacity-building efforts to a more heterogeneous group of participants (e.g., patient support groups, journalists, communication experts). Joint involvement of HCPs and OKPs would impact on other aspects, such as standardization of training programmes, and collaboration between countries and sharing of best experiences (4).

As in previous EU-funded training projects, such as ETPOD (25) and EMPODaT (26), the methodology used was blended learning defined in this project as the appropriate mix and use of face-to-face instructional methods and various learning technologies to support planned learning and foster subsequent learning outcomes (27). EUDONORGAN provided an innovative dimension with the use of an instructional delivery method consisting of computer-based training or WebApp with the application of the main adult learning principles in that consider the learner's role is not only to receive knowledge but also to search, challenge, construct knowledge and change their own perception, views, and beliefs (28). Innovation came by offering game elements, animated characters and scenarios in each of the seven modules on organ and tissue donation following an interactive, enjoyable, and easy to understand manner.

TABLE 7 | Attitudes regarding organ and tissue donation in all participants.

Questions	Healthcare professionals			Other relevant key players		
	Pre-test (*n* = 79)	Post-test (n = 64)	*p*-value	Pre-test (*n* = 17)	Post-test (*n* = 13)	*p*-value
Would you donate your organs after death?						
Yes	78 (98.7)	64 (100)	0.321	13 (76.5)	13 (100)	NA
No	0	0		1 (5.9)		
I do not know	1 (1.3)	0		3 (17.6)		
Would you donate the organs of your relatives after death?						
Yes	69 (87.3)	60 (93.7)	<0.0001	16 (94.1)	13 (100)	NA
No	1 (1.3)	0		1 (5.9)		
I do not know	10 (12.7)	4 (6.2)		0		
If you choose "No" or "I do not know" in the previous question, why? (more than one answer is accepted)						
Religious reasons	0	1 (1.6)	NA	0	13 (100)	NA
Lack of trust in the health system	2 (2.5)	1 (1.6)		0		
Not knowing the wish of the deceased	14 (17.8)	4 (6.3)		3 (17.6)		
Ethical reasons	1 (1.3)	0		0		
Fear of body disfiguration	0	0		1 (5.9)		
Other reasons	25 (31.6)	0		4 (23.5)		
Organ and tissue donation should be part of the end of life care						
Yes	75 (94.9)	64 (100)	0.182	13 (76.4)	12 (92.3)	0.689
No	3 (3.8)	0		1 (5.9)	0	
I do not know	1 (1.3)	0		3 (17.6)	1 (7.7)	
When do you consider that it is the most appropriate moment to talk about organ and tissue donation?						
Anytime	29 (36.7)	24 (37.5)	<0.0001	15 (88.2)	7 (53.8)	0.246
When the death of the patient is predictable	28 (35.4)	22 (34.3)		2 (11.8)	2 (15.4)	
After the patient's death	22 (27.9)	18 (28.1)		0	4 (30.8)	
Do you agree with the admission to the intensive care unit (ICU) of patients with devastating injuries in whom the treatment has deemed futile, for the solely reason of facilitating organ and tissue donation?						
Yes	70 (88.6)	60 (93.7)	0.810	13 (76.5)	12 (92.3)	0.494
No	4 (5.1)	0		2 (11.8)	1 (7.7)	
I do not know	5 (6.3)	4 (6.2)		2 (11.8)	0	
Do you consider appropriate to employ the same resources to maintain a potential brain dead donor as in any other critical patient?						
Yes	75 (94.9)	61 (95.3)	<0.0001	10 (58.9)	12 (92.3)	0.559
No	0	0		3 (17.6)	1 (7.7)	
I do not know	4 (5.1)	3 (4.7)		4 (23.5)	0	

As shown in the satisfaction results, the online educational modules were scored with high values and so it was the methodology used during the face-to-face sessions that boosted hands-on learning, networking, best practice exchange and promoted great interactivity between both groups of participants. They found the training very useful to improve their teaching and communication skills and to organize both trainings and raising awareness events in their daily work context: hospitals, national transplant organizations and/or patients' associations. Learning results indicated that the training was successfully implemented involving a total of 96 participants from 24 different countries that passed the program with a pass mark of 95%, which is a relevant indicator of a significant increase of knowledge acquisition. These outcomes are even more remarkable in the group of RN as part of the HCPs as professionals active in the field of organ donation and transplantation that resulted as a major factor in maximizing deceased donor potential and eventually increase donation rates (25) and an asset to replicate the training at a national level (28).

Results were also positive in the group of OKPs that become a pool of professionals trained that are part of the entire donation and transplantation chain. In both groups of participants, a change of attitude on their willingness to donate their organs or their relatives was observed. Training also helped improvement towards a positive perception that was noticeable by the increase of positive terms in the post-test. Moreover, both groups could also benefit from further education on various aspects of organ donation and transplantation (4) and on communication skills to support the implementation of public awareness actions and how to communicate with the families of patients, education in schools, generating overall public awareness, and the use of social media (4).

Some limitations of the study should be mentioned. The implementation of "Train the Trainers" program was analyzed, but only at satisfaction and learning levels. The requirements of the EU tender did not foresee the implementation of trainings at local level or regional level, directly related to behavior and result evaluation levels. A post-survey was proposed to optimize the

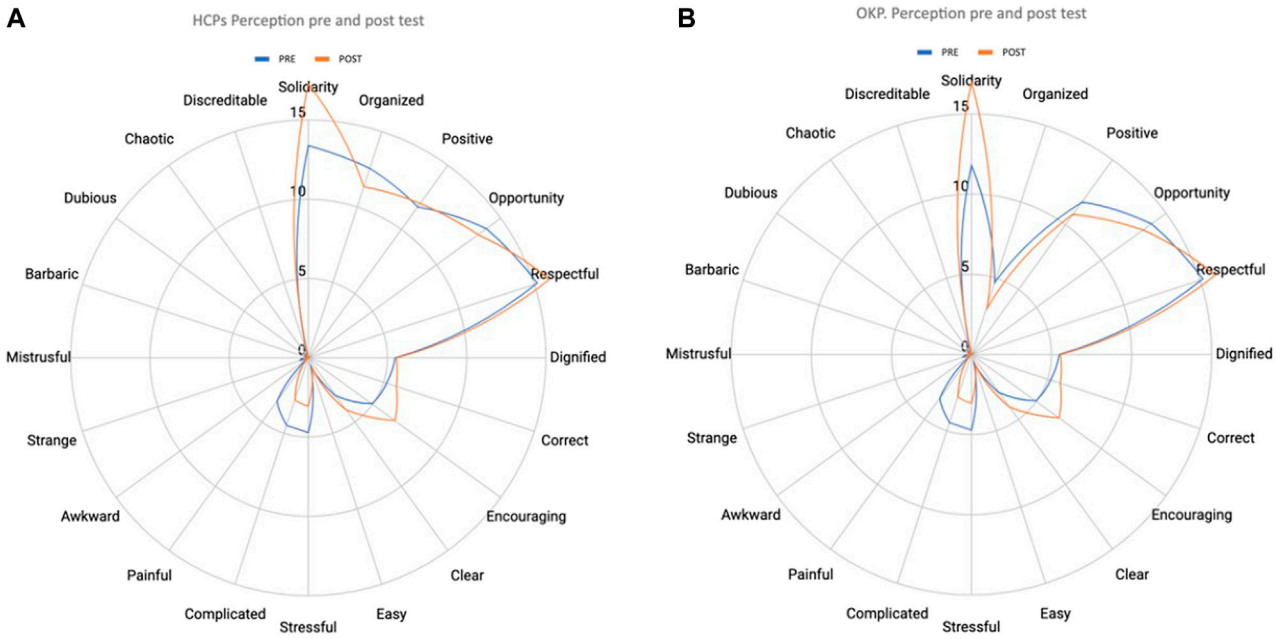


FIGURE 1 | Comparison on perception pre- and post-training in HCPs **(A)** and OKPs **(B)**.

impact of training provided but, the study did not measure the effectiveness of the post-training activities performed by both groups of participants. Assessment of the direct impact of the training program on donation rates was not feasible. However, EUDONORGAN responded very positively to the Action Plan and contributed to promote awareness rising among population with the ultimately improve organ donation rates in the EU and neighbouring countries.

The "Train the Trainers" program was a source of learning and motivation for the professionals. It provided a whole educational framework that allowed a multiplying impact at different levels and types of entities and human supports. The professionals who participated in the study were prepared to organize training actions and events at the local level (university, hospital and/or patient organizations, etc.) and aimed at the target audience. Some of them reported that they had started to implement training actions and a Facebook group was set up in which they continued to interact (https://www.facebook.com/groups/340412829742498/). An evaluation at the clinical and social level could be performed through a follow-up study conducted in European hospitals 2–3 years after the implementation of the training. It would allow to measure whether changes in donation and transplantation occurred in that period.

In summary, organ donation remains a multicomplex process that affects both healthcare professionals and the entire society. Training is a key enabler in healthcare to increase knowledge and skills. This study proves that the methodology used classically in HCPs also applies in OKPs. We identified a significant increase in knowledge and change of attitude and perception that underline the need of permanent education at different levels in relation to organ and tissue donation.

EUDONORGAN CONSORTIUM

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All participants were informed of the content. They also signed their consent to fulfil voluntarily the surveys that guaranteed the protection of their data, the anonymity and the confidentiality according to the information collected.

AUTHOR CONTRIBUTIONS

PP: Design, analysis and writing of the manuscript; MI: Collecting data and intellectual review; CB: Methodological

advice and intellectual review; MM: Supervision, methodological advice, and intellectual review; RV: Design, data analysis, writing the manuscript and intellectual review. All authors have seen and approved the final draft.

CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10878/full#supplementary-material

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ORIGINAL RESEARCH
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Organ Donation After Euthanasia in Patients Suffering From Psychiatric Disorders: 10-Years of Preliminary Experiences in the Netherlands

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Euthanasia based on psychiatric suffering, followed by subsequent organ donation, is considered medically and legally permissible in the Netherlands. Although organ donation after euthanasia (ODE) in patients suffering from unbearable psychiatric illness is performed, it is not specifically addressed in the Dutch guideline on organ donation after euthanasia, and national data on ODE in psychiatric patients have not yet been published. In this article, the preliminary results of the 10-year Dutch case series of psychiatric patients who choose ODE are presented and potential factors influencing opportunities for donation in this population are discussed. We conclude that further future in-depth qualitative exploration of ODE in patients suffering from psychiatric illness and its associated ethical and practical dilemmas, including the consequences for the patient and their family and healthcare professionals, will be important to help make sense of potential barriers to donation for people undergoing euthanasia as a result of psychiatric suffering.

Keywords: organ donation, euthanasia, psychiatric illness, disorder, suffering

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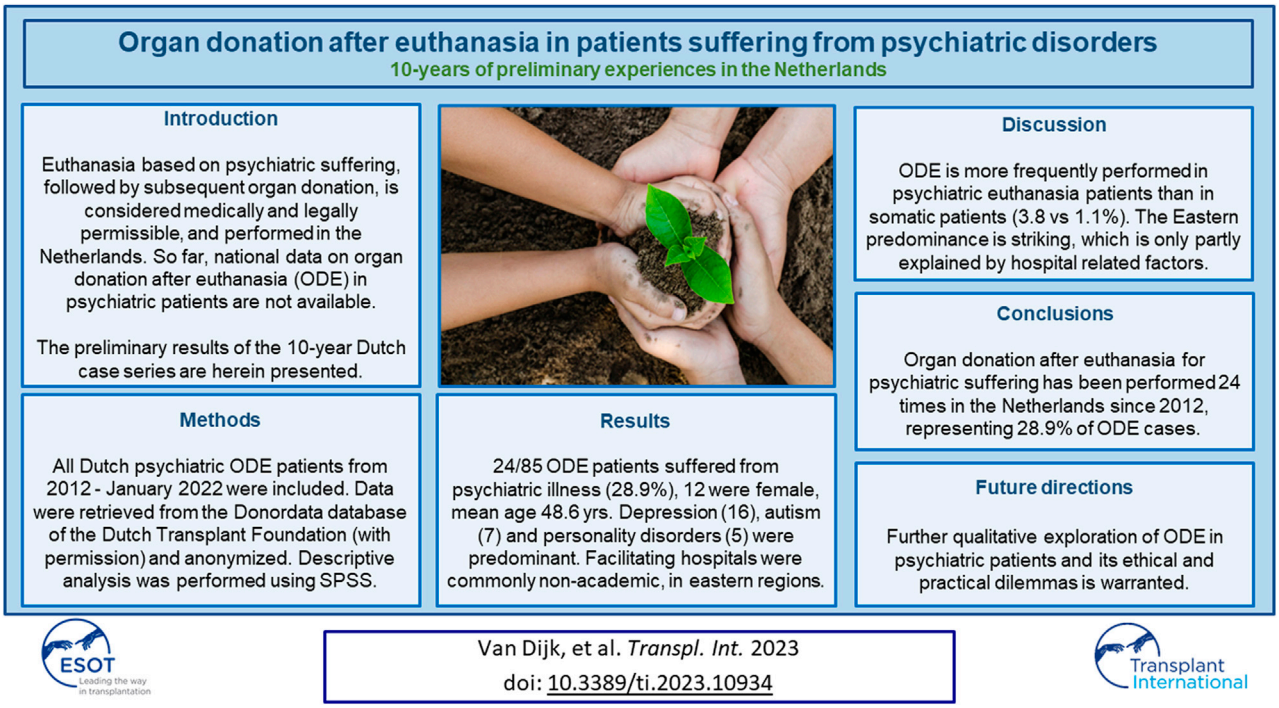
INTRODUCTION

Euthanasia

Physician assisted dying makes it possible for a patient to die a self-chosen, peaceful death. Euthanasia, where a physician administers a lethal drug intravenously to a patient, is currently legally permitted in Belgium, the Netherlands, Luxemburg, Colombia, Canada and parts of Australia (1, 2). Recently, euthanasia was allowed in Spain since June 2021 and in New Zealand since November 2021. In the Netherlands, the societal, judicial, ethical and medical debate resulted in the Termination of Life on Request and Assisted Suicide Act in 2002. Since its legalization, euthanasia has been allowed following a voluntary request if the mentally competent patient is suffering hopelessly, irreversibly and unbearably, while no other reasonable options are available, and only after a second, independent physician is consulted.



GRAPHICAL ABSTRACT |

82,963 patients underwent euthanasia between 2002 and 2021 in the Netherlands, most commonly because of end-stage cancer (3). In general, the patient requests euthanasia *via* his general practitioner.

In 1994, a Dutch psychiatrist facilitated physician assisted suicide in a patient with underlying psychiatric suffering for the first time, in the so-called Chabot case. The Dutch supreme court reasoned that unbearable and irreversible psychiatric suffering could justify physician assisted death, but mandated consultation of a second independent psychiatrist in future cases (4). Although extra due diligence criteria for patients with a psychiatric cause of suffering are not included in the 2002 Termination of Life on Request and Assisted Suicide Act, they are formulated in the specific guideline by the Dutch Society of Psychiatrists, and currently applied in practice: if the cause of suffering is psychiatric in nature, either the treating physician or the second independent physician needs to be a psychiatrist, and a second opinion by a psychiatrist competent to assess the patient's specific pathology is mandatory (5).

It should be underscored that euthanasia in psychiatric suffering is only allowed when the patient has maximally pursued all reasonably possible treatments. Consequently, it takes several years before a euthanasia request is granted. This time period is essential to assess whether previously attempted treatments were (in)efficacious, to explore potential new medical treatment strategies, and to evaluate the patient's perseverance. Ultimately, an estimated 10% of euthanasia requests in psychiatric patients is granted (6).

Every euthanasia case is reviewed by a Regional Euthanasia Review Committee consisting of a lawyer, an ethicist and a doctor 6 weeks after the procedure, supported and advised by a secretary (also a lawyer). While a psychiatrist is required at the earlier stage of assessment for euthanasia, this is not the case in the *post hoc* review of the eventual procedure. If the review committee determines that the due diligence requirements were not fulfilled, the performing physician can be prosecuted. The additional requirements for patients suffering from a psychiatric illness formulated in the Dutch Society of Psychiatrists guideline are also used in the *post hoc* analysis (see below).

In 2021, 7666 patients underwent euthanasia in the Netherlands, of whom 115 (1.5%) suffered primarily from a psychiatric disease. Since 2002, *post hoc* analysis confirmed procedural correctness in all cases, and prosecution in cases in which psychiatric suffering was the basis for the euthanasia request, has so far not occurred.

Organ Donation After Euthanasia (ODE)

Organ Donation after Euthanasia is currently being performed within legal boundaries in Belgium, the Netherlands, Canada and Spain. Neither the Dutch law on organ donation nor Dutch Termination of Life on Request and Assisted Suicide Act preclude organ donation after euthanasia, although these laws were drafted independently. The subject of organ donation can only be raised by the patient after the euthanasia request is approved. This sequential order, carefully laid down in the National

Dutch Organ Donation after Euthanasia guideline (7) aims to ensure the separation of the euthanasia assessment procedure and subsequent organ donation request, and to prevent any influence that the euthanasia request could have on the organ donation request, and *vice versa*. In case a patient raises the issue of organ donation prematurely, the treating physician should postpone discussing the topic until after the euthanasia assessment procedure has been correctly completed, and the euthanasia requests has been granted. If the patient however does not raise the issue of organ donation, the physician is legally obligated to check the patient's registration in the Dutch donor registry, in which patient preferences regarding donation are newly documented since the introduction of the national Dutch op-out system in July 2020 (8). In the absence of a patient's registered active refusal to donate, the patient's presumed consent may scaffold further dialogue facilitating shared decision making and respecting the patient's autonomy regarding organ donation after euthanasia.

Organ donation after euthanasia is a donation after circulatory death (DCD) procedure (9). DCD is possible in the absence of medical contraindications, and after fulfilling all criteria in the Dutch Organ Donation Act, including the guideline regarding death determination by the Dutch Health Council (10). The most common general contraindication for organ donation is malignancy. Of all patients who underwent euthanasia an estimated 10% are potentially medically eligible to donate their organs (11). Most commonly these patients suffer from underlying neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Huntington's disease (11). From 2012, when the first ODE was performed, to January 2022, 85 patients donated their organs following euthanasia in the Netherlands. Following DCD donation, it is possible to donate lungs, kidneys, pancreas and liver (12). In March 2021, DCD heart donation has been introduced in the Netherlands (13), which recently resulted in the first heart donated by a patient suffering from an unbearable psychiatric disease who requested for euthanasia, which was subsequently successfully transplanted. The euthanasia procedure is most commonly performed in an intensive care, medium care or post anesthesia care unit by the treating physician (14). This environment can facilitate distantly monitoring the patient using an arterial line, and these units are often located a short distance from the operation theatre, which helps limiting ischemic damage to the procured organs. Comparable to the strict separation of the euthanasia and organ donation requests procedures, the transplantation procedure is strictly separated from the organ donation procedure, to avoid any conflict of interest. More detailed information about the practical aspects and governance of ODE has been published previously (7).

Currently, organ donation after euthanasia in patients suffering from unbearable psychiatric illness is mentioned, yet not addressed in detail, in the Dutch guideline on organ donation, presumably because of the perceived limited occurrence and unfamiliarity with ODE requests in this category of patients.

So far, experiences with organ donation after euthanasia in patients suffering from a psychiatric disease are limited (14) and not previously systematically explored in the literature. In this article, in order to identify temporal trends in organ donation after euthanasia, an overview of the preliminary data of *all* cases of organ donation after euthanasia due to psychiatric suffering in the Netherlands is presented, and compared to data on euthanasia and ODE in somatic patients, followed by a review of the practical hurdles patients and healthcare professionals may encounter when they encounter a request for this specific combined procedure.

The aim of this study is to increase the understanding of both euthanasia in patients with psychiatric suffering and its combination with organ donation.

PATIENTS AND METHODS

All patients who underwent organ donation after euthanasia due to a psychiatric illness from 2012 until January 2022 in the Netherlands were included in the analysis. Data on gender, age, and underlying psychiatric disorder, as well as the facilitating hospitals were retrieved from the Donordata database of the Dutch Transplant Foundation database (with permission) and anonymized. Retrospective analysis of prospectively collected data were performed with descriptive analysis using SPSS version 26.0 (15).

RESULTS

This section will subsequently discuss the eligibility for euthanasia and organ donation, the background of the euthanizing physicians, the patients' underlying psychiatric conditions, the types of facilitating hospitals, the geographical distribution of the ODE procedures, and the nature and number of organs transplanted resulting from ODE procedures in patients with underlying psychiatric disorders in the Netherlands.

Eligibility for Euthanasia and Organ Donation

Over the ten-year study period 2012–2021 59,546 patients underwent euthanasia of whom 58,912 suffered from a somatic disorder. The number of patients that underwent euthanasia for an underlying psychiatric disorder was 634 (1.1%). An estimated 10% (5955) of patients who undergo euthanasia in general are medically eligible to donate one or more organs (11).

An estimated 5321 (5955–634) somatic patients would theoretically be eligible for organ donation, and 61 actually donated after euthanasia (61/5321; 1.1%). Assuming contraindications such as malignancy are absent, theoretically, all patients who undergo euthanasia for psychiatric reasons are medically eligible to donate their organs. The percentage of patients with an underlying

TABLE 1 | Evolution of ODE in patients suffering from somatic respectively psychiatric disorders over time.

Year	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	Total
Somatic disorders	1	2	2	9	7	11	9	8	5	7	61
Psychiatric disorders		1			1	2	3	5	7	5	24

psychiatric disorder that underwent organ donation after euthanasia (24/634; 3.8%) in perspective of the percentage of patients who donated after euthanasia based on an underlying somatic disorder, is thus high.

Since 2012, 85 patients underwent organ donation after euthanasia, of which 24 (28.9%) because of psychiatric suffering and 61 because of somatic suffering (see **Table 1**).

In the 24 cases, there was an equal division in gender, with an average age of 48.6 (range 21–72) years.

Euthanising Physicians

The physicians performing euthanasia in the procedures related to psychiatric suffering were physicians from the Dutch Euthanasia Expertise Centre in half the cases (13), followed by psychiatrists (6), general practitioners (4) and specialist in elderly care (specialist in geriatric care e.g. geriatric patients in nursing homes) (1).

Underlying Psychiatric Conditions

The underlying psychiatric conditions, as documented in, and retrieved from the database, were personality disorders, depression, autism, posttraumatic stress disorders (PTSS), and Attention Deficit Hyperactivity Disorder (ADHD). Depression (16 pts), autism (7 pts) and personality disorders (5 pts) were predominant. In 2 cases a combination of a somatic disorder with a psychiatric disorder was reported. In 11 cases a combination of psychiatric disorders was documented.

Types of Facilitating Hospitals

The procedures in patients with underlying psychiatric disorders was performed in 4 out of the total of seven university medical centers in 9 out of 24 cases, and affiliated hospitals in the others. The four university medical centers facilitated the procedures 4, 3, 1 and 1 times, respectively. One affiliated hospital performed the procedure four times, another affiliated hospital three times, and two hospitals two times. Four affiliated hospitals performed the procedure once.

Geographical Distribution

Geographically, the facilitating hospitals were predominantly located in the Eastern part of the Netherlands. Nearly all euthanasia cases were performed in a medium or intensive care setting. The remaining 2 cases in an outpatient treatment (day care) setting, respectively post anesthesia care setting. All cases were facilitated by intensivists.

Organs Transplanted

The 24 cases lead to 107 (mean 4.5, range 1–7) organ transplantations. The transplanted organs were kidneys (46), lungs (35), liver (16), pancreas (5) and heart (2).

DISCUSSION

Until January 1st 2022, 24 patients suffering from an underlying psychiatric disorder have chosen to donate their organs following euthanasia (from a total of 85, 27%). The primary goal of organ donation after euthanasia is to facilitate the patient's last wish. The positive consequences for the transplant waiting lists are subordinate. Nevertheless, the preliminary results of this unique study do demonstrate that patients with psychiatric suffering who underwent ODE have improved and/or extended the lives of dozens of patients who were on the waiting list for an organ transplantation.

Since 2012 the incidence of ODE due to somatic disorders peaked in 2017 (in which 11 cases were documented), where after it more or less remained stable (see **Table 1**). The first case of ODE in a patient suffering from a psychiatric disorder was reported in 2013, and the number of cases annually seems to increase since 2016. In 2020 the number of patients that underwent ODE for psychiatric disease was higher than that for somatic disorders. Over recent years there has been increasing interest in patients' journeys as illustrated by publications in the non-scientific, popular media. It is, however, difficult to predict whether the number of requests for ODE will consequently potentially increase (17).

A remarkably higher proportion of patients with psychiatric conditions donated their organs than among patients who sought euthanasia for other reasons. Over the 10 years study period 2012–2021 59,546 patients underwent euthanasia of which 58,912 suffered from a somatic disorder, the number of patients that underwent euthanasia for an underlying psychiatric disorder was 634 (1.1%). An estimated 10% (5955) of patients who undergo euthanasia in general are medically eligible to donate one or more organs (11). We assume that all patients who undergo euthanasia for psychiatric reasons are medically eligible to donate their organs. An estimated 5321 (5955-634) somatic patients would theoretically be eligible for organ donation, and 61 actually donated after euthanasia (61/5321; 1.1%). The percentage of patients with an underlying psychiatric disorder that underwent organ donation after euthanasia (24/634; 3.8%) in perspective of the percentage of patients who donated after euthanasia based on an underlying somatic disorder is thus remarkably high.

In addition, patients with ODE based on an underlying psychiatric disease were 5 years younger than the average population of patients who underwent ODE (48.8 years versus 53.8 years (latter data are not shown). Patients who undergo organ donation after euthanasia appear to be younger than the general euthanasia population, although an average age for the latter group is not reported (18). However, the vast majority of patients in 2020 and 2021 (87.6% resp. 89%) was reportedly aged over 60 (17, 18).

How can these results be interpreted and what do they mean for the practice of organ donation after euthanasia?

It should be acknowledged that the analysis herein presented represents the willingness to donate of a small subgroup of psychiatric patients whose euthanasia and donation requests were both granted. A recent 2019 study of a stratified sample of 5361 non-sudden deaths from the central Dutch Registry of Statistics revealed that 3.4% had a psychiatric disorder (16). The frequency of euthanasia and assisted suicide (EAS) requests was 11.4%, compared to 11.2% in the whole population, and 8% among people with an accumulation of health problems (16).

Six percent of all deceased patients actually received EAS. This percentage was lower among psychiatric patients (4.8%). In case of a psychiatric disorder, the presence of (severe) symptoms other than pain (75.4%) and expected suffering (53.5%) were important reasons to grant EAS. Across the full sample, the two most important reasons to grant the request were the lack of prospect of improvement (81.9%–94.6%) and the autonomy of the patient (72.4%–85.8%) (16).

The main reason for refusal of EAS among *all* deceased patients, was death of the patients before the request was granted. The most important reason to refuse the request in psychiatric patients was that the due diligence criteria were not met, particularly regarding the well-considered nature of the request (16).

Comparison of donation rates in the general group of patients who underwent euthanasia with patients who underwent euthanasia due to psychiatric suffering is interesting. It can potentially answer the question regarding whether consent for donation and actual donation rates were higher in the euthanasia due to psychiatric suffering group, and thus whether rates to withdraw consent for either the euthanasia or organ donation procedure. However, data on donation consent and subsequent withdrawal rates in psychiatric versus non-psychiatric patients in a larger population than herein presented are unavailable, to the best of our knowledge.

One can thus only speculate on the reasons contributory to the herein observed differences in donation after euthanasia in somatic versus psychiatric patients. Possible explanations may be that psychiatric patients, for example compared to patients suffering from ALS, are physically more able to gather information, e.g. on the internet, on the possibilities of organ donation after euthanasia, or that they are more physically able to undergo the preparatory examinations necessary for donation. Another possibility is that patients suffering from psychiatric illness are more altruistic, or have become more altruistic, due to reflections about their own lives, or experiences with organ donation in their surroundings, and consequently want to finalize their lives with an altruistic gift to, for them unknown others (17). Theoretically, the physicians performing the euthanasia could also more frequently raise the issue of organ donation after euthanasia in psychiatric patients than somatic patients. However, the Dutch ODE guideline advises against proactively raising the option of donation after euthanasia in general (14). In addition, greater caution regarding competency is warranted and attributed in patients with mental conditions.

Furthermore, it is possible that psychiatric patients assume, more frequently than somatic patients, that their organs will be medically suitable to donate organs (see below). Based on their relatively young age and underlying psychiatric disorders (in comparison with patients with underlying somatic disorders), it indeed might be assumed that these patients have healthy organs that are more likely to be suitable for transplantation. Although 107 organs were successfully transplanted from these 24 donors, it is however premature to draw firm conclusions whether the organs of patients with underlying psychiatric disorders are indeed in better condition. In contrast to the general population, people with mental disorders for example have high rates of adverse health behaviors, including tobacco smoking, substance use, physical inactivity, and poor diet, e.g. resulting in cardiovascular disorders and diabetes (20). Although the preliminary results of transplantation following organ donation after euthanasia *in general* demonstrate good functional results (21,22,23), further research on the transplantation results following organ donation after euthanasia in psychiatric suffering still has to be performed.

Despite occurring relatively frequently, organ donation after euthanasia in psychiatric suffering is nevertheless surrounded by several mainly ethical challenges, related primarily to the euthanasia, such as the important issue of competency relating to both the euthanasia and organ donation requests, but also to the subsequent organ donation procedure in these patients, such as the impact on the health professionals involved in the procedures. Discussing these challenges is beyond the scope of this article.

In summary, patients can suffer unbearably and hopelessly from a psychiatric disease, comparable to patients with physical underlying disorders. Any request for euthanasia should be carefully considered, with particular attention paid to the patient's decision-making capacity, given the context of psychiatric issues. Any subsequent wish to donate organs is an extremely altruistic act, should likewise be subject of careful and deliberate consideration, critically considering any conflicts of interest. The strict separation between the euthanasia and organ donation requests on the one hand, and the organ donation and transplantation stages, with different physicians involved in the different stages on the other, aims to prevent such conflicts of interest. The effects of the requested organ donation on the transplantation waiting lists are principally irrelevant in this regard.

This study has strengths but also several limitations. The study is the first to provide a unique, preliminary insight into ODE in patients with psychiatric illness. The data reported here are however limited to the data formally accessible for the research group. The granularity of the data is thus limited. The information as retrieved from the database, e.g. the categorization of psychiatric illness in several cases, was not debated by the researchers. Somatoform complaints not primarily documented as a psychiatric disease in the database are thus not represented in this current case series. More detailed information on the psychiatric illness, treatments attempted and the duration of both illness and treatment was not available in the database.

Although the Eastern geographical predominance of ODE in psychiatric illness is striking, data which may provide insights into the reasons for this observation are likewise not available. It is known that not all hospitals in the Netherlands are willing to honor a patient's wish for organ donation after euthanasia. Such local and regional differences may perhaps at least partly explain some of the observed geographical differences. A recent cross sectional study on the crude rates of euthanasia (not followed by organ donation) indeed revealed considerable geographical variation across the Netherlands (24), however with a seemingly Western predominance. Associated factors herein were age, church attendance, political orientation, income, self-experienced health and availability of voluntary workers. After adjustment for these characteristics a considerable amount of geographical variation remained, which also calls for further exploration.

The preliminary results of this study nevertheless provide sufficient anchors for further qualitative exploratory interview studies among the different healthcare professionals involved and perhaps the patients' relatives to elucidate the reasons underlying some of the findings in this study. Herein their experiences regarding the timing and initiation of the donation request by patients and/or physicians, the use and usefulness of potential familiarization and/or exploration meetings between the patient and the facilitating hospital's staff, the option to start the ODE procedure from home (23–25), and the emotional burden for patients and their relatives to die in hospital(14), as well as the emotional burden of healthcare professionals facilitating the procedures can be explored more in depth. In addition, the transplantation results of the donated organs necessitate further short-, mid- and long-term follow up. Such follow up studies are currently performed.

In conclusion: euthanasia because of psychiatric suffering, followed by organ donation, is medically and legally permissible and performed in Dutch healthcare practice. The preliminary results of this 10-year Dutch case series contribute to increasing the understanding of both euthanasia in patients with psychiatric suffering and its combination with organ donation. Organ donation after euthanasia in psychiatric suffering has been performed 24 times in the Netherlands since 2012, representing 28.9% of cases. So far, this patient category is not specifically addressed in the national Dutch guideline on organ donation, since the ethical debate hereon is ongoing and data on ODE were so far completely lacking. This specific type of organ donation after euthanasia, and its associated ethical and practical dilemmas, including competency and consent issues, as well the consequences for the patient's family and healthcare professionals, necessitate further careful qualitative exploration before this topic can potentially become part of a revised version of the existing national guideline on organ donation after euthanasia.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Dutch Transplant Foundation. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

WM, JB, and WJ designed the study. ND, WJ, JB, and WM wrote the initial draft of the manuscript. NJ provided input on the final versions from a holistic national level, PS provided input as a psychiatrist from the Euthanasia Expertise Center, DS provided input for a holistic, ethical perspective.

CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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Very Low Dose Anti-Thymocyte Globulins Versus Basiliximab in Non-Immunized Kidney Transplant Recipients

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The choice between Basiliximab (BSX) or Anti-Thymocyte Globulin (ATG) as induction therapy in non-immunized kidney transplant recipients remains uncertain. Whilst ATG may allow steroid withdrawal and a decrease in tacrolimus, it also increases infectious complications. We investigated outcomes in non-immunized patients receiving a very low dosage of ATG versus BSX as induction. Study outcomes were patient/graft survival, cumulative probabilities of biopsy proven acute rejection (BPAR), infectious episode including CMV and post-transplant diabetes (PTD). Cox, logistic or linear statistical models were used depending on the studied outcome and models were weighted on propensity scores. 100 patients received ATG (mean total dose of 2.0 mg/kg) and 83 received BSX. Maintenance therapy was comparable. Patient and graft survival did not differ between groups, nor did infectious complications. There was a trend for a higher occurrence of a first BPAR in the BSX group (HR at 1.92; 95%CI: [0.77; 4.78]; $p = 0.15$) with a significantly higher BPAR episodes (17% vs 7.3%, $p = 0.01$). PTD occurrence was significantly higher in the BSX group (HR at 2.44; 95%CI: [1.09; 5.46]; $p = 0.03$). Induction with a very low dose of ATG in non-immunized recipients was safe and associated with a lower rate of BPAR and PTD without increasing infectious complications.

Keywords: diabetes mellitus, rejection, induction therapy, low immunological risk, low dose thymoglobulin

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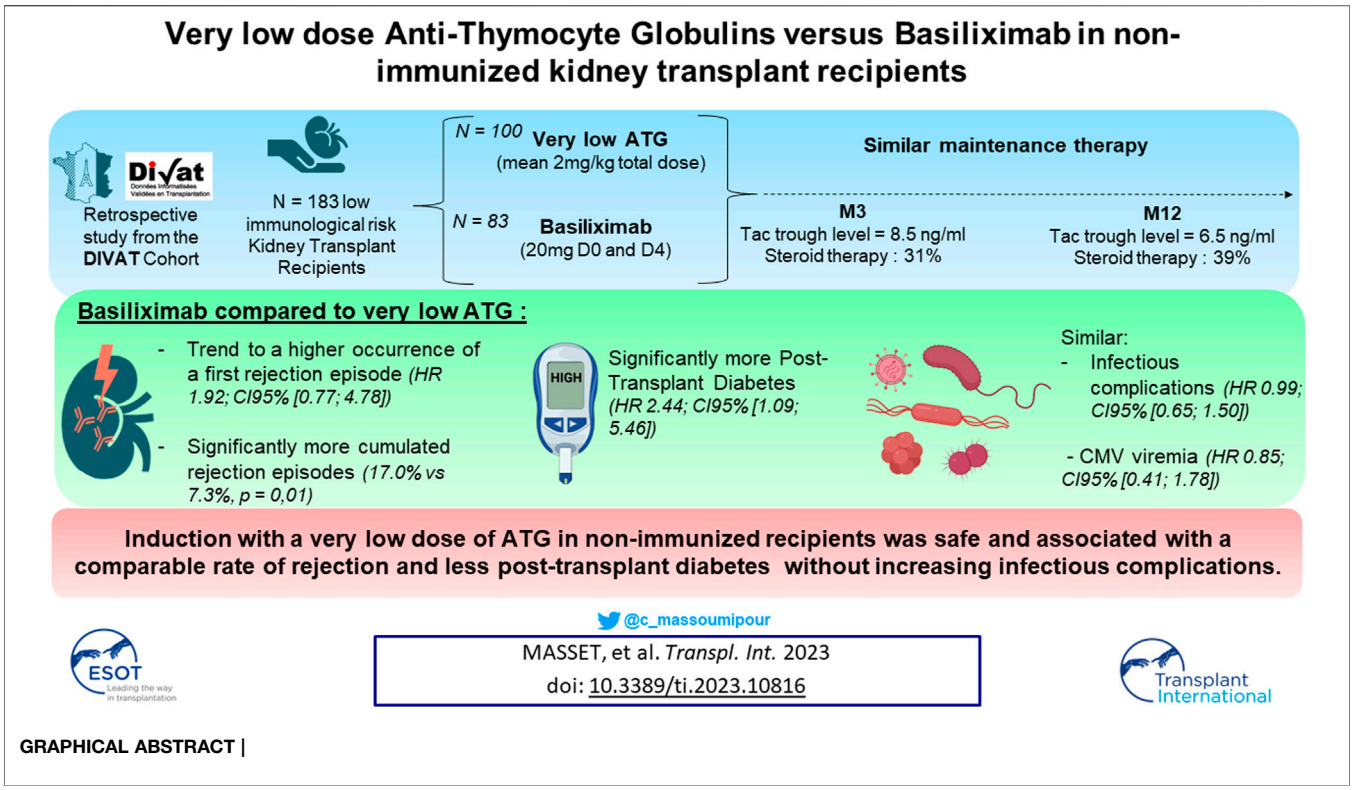
Abbreviations: 95%CI, 95% Confidence Interval; ATG, anti-thymocyte globulins; BMI, body mass index; BPAR, biopsy proven acute rejection; BSX, basiliximab; CIT, cold ischemia time; CMV, cytomegalovirus; CNI, calcineurin inhibitors; DGF, delayed graft function; DSA, donor-specific antibodies; ECD, expanded criteria donor; eGFR, estimated glomerular filtration rate; HLA, human leucocyte antigen; HR, hazard ratio; KDIGO, kidney disease improving global outcomes; MMF, mycophenolate mofetil; MPA, mycophenolic acid; OR, odds-ratio; SCr, serum creatinine; sd, standard deviation; RMST, restricted mean survival time; RMTL, restricted mean time lost.



GRAPHICAL ABSTRACT |

INTRODUCTION

The choice for induction therapy in patients with low immunological risk remains uncertain. Basiliximab (Simulect®) is a monoclonal antibody targeting the IL-2 receptor. It results in decreased T cell activation without inducing T cell depletion which has proven beneficial versus placebo controls in induction therapy of kidney transplant recipients (1). Anti-thymocyte Globulins (ATG) consists of polyclonal globulins exerting a strong T cell depleting effect which has proven beneficial against the occurrence of biopsy proven acute rejection (BPAR) in high immunological risk recipients compared to Basiliximab (2). However, this T cell depletion has also been associated with an increased risk of infectious complications, and notably, CMV reactivation (3). Thus, the kidney disease improving global outcomes (KDIGO) guidelines recommends Basiliximab as first line induction therapy in patients with low immunological risk in association with a triple maintenance therapy consisting of calcineurin inhibitors (CNI)—mainly tacrolimus –, antiproliferative drugs (mycophenolate mofetil—MMF; or mycophenolic acid—MPA) and steroids (4). However, ATG remains widely used due to its good protective effect against allograft rejection and other benefits such as a decrease in delayed graft function (5) or the possibility of rapid steroid withdrawal, thus permitting reduction in side effects such as post-transplant diabetes (6, 7). Moreover, ATG's depleting effect has been proven to depend on the total administered dose, which is currently lower than when first introduced several years ago (8).

In our institution, induction therapy is mainly standardized, even though transplant physicians are free to modify treatment according to the patients' history. Briefly, induction of non-immunized patients receiving a first kidney transplant consisted mostly of Basiliximab until 2016; and of a very low dose of ATG since 2017. The objective of this study was to retrospectively evaluate the impact of this induction modification on post-transplant outcomes, notably, immunological complications (BPAR and occurrence of *de novo* donor specific antibodies—dnDSA), infectious complications and side effects such as occurrence of post-transplant diabetes (PTD).

MATERIALS AND METHODS

Studied Population

The included patients were adults ≥18 years receiving a first kidney transplantation from heart beating deceased donors, treated with either ATG or Basiliximab (BSX) as induction therapy between 2015 and 2020. Multiple organ transplant recipients were not considered. We only included patients without anti-HLA class I and/or class II antibodies as determined by Luminex® assay (i.e., mean fluorescence index <2000) and without pretransplant DSA determined at a MFI threshold of 1000 at the time of transplantation. The patients in the BSX group received 20 mg of Simulect® (Novartis) intravenously at day 0 and day 4. The patients in the ATG group received 75 mg of thymoglobulin (Sanofi) for 2 days (day 0 and day 1); however, if they weighed 50 kg or less they

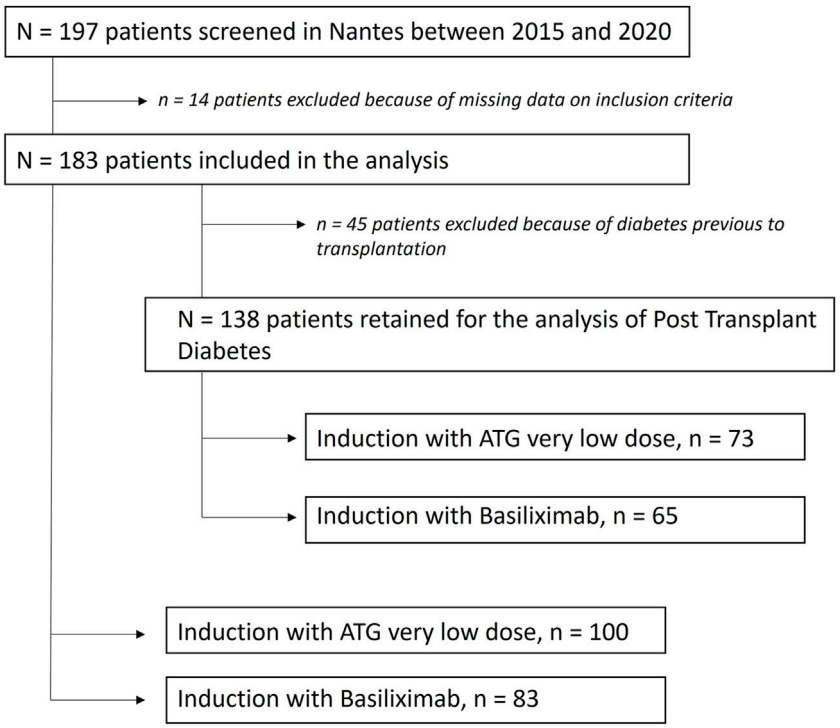


FIGURE 1 | Flow chart of the study.

received only 50 mg of thymoglobulin. All patients received initial corticotherapy during the first days following transplantation (one 500 mg pulse in the BSX group, two 250 mg pulses in the ATG group, followed by oral steroid therapy which was rapidly reduced and withdrawn according to physician's choice) associated with a maintenance immunosuppressive therapy consisting mainly of tacrolimus with antiproliferative drugs (mycophenolate mofetil or mycophenolic acid). Infectious prophylaxis consisted of trimethoprim + sulfamethoxazole (Bactrim®) during at least the first 6 months post transplantation, and until $CD4^+$ T cells counts were ≥200/mm^3, associated with valganciclovir depending on the risk of post-transplant CMV viremia (3 months in case of recipient's positive serological assay; 6 months in case of recipient's negative serological assay associated with a transplant from a CMV seropositive donor).

Available Data

Donor features included allograft status (extended criteria donor or standard criteria donor), donor age, donor sera creatininemia, cause of death, and CMV status. Recipient characteristics included age, gender, BMI, comorbidities (diabetes, hypertension, dyslipidaemia, neoplasia, vascular, cardiovascular history), duration on waiting list, pre-emptive transplantation, and CMV serology status. Transplantation parameters were the cold ischemia time (CIT), use of machine perfusion and the number of HLA-A-B-DR incompatibilities. Patients lost during follow-up were right-censored for mid- or long-term time-to-event. We assumed that the corresponding information were non-informative. For missing data, we voluntarily excluded patients for which the value spread from the initial date was too high (>3 months for 1-year analysis).

Outcomes

The principal outcome was patient and graft survival, defined by the time between the transplant and the first event requiring return to dialysis, pre-emptive re-transplantation, or death with a functioning graft. Secondly, we studied cumulative probabilities of all infectious complications (bacterial, BkV viremia or BkV nephropathy, CMV viremia or fungal infection), CMV viremia only, occurrence of the first biopsy proven acute rejection episode [BPAR according to the Banff classification (9)], occurrence of post-transplant malignancy and occurrence of post-transplant diabetes (PTD) (for this latter analysis, patients with diabetes before transplantation were excluded).

Protocol biopsies were performed at 3- and 12-month post transplantation. According to KDIGO recommendations, we considered indicated biopsies for suspicion of rejection by the occurrence of one criterion amongst the following: increased creatininemia (>25%) without any explanation, delayed graft function >10 days, occurrence of *de novo* donor specific anti-HLA antibody, new onset of proteinuria, or unexplained proteinuria >3 g per day. Occurrence of *de novo* DSA detected by Luminex® assay, and the eGFR (estimated by MDRD) at 1-year post transplantation were also evaluated (patients that died or were lost during follow-up before the first anniversary were excluded).

TABLE 1 | Description of the entire cohort according to induction therapy.

	Whole sample (*n* = 183)			ATG (*n* = 100)			Basiliximab (*n* = 83)			*p*-value
	NA	n	%	NA	n	%	NA	n	%	
Male recipient	0	131	71.6	0	76	76.0	0	55	66.3	0.1460
Preemptive transplantation	0	33	18.0	0	15	15.0	0	18	21.7	0.2415
Etiology of ESRD										
Glomerulonephritis	0	53	29.0	0	28	28.0	0	25	30.1	0.7529
Tubulo-interstitial (including PKD)	0	60	32.8	0	32	32.0	0	28	33.7	0.8034
Vascular (including Hypertension)	0	34	18.6	0	21	21.0	0	13	15.7	0.3554
Diabetes	0	19	10.4	0	11	11.0	0	8	9.7	0.7637
Undetermined	0	17	9.2	0	8	8.0	0	9	10.8	0.5095
History of diabetes	0	45	24.6	0	27	27.0	0	18	21.7	0.4060
History of vascular disease	0	72	39.3	0	41	41.0	0	31	37.3	0.6148
History of cardiac disease	0	65	35.5	0	43	43.0	0	22	26.5	0.0203
History of cardiovascular disease	0	100	54.6	0	60	60.0	0	40	48.2	0.1102
History of pregnancy	0	37	20.2	0	17	17.0	0	20	24.1	0.2341
History of malignancy	0	45	24.6	0	24	24.0	0	21	25.3	0.8387
History of dyslipidemia	0	114	62.3	0	60	60.0	0	54	65.1	0.4820
Recipient BMI ≥ 25 kg/m^2	0	92	50.3	0	52	52.0	0	40	48.2	0.6081
Positive recipient CMV serology	1	75	41.2	1	45	45.5	0	30	36.1	0.2038
HLA incompatibilities > 4	0	52	28.4	0	32	32.0	0	20	24.1	0.2379
Use of machine perfusion	0	111	60.6	0	62	62.0	0	49	59.0	0.6516
Male donor	0	108	59.0	0	56	56.0	0	52	62.7	0.3624
ECD donor	0	112	61.2	0	60	60.0	0	52	62.7	0.7141
Vascular cause of death	0	115	62.8	0	63	63.0	0	52	62.7	0.9612
Donor hypertension	5	51	28.7	5	17	17.9	0	34	41.0	0.0007
Positive donor CMV serology	0	87	47.5	0	40	40.0	0	47	56.6	0.0249
	NA	m	SD	NA	m	SD	NA	m	SD	
Recipient age (years)	0	58.2	15.6	0	58.5	16.0	0	57.9	15.1	0.8069
Donor age (years)	1	59.7	17.3	1	60.3	17.0	0	59.0	17.6	0.7992
Donor creatininemia (µmol/L)	0	88.3	51.0	0	89.2	61.3	0	87.3	46.8	0.6285
Duration on waiting list (months)	0	22.0	20.3	0	21.9	21.6	0	22.1	18.6	0.9632
Cold ischemia time (hours)	0	13.3	5.6	0	12.7	5.8	0	13.9	5.1	0.1377

Abbreviations: BMI, body mass index; CMV, cytomegalovirus; EBV, Epstein-Barr virus; ECD, expanded criteria donor; HLA, human leucocyte antigen; NA, not available (missing); sd, standard deviation; ESRD, end stage renal disease; PKD, polycystic kidney disease.

Statistical Analysis

The characteristics at the time of transplantation between ATG and BSX groups were compared using Chi-square tests or Fisher's exact test for categorical variables and using Student t-tests for continuous variables. To consider possible confounding variables, we weighted the models on the propensity scores (10), which were obtained by a multivariable logistic regression. To consider possible confounders, we weighted the contribution of individuals according to inverse probability (inverse probability weighting - IPW) of the propensity score (PS) (11). The PS was estimated by a multivariable logistic regression with splines on continuous covariates to ensure the log-linearity assumption. If the splines had an OR greater than 10 or less than 0.1 in univariate analysis, the variable was categorized. Stabilized weights were used in order to obtain a pseudo dataset with a similar sample size to the original one and to estimate the average treatment effect in the entire population (ATE) of the exposure (12). The goodness-of-fit of the model was assessed by graphically checking the positivity assumption (**Supplementary Figure S1**) and studying the standardized differences (**Supplementary Tables S1–S7**). The adjusted survival curves were obtained using the weighted Kaplan-Meier estimator and compared using the adjusted log-rank test (13). To provide a relative measure of the effect, a Cox model was estimated maximizing the partial weighted likelihood and using a robust estimator for the variance (14). The corresponding hazard proportionality was graphically checked by plotting log-minus-log of the survival function for the variable of interest. Statistical analyses were performed using Plug-Stat® software (www.labcom-risca.com/plug-stat) based on the R software (15). Note, to respect the methodology in causal inference, we did not consider the maintenance therapy in the propensity scores as physicians may adapt the treatment according to the initial induction therapy.

Ethics Statement

Data were extracted from the French DIVAT cohort (www.divat.fr, approved by the CNIL, n°914184) consisting of recipients monitored in Nantes. The quality of the DIVAT data bank is validated by an annual cross-center audit. All participants gave informed consent. All patients were included and extracted from the DIVAT database, after informed consent. In order to respect confidential medical information, all data were anonymized before analysis.

RESULTS

Description of the Cohort

The study flow-chart is presented in **Figure 1**. The characteristics of the 183 studied patients at the time of transplantation are presented in **Table 1**. 83 patients were in the BSX group (45.4%) versus 100 in the ATG group (54.6%). The average total dose of thymoglobulin administered was 2.0 mg/kg, and no serious side effects were noted in this population (notably no serum sickness disease). Of note, 49 patients in the ATG group received ≤2.0 mg/kg of thymoglobulin, while the average dosage of the 51 other patients was 2.3 mg/kg of thymoglobulin.

The average recipient age was 58.5 years in the BSX group versus 57.9 years in the ATG group ($p = 0.80$) and a majority of them were male (71.6%). 18% had a preemptive transplantation, 24.6% had history of diabetes and 61.2% received an allograft from an extended criteria donor (ECD) without any significant difference between groups. 41.2% of the recipients had a positive CMV serology (45.5% in the ATG group and 36.1% in the BSX group, $p = 0.20$) and 47.5% of the donors had a positive CMV serology (40.0% in the ATG group and 56.6% in the BSX group, $p = 0.02$).

With respect to steroid maintenance therapy, 31.1% of patients received corticotherapy by month 3 (32.8% in the BSX group and 29.7% in the ATG group, $p = 0.67$) with an average dose of 7 mg/day; and 38.8% at 1 year (38.9% in the BSX group and 38.8% in the ATG group, $p = 0.16$) with an average dose of 7 mg/day. Tacrolimus therapy was the most common immunosuppressant in both groups (95.8% vs. 94.0%, $p = 0.72$ at 3 months and 86.3% vs. 89.4%, $p = 0.56$ at 12 months in the BSX and ATG groups, respectively) with similar trough levels (8.3 ng/mL vs. 8.7 ng/mL at 3 months, $p = 0.76$ and 6.2 ng/mL vs. 6.7 ng/mL at 12 months, $p = 0.19$ in the BSX and ATG groups, respectively). Finally, the use of antiproliferative drugs was similar between patients who underwent an induction by BSX or ATG (respectively 87.6% vs. 92.8% at 3 months, $p = 0.27$ and 78.7% vs. 81.5% at 12 months, $p = 0.67$), **Supplementary Figure S2**.

Patient and Graft Survival

During the follow-up, 18 deaths with a functioning allograft (9 in each group) and 8 returns to dialysis (including 6 in the group BSX) were observed. Median event-free follow-up time was 3.0 years (min: 0.0; max: 5.2). The patient and graft survival, illustrated in **Figure 2A**, was 95% at 1-year post-transplantation (95% CI: 91%; 99%) in ATG group versus 94% in the BSX group (95% CI: 90%; 100%). These results corresponded to an adjusted HR of 1.22 (95% CI: 0.53; 2.80, p-value = 0.63), between patients of the BSX group versus those of the ATG group.

Occurrence of BPAR Episodes and dnDSA

The cumulative adjusted probabilities of the occurrence of a first BPAR is illustrated in **Figure 2B**. Median event-free follow-up time was 3.0 years (min: 0.0; max: 5.2). The value for the ATG group was 5% at 1-year post-transplantation (95% CI: 1%; 9%) versus 11% for the BSX group (95% CI: 4%; 17%). These results corresponded to an adjusted HR of 1.92 (95% CI: 0.77; 4.78, p-value = 0.15), between BSX patients compared to ATG treated patients. Finally, at 1-year post transplantation, 2 patients in the ATG group and 2 patients in the BSX group presented a DSA with MFI >1000. However, the MFI were rather lower in patients from the ATG group (2147 and 1441) compared to the BSX group (4,900 and 14,000).

During the follow-up, 279 biopsies were performed, 150 in the ATG group and 129 in the BSX group.

Overall, and considering all rejection episodes (first and recurrent), there was significantly more biopsies concluding to a BPAR in the BSX group ($n = 22$, representing 17.0% of performed biopsies) than in the ATG group ($n = 11$, representing 7.3% of performed biopsies), $p = 0.0152$, **Supplementary Figure S3**.

In the ATG group, BPAR consisted of 7 Borderline lesions (BL) and 4 T Cell Mediated Rejection (TCMR); these later were all successfully treated except for one patient who died with a functional allograft. Of note, among the 11 patients who presented a BPAR in the ATG group, 5 had a total dose < to 2 mg/kg and 6 a total dose > to 2 mg/kg.

In the BSX group, BPAR consisted of 11 BL, 8 TCMR, 1 Antibody Mediated Rejection (ABMR) and 2 mixed rejection (ABMR + TCMR). While 15 BPAR would have require a treatment, only 13 received it (2 patients were considered too frailty). 6 on 13 treated episodes evolved favorably, 6 were refractory and evolved towards end stage renal disease, and one patient deceased with a functional allograft.

Cumulative Probability of Infectious Complications

During the follow-up, 95 events (including 45 in the group BSX) were observed. Median event-free follow-up time was 3.0 years (min: 0.0; max: 5.0). The cumulative adjusted probabilities of the occurrence of infection is presented in **Figure 2C**. The value for the ATG group was 43% at 1-year post-transplantation (95% CI: 32%; 52%) versus 47% in the BSX group (95% CI: 35%; 57%). These results corresponded to an adjusted HR of 0.99 (95% CI: 0.65; 1.50, p-value = 0.95), between BSX patients versus ATG treated patients.

Cumulative Probability of CMV Viremia

During the follow-up, 30 events (including 14 in the group BSX) were observed. Median event-free follow-up time was 3.0 years (min: 0.0; max: 5.1). The cumulative adjusted probabilities of the occurrence of CMV viremia is presented in **Figure 2D**. The value for the ATG group was 17% at 1-year post-transplantation (95% CI: 9%; 24%) versus 13% for the BSX group (95% CI: 6%; 20%). These results corresponded to an adjusted HR of 0.85 (95% CI: 0.41; 1.78, p-value = 0.6731), between the two groups of patients.

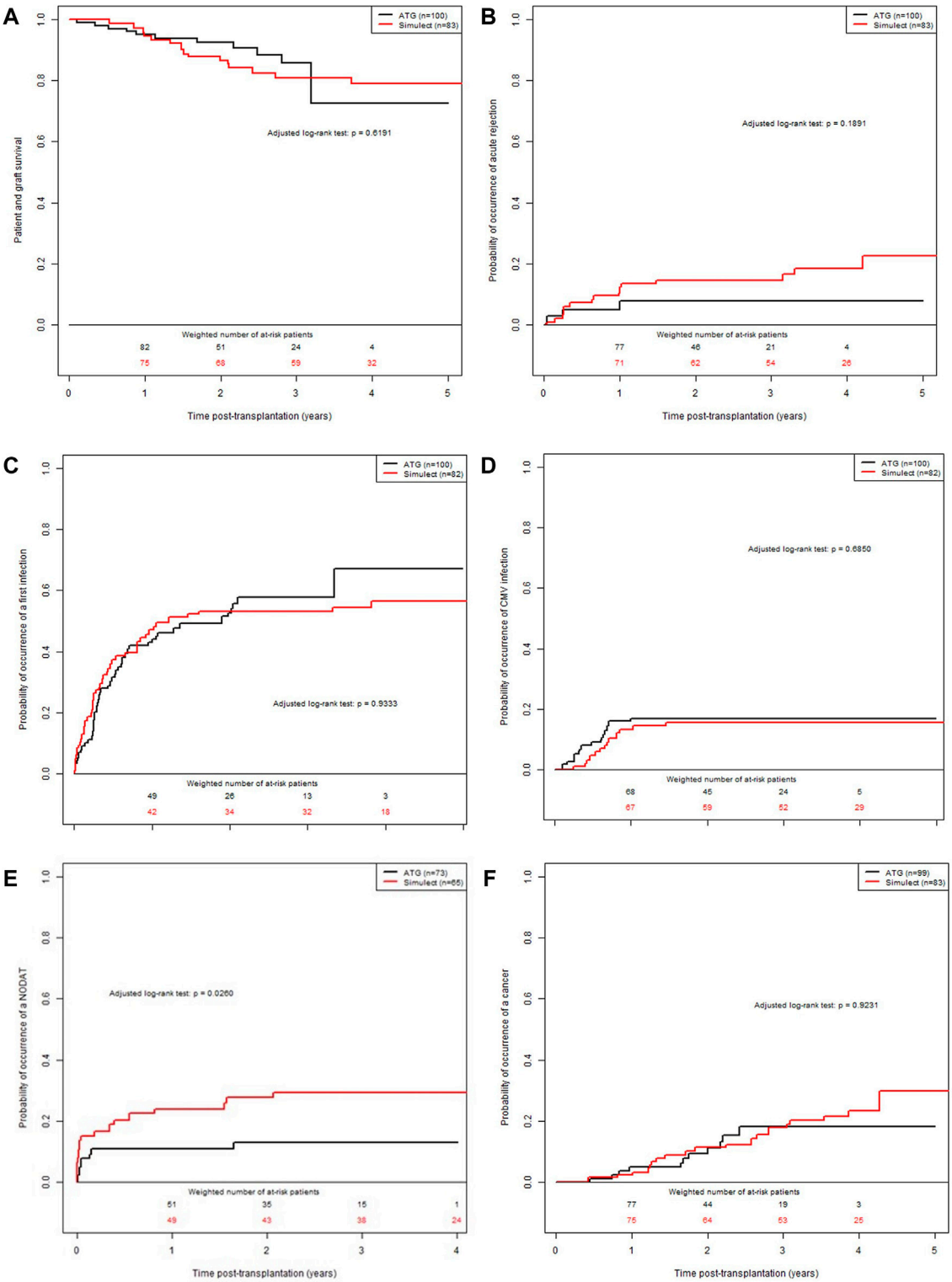


FIGURE 2 | Confounder-adjusted probabilities of events according to the time post-transplantation and the induction therapy. **(A)** patient and graft survival. **(B)** cumulative probability of a first episode of acute rejection episode. **(C)** cumulative probability of infection. **(D)** cumulative probability of CMV replication. **(E)** cumulative probability of PTD. **(F)** cumulative probability of post-transplantation malignancy.

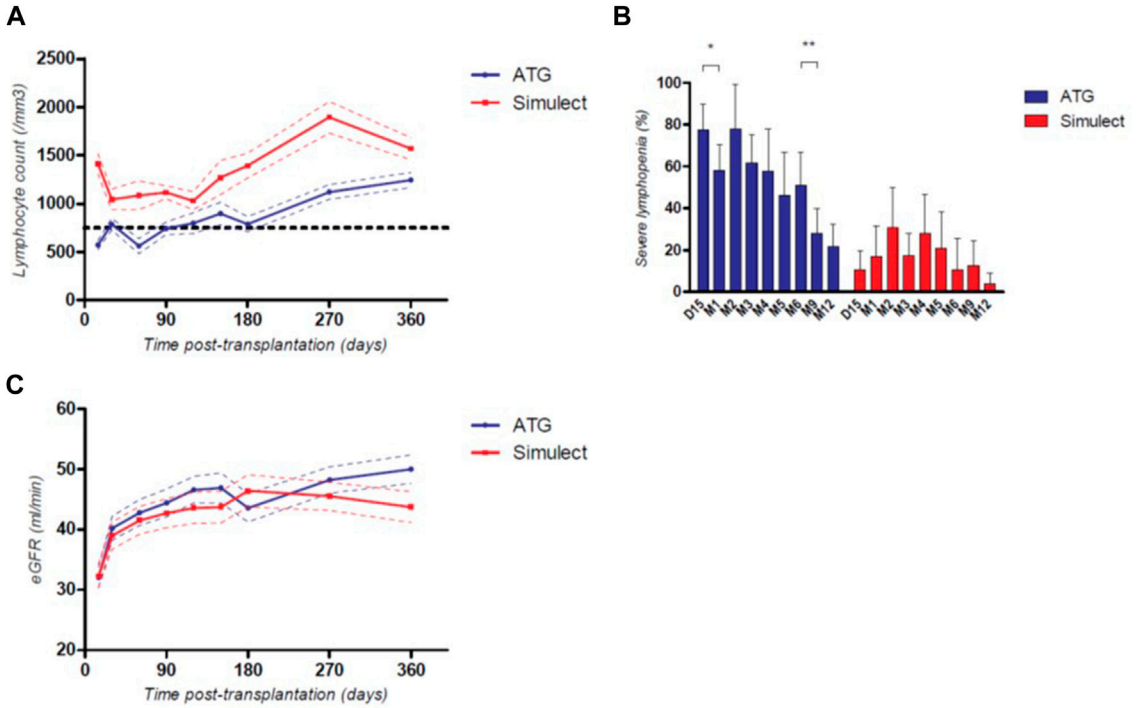


FIGURE 3 | Mean total lymphocyte count **(A)**, percentage of patients with severe lymphopenia (≤750/mm^3) **(B)** and mean eGFR **(C)** during the first-year post transplantation depending on their induction therapy; *: $p < 0.05$; **: $p < 0.01$; continuous variables are represented with their respective standard mean error, categorical variables are represented with their respective 95CI%.

Cumulative Probability of Post-Transplant Diabetes

The patient characteristics at the time of transplantation are presented in **Supplementary Table S6**. Among the 183 patients from the initial cohort, 45 were excluded because they were diabetic before transplantation, leading to a sub-cohort of 138 KTR: 65 patients were treated with BSX (47.1%) and 73 treated with ATG (52.9%). During the follow-up, 27 events were observed (including 18 of the BSX group). Median event-free follow-up time was 3.0 years (min: 0.4; max: 5.1). The cumulative adjusted probabilities of the occurrence of post-transplant diabetes is presented in **Figure 2E**. The value for the ATG group was 11% at 1-year post-transplantation (95% CI: 3%; 18%) versus 24% in the BSX group (95% CI: 13%; 34%). These results corresponded to an adjusted HR of 2.44 (95% CI: 1.09; 5.46, p-value = 0.03), between BSX versus ATG treated patients.

Cumulative Probability of Post-Transplant Malignancy

During the follow-up, 30 events were observed (19 in the group BSX). Median event-free follow-up time was 3.0 years (min: 0.0; max: 5.2). The cumulative adjusted probability of the occurrence of neoplastic (included squamous cell carcinoma and post-transplant lymphoma disease) is presented in **Figure 2F**. The value for the ATG group was 0.04 at 1-year post-transplantation (95% CI: 0.00; 0.08) versus 0.02 for the BSX group (95% CI: 0.00; 0.06). These results corresponded to an adjusted HR at 1.04 (95% CI: 0.48; 2.25, p-value = 0.9162), between BSX and ATG treated patient groups.

Evolution of Lymphocyte Count and Allograft Function

As expected, total lymphocyte counts were significantly lower during the first-year post-transplantation for patients in the ATG group (**Figure 3A**). However, after the sixth month post transplantation, the lymphocyte count in these patients increased, and the percentage of patients with severe lymphopenia (<750/mm^3) decreased significantly (51.1% of patients receiving ATG had a severe lymphopenia at 6-month post transplantation vs. 28.0% at 9-month post transplantation, p = 0.01, **Figure 3B**). In the ATG group, patients who presented a severe lymphopenia at 1-year post-transplantation despite very low doses of thymoglobulin were significantly older (69 years old vs. 53 years old, p = 0.0020). Allograft function was globally similar between ATG and BSX groups, however with a trend to a better eGFR at 1-year post transplantation in patients from the ATG group (50.0 vs. 43.7 mL/min, p = 0.07), **Figure 3C**. Of note, there was no significant difference in the occurrence of delayed graft function between groups (26.2% vs. 29.2% in ATG and BSX groups respectively, p = 0.74).

DISCUSSION

Despite multiple studies characterizing induction therapy in low immunological risk kidney transplant recipients, controversies still exist regarding the best treatment to provide for these patients. Basiliximab is currently recommended by the KDIGO guidelines based on studies performed several years ago comparing BSX to ATG (4), notably because of an increased risk of viral infection due to the prolonged T cell depletion induced by ATG. However, as ATG acts in a dose-dependent manner (6), numerous transplant physicians have decreased its dosage over the years in order to reduce its side effects (17, 18).

We demonstrated the consequence of drastically reduced ATG dosage (average of 2 mg/kg total dose) in non-immunized recipients receiving a first kidney transplant, compared to basiliximab-treated patients, undergoing a similar maintenance therapy. Similarly, previous studies on smaller cohorts reported the safety of a very low dose of ATG in low immunological risk patients (19, 20). Other investigated outcomes related to a low dose of ATG, which however remained higher than our and usually associated with long term steroid therapy (21, 22). In relation to immunological complications, we found that a very low dose of ATG seems safe in this low-risk population with a comparable occurrence of a first episode of rejection. Moreover, we even observed a trend in favor of low doses of ATG. This non-significant difference was probably due to an underpowering of the study, and larger series seems mandatory to confirm this later point. Nevertheless, investigation of all BPAR episodes demonstrated a significant lower number of episodes rejection in patients from the very low ATG group. Moreover, patients from the ATG group had a significantly lower occurrence of post-transplant diabetes. One hypothesis is the different number of treated BPAR among groups, as steroids are known to promote post-transplant diabetes. Other reports evidenced a difference in PTD occurrence depending on the induction, but the exact imputability rather than the consecutive maintenance therapy management is still unclear (6, 23, 24). Finally, the small number of studied events may induce a lack of power and validation data from other cohorts will be of interest. Whilst ATG did not significantly impact allograft survival (1, 25), despite a trend to a better 1-year allograft function, it is well known that allograft rejection negatively impacts long-term kidney transplant outcomes (26, 27). Based on our data, a steroid-sparing strategy appears safe in low-immunological risk patients who received a very low dose of Thymoglobulin and can thus be conduce without increasing the risk of allograft rejection.

The use of ATG by transplant physicians is often accompanied by an apprehension of viral infections. In our cohort, according to a well-controlled prophylaxis, there was no significant difference between global infectious complications, nor CMV viremia, using low doses of ATG compared to an induction with Basiliximab. This is concordant with a previous study by our group where we found no difference in infectious complications (notably CMV viremia) regarding ATG administration in elderly kidney transplant recipients (6). Indeed, CMV viremia may be more related to the presence—or not—of the specific CMV cellular immunity rather than the total lymphocyte count (28).

Obviously, the use of ATG resulted in deep lymphopenia in the first months' post-transplantation. However, the reduced dosage in our cohort led to a higher lymphocyte count at 1-year post transplantation than previously described (29). Moreover, after the sixth month post transplantation, the percentage of patients presenting a severe lymphopenia, which is known to impact patient survival (30), significantly decreased. This shorter time of deep lymphopenia may enhance the use of ATG at a very low dosage in non-immunized kidney transplant recipients, particularly in younger recipients.

Finally, the use of ATG at this very low dose also seemed to permit cost savings for our institution. In France, induction by Simulect® costs around 3,000 euros per patient and 25 mg of thymoglobulin costs around 250 euros. In our cohort, induction by ATG despite basiliximab for non-immunized patients permitted a total saving of 150,000 euros in 5 years (1,500 euros per patient). These results differs from others who found a cost saving using Basiliximab, because the highest dosage of ATG induced more infectious complications (31). However, as we did not perform a cost-effectiveness study assessing all cumulative costs (days of hospitalization, number of consultations, post-transplant complications ...), definitive conclusions are not possible. A recent American cost-effectiveness study revealed that use of ATG appeared to offer cost and outcomes advantages compared to no-induction in kidney transplant recipients (32).

Our study has some limitations, the main one being the relatively small monocentric sample size, with requirement of further validation, ideally based on a randomized clinical trial which remains the gold standard. However, retrospective data from other centers could not have been included as their total ATG dose is considerably higher. Also, based on recent improvements in detecting anti-HLA immunogenicity, determination of low-risk kidney transplant recipients (i.e., total absence of significant anti-HLA antibodies) is now more accurate, and some transplant teams are currently conducting clinical trials to assess the benefit of induction therapy in this low-risk population (33). In our cohort, ATG induced a better prevention of BPAR than Basiliximab, which supports the pursuit of induction therapy for these patients; which however needs to be tailored to provide a very low dose of ATG.

In conclusion, our report highlights that a very low dose of ATG in non-immunized recipients was safe and associated with a lower rate of rejection episodes and post-transplant diabetes, without increasing infectious complications probably because of a reduced duration of deep lymphopenia.

NANTES DIVAT CONSORTIUM

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DATA AVAILABILITY STATEMENT

Complete datasets are available upon reasonable 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

CM: collected the data, participated in the study analysis, and wrote the manuscript. CK: participated in the study analysis. JD: elaborated design and research project, supervised analysis, and critically revised the manuscript. AW: participated in collection and analysis of data, significantly participated in the reviewing process and approved the final version of the manuscript. All authors participated in writing and revising the manuscript.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10816/full#supplementary-material

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Paving the Way for Personalized Medicine in First Kidney Transplantation: Interest of a Creatininemia Latent Class Analysis in Early Post-transplantation

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Plasma creatinine is a marker of interest in renal transplantation but data on its kinetics in the first days following transplantation are scarce. The aim of this study was to identify clinically relevant subgroups of creatinine trajectories following renal transplantation and to test their association with graft outcome. Among 496 patients with a first kidney transplant included in the French ASTRE cohort at the Poitiers University hospital, 435 patients from donation after brain death were considered in a latent class modeling. Four distinct classes of creatinine trajectories were identified: "*poor recovery*" (6% of patients), "*intermediate recovery*" (47%), "good recovery" (10%) and "*optimal recovery*" (37%). Cold ischemia time was significantly lower in the "*optimal recovery*" class. Delayed graft function was more frequent and the number of hemodialysis sessions was higher in the "*poor recovery*" class. Incidence of graft loss was significantly lower in "*optimal recovery*" patients with an adjusted risk of graft loss 2.42 and 4.06 times higher in "*intermediate recovery*" and "*poor recovery*" patients, respectively. Our study highlights substantial heterogeneity in creatinine trajectories following renal transplantation that may help to identify patients who are more likely to experience a graft loss.

Keywords: donation after brain death, chronic kidney disease, latent class mixed model, kydney transplantation, trajectories, creatinine

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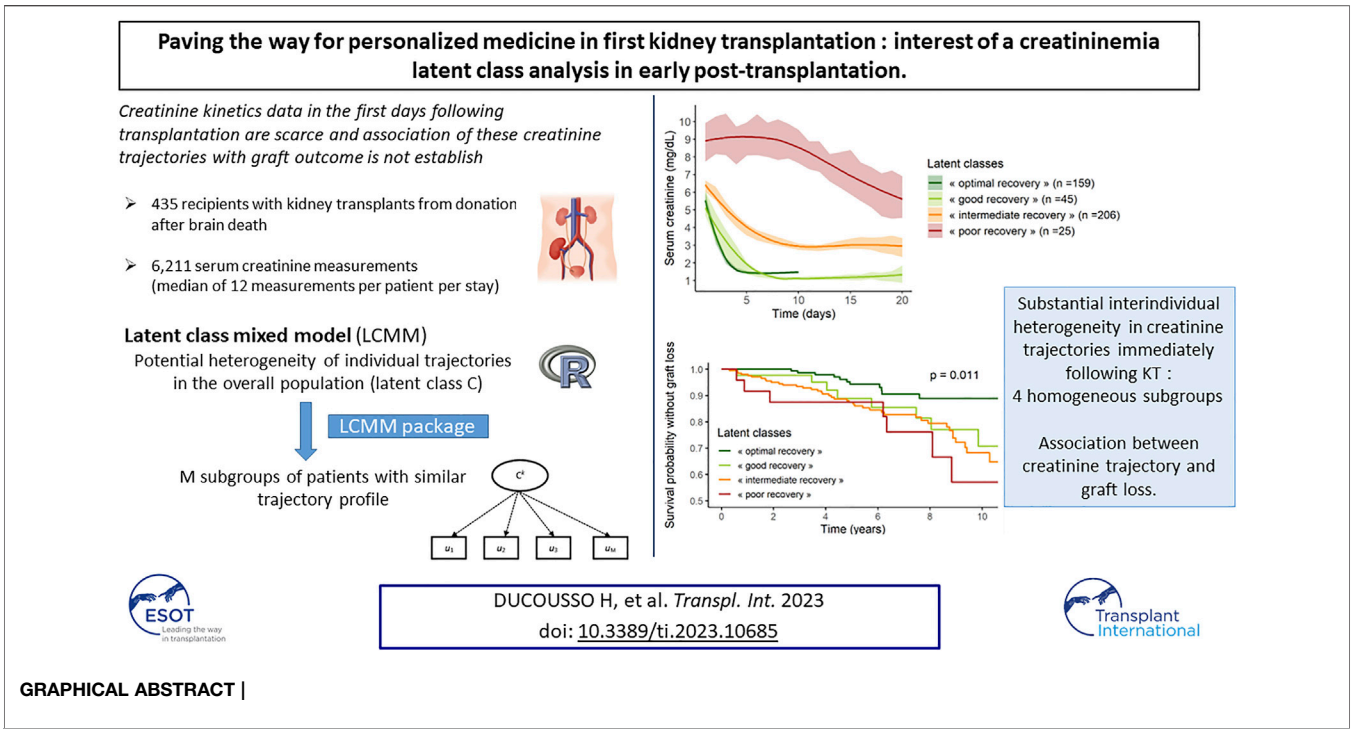
Abbreviations: BIC, Bayesian Information Criterion; BMI, Body Mass Index; CKD, Chronic Kidney Disease; DBD, Death after Brain Death; DGF, Delayed Graft Function; ECD, Expanded Criteria Donor; ESRD, End-Stage Renal Disease; GFR, Glomerular Filtration Rate; GL, Graft Loss; KT, Kidney Transplantation; LCMM, Latent Class Mixed Model.



GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney transplantation (KT) is optimal treatment for end-stage renal disease (ESRD), whether for the patient's survival, his quality of life or the cost of treatment (1,2,3).

Over the last two decades, the number of KTs has increased worldwide, in the number of transplantations from both living donors (4) and cadaveric donors (5,6). This phenomenon is due to an increase of patients with kidney failure, largely linked to longer life expectancy, and to an increase in prevalence of type 2 diabetes (7). As a result, there has been a situation of kidney transplant shortage for several years which led to a rise in the number of patients on KT waiting list (6).

Reducing the need for repeated transplantation by avoiding graft loss (GL) is a major objective. Despite progress in post-transplant management, particularly in terms of immunosuppressive treatments (8,9,10), long-term graft survival has only slightly improved over recent years (10,11), with a median estimated at 14 years in France (7).

Many predictive factors of long-term graft survival have been identified in recent years such as ischemia-reperfusion syndrome (12,13), delayed graft function (DGF) (14,15). Currently, post-transplant recovery assessment of the graft is based on consensual but not very reliable parameters. Plasma creatinine is a marker of interest for the clinician, but data on its kinetics in the first days following KT are scarce. Nadir creatinine and time to reach a creatinine level below 2.83 mg/dL during the transplant patient's hospital stay are parameters commonly used by clinicians since they are considered to be independent prognostic factors for graft survival.

Daily creatinine values during the transplant patient's hospital stay are available and consideration of their dynamic over time could yield additional predictive information.

Many statistical models have been developed to study the evolution of quantitative markers over time. One of them is the latent class mixed model (LCMM), which is an extension of the standard linear mixed model (16). The model computes the heterogeneity of individual trajectories and identifies subgroups of patients with similar trajectory profiles, independently of their observed characteristics. This method has shown its interest in identifying subgroups of trajectories of renal function in a cohort of patients with chronic kidney disease (CKD) (17) and, more recently, in diabetic patients (18,19). While the LCMM has been applied for repeated measures of creatinine or glomerular filtration rate (GFR) (20), it has never been used to describe subgroups of trajectories of renal recovery in the days following transplantation in renal recipients. LCMM could reveal unknown heterogeneity in post-KT trajectories of renal function which could help identify kidney recipients at high risk of GL.

Therefore, our aims were 1) to evaluate the ability of the LCMM to identify subgroups of trajectories of creatinine immediately following renal transplantation, 2) to identify the clinical factors associated with the different trajectories and 3) to test the association between subgroups of trajectories and graft outcomes.

MATERIALS AND METHODS

Study Population

Patients were included if they met the following criteria: age ≥18 years; first KT from donation after brain death

(DBD) between January 2008 and July 2017 at the Poitiers university hospital and enrollment in the French ASTRE cohort (21). Non-inclusion criteria were living donors, donors after cardiac death (DCD), retransplantation, early graft failure, defined by recipient death or GL within 3 months, or primary non-function.

All patients signed informed consent before inclusion. The study follows the STROBE statement and was conducted following the principles of the Declaration of Helsinki and approved by the CNIL (Authorization number DR-2012- 518 [ps2]).

Expanded criteria donors (ECD) were defined by age >60 years or by age between 50 and 59 years with the association of two comorbidities: hypertension, creatinine ≥1.511 mg/dL or a cerebrovascular death (22,23,24,25).

Clinical and biological data

The demographic, clinical and biological data of the recipients and donors were collected in the ASTRE database. Post-KT hemodialysis session follow-up data was obtained from the electronic health record system by the Department of Medical Information of Poitiers University Hospital. Serum creatinine was determined daily, by enzymatic method in the Biochemistry department of Poitiers University Hospital. To establish the renal function trajectories following transplantation, we considered all the creatinine determinations during the hospital stay from 24 h after the transplant to hospital discharge.

Follow-Up and Outcome

Patients were followed up as were all the patients included in the French ASTRE cohort and the subsequent information was reported in the ASTRE database.

The main study outcome was occurrence of a graft loss from 3-month post-KT to last updating in July 2020 or to patient death. Secondary outcomes were creatinine values and HLA antibodies at 1-year post transplant, as well as HLA antibodies and acute cell rejection at latest news.

Statistical analysis

LCMM separating the population into homogeneous subgroups of individuals according to their creatinine trajectory was computed with the R "lcmm" package. Models with one to five classes were estimated and the number of classes was chosen by minimization of the Bayesian Information Criterion (BIC) and according to the size of identified subgroups.

Regarding the expected non-linearity of the trajectories, polynomial functions of time (2–5) were considered in the models. The models were adjusted for cold ischemia time, ECD, preemptive transplant and hemodialysis session completion, which were factors known to influence the dynamics of creatinine trajectories. Recipients were *a posteriori* classified in the class where they had the highest class-membership probability. The LCMM results are reported following the published "Guidelines for Reporting on Latent Trajectory Studies" (26).

We performed a sensitivity analysis excluding creatinine values recorded within the 24 h following a hemodialysis session.

Clinical characteristics were compared between the different latent classes identified by Chi2 or Fisher tests for qualitative variables and by ANOVA or Kruskal-Wallis test for quantitative variables. Dunn's *post hoc* tests were performed in case of significance.

Association between latent classes and GL at one year and at the date of latest news was tested by a chi2 test. Time to GL was described by Kaplan-Meier curves in each latent class and compared using a logrank test. Adjustment for all factors found associated with GL was performed using a Cox model. Other qualitative outcomes were tested by a chi2 test and creatine values at one year were tested by ANOVA or Kruskal-Wallis test.

Statistical analyses were performed with R software (R Foundation for Statistical Computing, Vienna, Austria, version 4.0.3).

The results were considered significant for p values < 0.05.

RESULTS

In the ASTRE cohort, 496 patients underwent a rank 1 KT at the Poitiers University Hospital. After applying the selection criteria, 435 recipients (258 males, 177 females aged 56 years) with kidney transplants from a DBD donor were included in the analysis (**Figure 1**).

Recipients, donors and transplants background characteristics are displayed in **Table 1**.

Between 24 h post-KT and hospital discharge, 6,211 serum creatinine measurements were recorded in the 435 patients corresponding to a median of 12 (11,12,13,14,15) measurements per patient per stay in agreement with median hospital stay duration. As shown in **Table 2**, delayed graft function was observed in 80 patients (18%) and a median number of 2 (2,3,4) hemodialysis sessions was needed during the hospital stay.

During median follow-up of 73 (48–107) months, 68 patients (16%) lost their graft. At year one 7 GL (2%) and 39 T cell acute rejections (9%) were recorded.

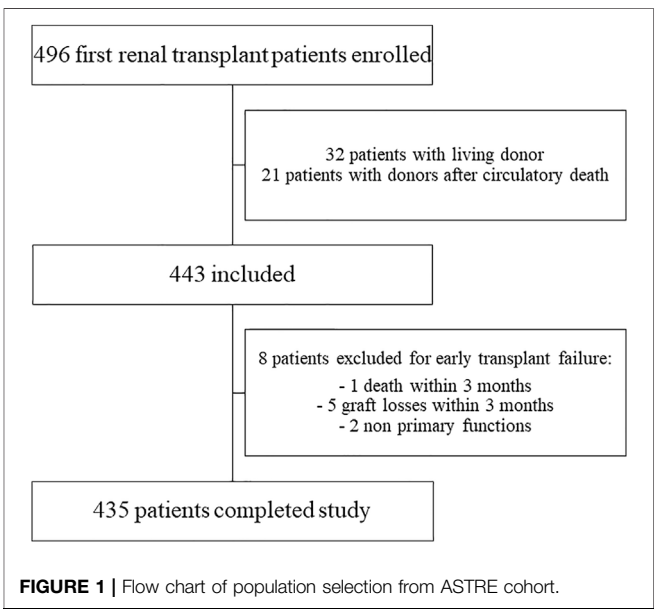


FIGURE 1 | Flow chart of population selection from ASTRE cohort.

TABLE 1 | Recipient, donor and transplant background characteristics in the overall population according to latent classes.

	All (*n* = 435)	Class 1 (*n* = 25) *poor recovery*	Class 2 (*n* = 206) *intermediate recovery*	Class 3 (*n* = 45) *good recovery*	Class 4 (*n* = 159) *optimal recovery*	*p*
Recipient characteristics						
Age (years)	56 (47–63)	58 (49–65)	56 (48–64)	57 (46–62)	56 (45–62)	0.3989
Male	258 (59%)	23 (92%)	129 (63%)	16 (36%)	90 (57%)	**<0.0001**
BMI, kg/m^2	25 (22–28)	27 (24–31)	25 (23–29)	24 (20–28)	24 (21–27)	**0.0009**
CV disease	389 (89%)	24 (96%)	181 (88%)	38 (84%)	146 (92%)	0.2988
Hypertension	368 (85%)	22 (88%)	175 (85%)	36 (80%)	135 (85%)	0.8224
Diabetes	61 (14%)	2 (8%)	31 (15%)	7 (16%)	21 (13%)	0.8334
PRA status						**0.0459**
0 to 25	367 (84%)	22 (88%)	177 (66%)	31 (69%)	137 (86%)	
25 to 50	20 (5%)	2 (8%)	10 (5%)	2 (4%)	6 (4%)	
50 to 100	48 (11%)	1 (4%)	19 (9%)	12 (3%)	16 (10%)	
Preemptive transplantation	80 (18%)	3 (12%)	38 (18%)	4 (9%)	35 (22%)	0.2009
Waiting time on dialysis (month) (n = 355)	15 (5–29)	15 (9–30)	14 (4–28)	19 (10–34)	16 (2–29)	0.1005
Pre-operative hemodialysis	73 (16%)	7 (28%)	26 (13%)	14 (31%)	26 (16%)	**0.0109**
Donor characteristics						
Age (years)	55 (45–64)	56 (52–64)	56 (48–65)	54 (40–62)	55 (40–64)	0.1389
Male	255 (59%)	18 (72%)	116 (56%)	30 (67%)	91 (57%)	0.3049
BMI, kg/m^2	25 (22–29)	25 (21–29)	25 (23–29)	25 (22–29)	25 (22–28)	0.6943
Expanded criteria donor	202 (46%)	12 (48%)	104 (50%)	16 (36%)	70 (44%)	0.2729
Hypertension	137 (31%)	7 (28%)	81 (39%)	11 (24%)	38 (24%)	**0.0082**
Diabetes	32 (7%)	3 (12%)	17 (8%)	2 (4%)	10 (6%)	0.5918
Transplantation characteristics						
Cold ischemia time (hours)	15 (12–18)	16 (13–18)	15 (13–18)	17 (13–22)	14 (12–17)	**0.0093**
Hypothermic machine perfusion	113 (26%)	5 (20%)	53 (26%)	7 (16%)	48 (30%)	0.2259
HLA-A mismatches						0.314
0	56 (13%)	3 (12%)	28 (14%)	10 (22%)	15 (9%)	
1	236 (54%)	16 (64%)	114 (55%)	21 (47%)	85 (53%)	
2	143 (33%)	6 (24%)	64 (31%)	14 (31%)	59 (37%)	
HLA-B mismatches						0.7986
0	24 (6%)	2 (8%)	9 (4%)	4 (9%)	9 (6%)	
1	201 (46%)	10 (40%)	97 (47%)	22 (49%)	72 (45%)	
2	210 (48%)	13 (52%)	100 (49%)	19 (42%)	78 (49%)	
HLA-DR mismatches						0.5899
0	106 (24%)	2 (7%)	50 (24%)	11 (25%)	43 (28%)	
1	240 (55%)	17 (63%)	116 (56%)	25 (57%)	82 (53%)	
2	89 (20%)	8 (30%)	42 (20%)	8 (18%)	31 (20%)	

Numbers are median (25th-75th percentiles) or effective (%); BMI, body mass index; PRA, panel reactive antibody.
The bold values are the significant values.

TABLE 2 | Post-transplant characteristics in the overall population according to latent classes.

	All (*n* = 435)	Class 1 (*n* = 25) *poor recovery*	Class 2 (*n* = 206) *intermediate recovery*	Class 3 (*n* = 45) *good recovery*	Class 4 (*n* = 159) *optimal recovery*	*p*
Postgraft creatininemia at 24 h, mg/dL	6 (4–8)	9 (7–11)	6 (5–8)	5 (4–7)	6 (4–7)	**<0.0001**
Delayed graft function	80 (18%)	19 (76%)	43 (21%)	6 (13%)	12 (8%)	**<0.0001**
Nb. of hemodialysis sessions	2 (2–4)	4 (3–8)	2 (1–4)	2 (2–3)	2 (2–2)	**0.0057**
Time to reach the 2.83 mg/dL threshold (days)	3 (2–5)	33 (27–39)	4 (3–8)	3 (2–4)	3 (2–3)	**<0.0001**
Nadir creatinine	138 (103–202)	396 (302–450)	184 (140–237)	88 (71–106)	107 (90–135)	**<0.0001**
Patient's hospital stay duration	12 (11–15)	20 (17–24)	13 (12–17)	13 (11–15)	11 (10–12)	**<0.0001**

Numbers are median (25th-75th percentiles) or effective (%); Delayed Graft Function: the need for a hemodialysis session in the first 7 days post-transplantation.
The bold values are the significant values.

Subgroups of Serum Creatinine Trajectories

The choice of the various LCMM parameters are detailed in **Supplementary Table S1**. The best model, with the lowest BIC and a high entropy, determined 4 latent classes, whether there were adjustment variables or not (**Supplementary Table S2**). The mean posterior probabilities of class membership for individuals ranged from 81% to 87%, indicating good overall discrimination ability regarding the adjusted model.

The mean trajectories of creatinine in the four latent classes are shown on **Figure 2**.

Class 1 ("*poor recovery*") contained 25 patients (6%) with high values of serum creatinine at day 1, around 9 mg/dL, which decreased slowly over time, ending at 5.5 mg/dL at day 20 post KT. Class 2 ("*intermediate recovery*") was the more numerous one with 206 patients (47%) showing moderate values of creatinine at day 1, around 6.5 mg/dL, which fell to 3 mg/dL within 10 days post-KT. Patients classified in Class 3 ("*good recovery*", n = 45, 10%) and patients in Class 4 ("*optimal recovery*," n = 159, 37%) had the lowest creatinine values at day 1, 5, and 5.5 mg/dL respectively; they both dropped to approximately 1.1–1.3 mg/dL at day 8 and day 4 respectively. After this pronounced diminution, "good recovery" patients' creatinine remained stable, whereas "*optimal recovery*" patients' creatinine showed a small rise of approximately 0.5 mg/dL from day 5 to day 10.

LCMM performed on the database excluding creatinine values reported within the 24 h following a hemodialysis session gave comparable results, identifying four classes with similar trajectories (n = 23 in Class 1, n = 204 in Class 2, n = 44 in Class 3 and n = 164 in Class 4).

Characteristics of recipients and donors according to renal function trajectories

The characteristics of the recipients, donors and transplants according to the 4 latent classes are shown in **Table 1**. Recipients did not differ in age, history of hypertension, diabetes or immunization. Men and recipients with high body mass index (BMI) were more represented in the "*poor recovery*" class (92% men, median BMI value: 27 (24,25,26,27,28,29,30,31) kg/m^2). Frequency of pre-emptive transplantation and waiting time on dialysis did not differ between classes.

Characteristics of donors did not differ between the four groups, including ECD criteria.

Regarding transplant characteristics, the median of cold ischemia time was different between the four classes (p = 0.0093); it was significantly lower in the "*optimal recovery*" group compared to the "good recovery" group (p = 0.0074).

Multivariate polynomial logistic regression showed that donor hypertension and cold ischemia time were the only independent predictors of latent classes with R-squared (R2) value not exceeding 6%.

In post-KT (**Table 2**), 24-hour creatinine values were significantly lower in "*optimal recovery*" and "good recovery" classes than in the others. DGF was more frequent and the number of hemodialysis sessions was higher in "*poor recovery*" recipients than in the others. Moreover, the time to reach the 2.83 mg/dL creatinine threshold and the nadir creatinine were significantly higher for "*poor recovery*" recipients. The latter had a significantly longer hospital stay than the others.

Prognosis values of latent classes of creatinine

The proportion of recipients with GL during follow-up was significantly lower in "*optimal recovery*" patients ($p_{chi\text{-}deux}$ = 0.0015) (**Table 3**). Considering time to event, incidence of GL was also lower in "*optimal recovery*" patients ($p_{logrank}$ = 0.011) (**Figures 3**, **4**). After adjustment for all factors found associated with GL in a univariate analysis (**Supplementary Table S3**), trajectories of creatinine were still significantly associated with GL: Compared with "*optimal recovery*" class, the risk of graft loss was 2.42 times higher for patients classified in "*intermediate recovery*" class and 4.06 times higher for patients classified in "*poor recovery*" class (**Table 4**). of note, nadir creatinine during hospital stay did not remain associated with GL when latent classes were considered in the model (p > 0.9).

Serum creatinine levels one year after KT were significantly different between the four classes with higher levels in "*poor recovery*" recipients (median of 2 (1,2) mg/dL) and lower levels in "*optimal recovery*" and "good recovery" recipients (medians of 1 (1) mg/dL and 1 (1,2) mg/dL respectively).

Biopsy-proven acute T cell rejection was found in 39 patients (9%) one year after KT and did not differ between the four latent classes.

Anti-HLA antibodies were not found significantly different between the latent classes neither at one year after KT nor at the latest news.

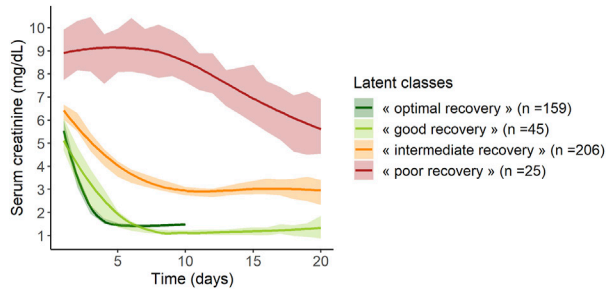


FIGURE 2 | Mean trajectories of serum creatinine according to the 4 classes of the mixed latent class model (n = 435). Lines represent mean values and areas represent their 95% confidence intervals.

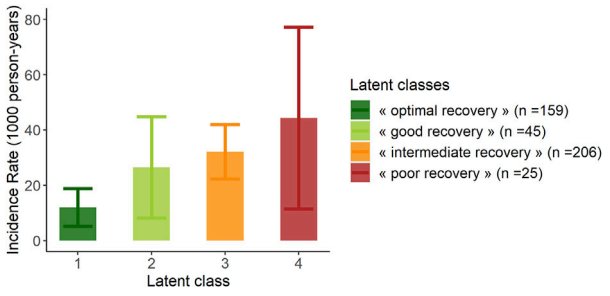


FIGURE 3 | Incidence rate of graft loss at latest news according to the 4 classes of the mixed latent class model (n = 435). Error bars represent confidence intervals.

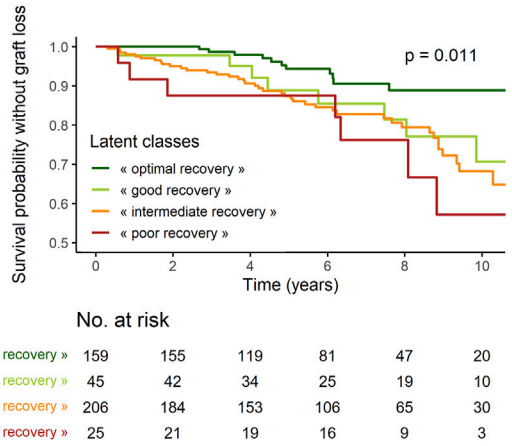


FIGURE 4 | Survival probabilities without graft loss according to latent classes.

DISCUSSION

In the present study, using a retrospective cohort of 435 kidney transplant recipients, we performed a LCMM and highlighted substantial heterogeneity in renal function recovery immediately following KT from DBD. Interestingly, we identified four distinct trajectories showing non-linear decrease in daily creatinine levels during the initial hospitalization for transplantation.

These trajectories showed heterogeneity between patients regarding both creatinine level at day 1 and speed of the decrease, which reflects graft recovery. Patients with "optimal" or "good recovery" represented 37% and 10% of the study population, respectively, and their creatinine values dropped quickly. Patients with "*intermediate recovery*" had higher creatinine values at day one and a slower decrease over time, and they were the most numerous, representing 47% of the population. Finally, 6% of patients presented a trajectory markedly different from the others, with high initial values and a delayed and small decrease ("*poor recovery*"). The slight increase observed in the trajectory of the "*optimal recovery*" group could be due to the renal toxicity of the calcineurin inhibitors.

Although baseline characteristics were poor predictors of these trajectories, some clinically relevant variables such as cold ischemia time were associated with the trajectories. Interestingly, kidney donor hypertension, but not ECD, was also associated with the trajectories: in the "*optimal recovery*" trajectory, the lowest cold ischemia time and rate of hypertensive donors were found. The use of machines perfusion did not differ significantly between the different latent classes.

The four trajectories were also associated with relevant post-KT characteristics. As expected, patients classified in the "*poor recovery*" class were more likely to have DGF. Furthermore, they had the highest nadir creatinine and the longest time in days needed to reach the creatinine threshold of 2.83 mg/dL. "Good" and "*optimal recovery*" trajectories did not differ significantly in terms of time needed to reach the 2.83 mg/dL creatinine threshold.

Regarding graft outcome, the four trajectories were found to be an independent prognostic factor. In the literature, there are a few risk scores predicting graft loss, which include mainly donor and baseline transplant characteristics as prognostic factors (27,28). Cold ischemia time is identified as a risk factor for reduced graft and patient survival and DGF (29). The hypothermic perfusion machine also reduces the DGF incidence from 38% to 24% (30). Moreover, it is demonstrated that the survival of grafts at 1 year is 92.3% for grafts placed on a hypothermic machine perfusion, versus 80.2% for grafts with cold storage (31). During the first few months post-KT, 3-, 6- and 12-month eGFR have been reported as prognostic values (32) such as creatinine and eGFR trajectories beyond one year of follow-up (20,33). Nevertheless, none of these risk scores takes into consideration the evolution of daily creatinine levels in early post-KT. To our knowledge, our study is the first to characterize renal recovery trajectories immediately following KT, using the latent class analysis tool. Consideration of these daily determinations of creatinine during the hospital stay could be more relevant to predict GL than isolated values such as nadir of creatinine level.

The use of LCMM for the identification of trajectories of renal function evolution has raised a lot of interest in recent years (17,34). This novel unsupervised approach overlooks prior

TABLE 3 | Graft outcomes in the overall population according to the different latent classes.

	All (n = 435)	Class 1 (*n* = 25) *poor recovery*	Class 2 (*n* = 206) *intermediate recovery*	Class 3 (*n* = 45) *good recovery*	Class 4 (*n* = 159) *optimal recovery*	*p*
Follow-up duration (months)	73 (48–107)	83 (49–106)	72 (48–107)	85 (48–118)	73 (48–98)	0.773
Graft loss, n (%)						
latest news	68 (16%)	7 (28%)	41 (20%)	8 (18%)	12 (8%)	**0.0015**
at year 1	7 (2%)	2 (8%)	4 (2%)	1 (2%)	0 (0%)	**0.0264**
Postgraft creatininemia at year 1, mg/dL (n = 428)*	1 (1–2)	2 (1–2)	2 (1–2)	1 (1–1)	1 (1–2)	**<0.0001**
T cell acute rejection at year 1, n (%)	39 (9%)	4 (16%)	18 (9%)	2 (4%)	15 (9%)	0.4478
Anti-HLA antibodies, n (%)						
at year 1 (n = 412)*	55 (13%)	4 (16%)	22 (11%)	6 (13%)	23 (14%)	0.5726
latest news (n = 415)*	76 (17%)	6 (24%)	37 (18%)	7 (16%)	26 (16%)	0.6626

*Numbers are median (25th-75th percentiles) or effective (%); *7 patients lost their graft during the first year.*
The bold values are the significant values.

TABLE 4 | Predictive factors of graft loss: Cox regression model.

Characteristic	HR[a]	95% CI[a]	*p*
Pre-emptive graft			**0.028**
No	—	—	
Yes	0.43	0.18, 1.00	
BMI donor, kg/m^2	1.05	1.01, 1.10	**0.014**
Expanded criteria donor			**<0.001**
No	—	—	
Yes	2.54	1.49, 4.35	
HLA-B mismatches			**0.033**
0	—	—	
1	0.29	0.12, 0.68	
2	0.41	0.18, 0.93	
Latent classes			**0.010**
« *optimal recovery* »	—	—	
« good recovery »	2.07	0.84, 5.11	
« *intermediate recovery* »	2.42	1.26, 4.63	
« *poor recovery* »	4.06	1.59, 10.4	

[a]*HR, adjusted Hazard Ratio; CI, confidence interval.*
The bold values are the significant values.

assumptions on the evolution of creatinine as a means of classifying patients. Our model was fitted on several covariates known to influence renal recovery, and this statistical fitting results in independency between these covariates and the identified trajectories. More specifically, the impact of hemodialysis sessions was taken into account as a time-dependent variable since it induces a drop in creatinine levels that gradually affects the creatinine levels, up to 24–48 h after the session. In a sensitivity analysis, we applied the model on the subset of data free of post-hemodialysis creatinine values and obtained four latent classes comparable to the previous ones.

Regarding the clinical applicability of our models, the type of recovery for a given recipient could be estimated outside the framework of this study. Indeed, the available hospital stay creatinine values and the covariates present in the model could be calculated in a dedicated application which would indicate the class membership probabilities. Hence, transplant recipients at higher risk of graft loss could be identified upon their hospital discharge, enabling personalized follow-up frequency and better management.

This study has several limitations, particularly its monocentric design. Selection bias regarding recipients and donors might have conditioned class-membership. External validation is needed to confirm the general applicability of a LCMM. Moreover, an analysis conducted on Maastricht-III and living donors could be of particular interest.

In conclusion, substantial inter-individual heterogeneity in creatinine trajectories immediately following KT was highlighted. Insight into a recovery class might lead to more precise estimation of risk of graft loss for a given patient and be conducive to optimized post-KT management.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Commission nationale de l'informatique et des libertés (CNIL). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HD, SR, and AT contributed to conception and design of the study. FD and IC organized the database. IC performed the statistical analysis. HD wrote the first draft of the manuscript. MV, TK, SR, IC, and AT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10685/full#supplementary-material

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Renal Vessel Extension With Cryopreserved Vascular Grafts: Overcoming Surgical Pitfalls in Living Donor Kidney Transplant

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In LDKT, right kidneys and kidneys with anomalous vascularization are often deferred because of concerns on complications and vascular reconstructions. To date, only few reports have examined renal vessel extension with cryopreserved vascular grafts in LDKT. The aim of this study is to investigate the effect of renal vessel extension on short-term outcomes and ischemia times in LDKT. From 2012 to 2020, recipients of LDKT with renal vessels extension were compared with standard LDKT recipients. Subset analysis of rights grafts and grafts with anomalous vascularization, with or without renal vessel extension, was performed. Recipients of LDKT with (n = 54) and without (n = 91) vascular extension experienced similar hospital stays, surgical complications and DGF rates. For grafts with multiple vessels, renal vessel extension granted a faster implantation time (44±5 vs. 72±14 min), which resulted comparable to that of standard anatomy grafts. Right kidney grafts with vascular extension had a faster implantation time compared to right kidney grafts without vascular lengthening (43±5 vs. 58±9 min), and a comparable implantation time to left kidney grafts. Renal vessel extension with cryopreserved vascular grafts allows faster implantation time in right kidney grafts or grafts with anomalous vascularization, maintaining similar surgical and functional outcomes.

Keywords: living donor kidney transplant, renal vessel extension, cryopreserved vascular allografts, right kidney donation, abnormal kidney vascularization

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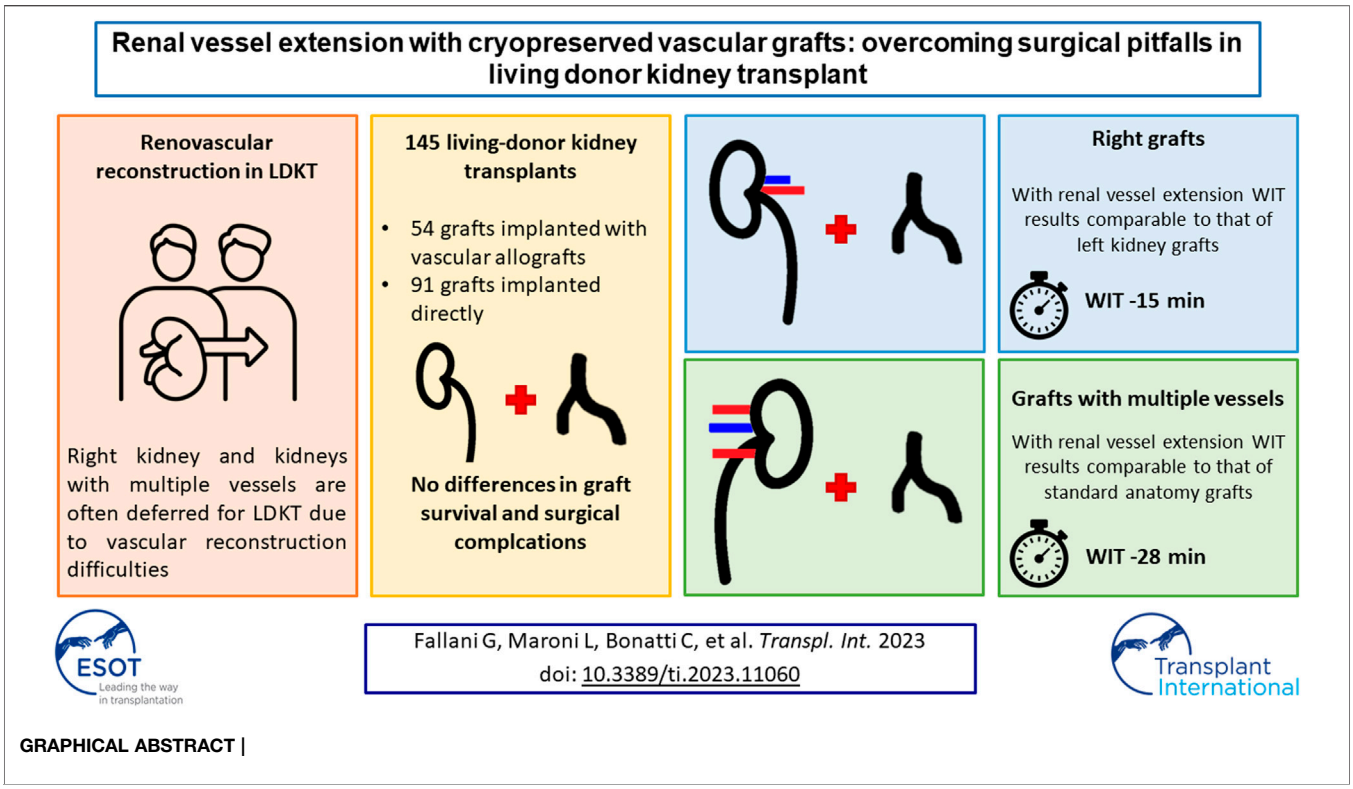
Abbreviations: ASA, American society of anesthesiologists; BMI, body mass index; CACI, Charlson age-comorbidity index; CCI®, comprehensive complication index®; CIT, cold ischemia time; DBD, donor after brain death; DCD, donor after cardiac death; DGF, delayed graft function; DMSO, dimethyl sulfoxide; ESRD, end-stage renal disease; HLA, human leukocyte antigen; LDKT, living donor kidney transplant; LOS, length of stay; POD, postoperative day; PRBC, packed red blood cells; RPMI, Roswell Park Memorial Institute growth medium; WIT, warm ischemia time.



GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney transplantation is the treatment of choice for end-stage renal disease (ESRD). The introduction of living donor kidney transplantation (LDKT) has allowed to extend the donor pool and to face the issues related to increasing waiting lists before transplant; nevertheless, the blanket is still short.

Although the choice of the kidney to procure should be based on "leaving the better kidney to the donor," left donor nephrectomy is still largely predominant and often represents the default option (1). Right donor nephrectomy has been discouraged in the past, as the shorter renal vein increases the risk of renal vein thrombosis and subsequent graft loss, as well as the risk of bleeding from the inferior vena cava during procurement (2–4). A parallel surgical issue in the field of LDKT is represented by grafts with multiple arteries or veins, whose utilization has raised concerns over increased difficulty in performing more vascular anastomoses, prolonged ischemia time to at least a portion of the graft when performing more than one arterial anastomosis, poorly controlled hypertension in the post-transplant period after segmental graft ischemia or infarctions and increased rates of ureteral complications (5). Therefore, as short-term outcomes of multiple vessel renal grafts have been demonstrated worse due to higher complication and DGF rates (6), the presence of anomalous vascularization still represents a common reason to defer an organ for living donor nephrectomy.

Extension of the renal vessels during bench surgery has been advocated as a strategy to increase the straightforwardness of kidney implantation, to reconstruct multiple vessels, to facilitate positioning of the graft into the iliac fossa without kinking or twisting of the renal vessels (especially in case of obese recipients or narrow surgical field) and subsequently to reduce post-transplant complications. Different techniques have been reported in medical literature, including the use of donor gonadal vein, recipient internal iliac artery patches or latero-lateral anastomoses (7–9).

To date, few reports have addressed in detail the use of renal vessel lengthening in living donor kidney transplant. This study aims to evaluate the effects of renal vessel extension with third-party cryopreserved vascular allograft on procedural straightforwardness and short-term complications of LDKT.

PATIENTS AND METHODS

Study Design

Consecutive recipients of living donor kidney transplantation from January 2012 to December 2020 were prospectively enrolled for the study. The population was stratified according to the use of third-party cryopreserved vascular allografts to extend the renal vessels before implantation. The clinical, demographic, and intraoperative characteristics of the two groups were compared. Subset analysis of grafts with multiple vessels, with or without vascular extension (compared to standard anatomy grafts without vascular extension as reference), and right kidney grafts with or without vascular extension (compared to left kidney grafts without vascular extension as reference) was performed to assess ischemia times and operative times.

Informed consent for study enrollment has been obtained from the subjects. The study was conducted in accordance with the principles of the 1964 Helsinki Declaration and its following revisions and was approved by the Institutional Review Board of the promoting center (Comitato Etico—Area Vasta Emilia Centro, CE-AVEC, protocol n. 312/2021/Oss/AOUBo).

Data Collection

A database was created for the purpose of this study in a typical Excel (Microsoft Corporation—Redmond, WA, United States) spreadsheet. To ensure consistency in data entry, free-text entries were avoided as much as possible, and the admitted values for each relevant variable were restricted to a predefined cluster. Before the statistical tests were conducted the database was checked for quality, and in cases of missing, unexpected, or ambiguous data, the data were re-examined.

The data collected concerned: age, sex, comorbidities, body mass index (BMI), indications for LDKT, side and characteristics of the donated kidneys, warm and cold ischemia times, duration of surgery, postoperative complications, length of postoperative hospital stay (LOS), hospital readmissions, and postoperative mortality.

Variable Definition, Outcome Measurements and Surgical Technique

To allow the individual risk stratification in association with concomitant diseases and physical status, the Charlson Age–Comorbidity Index (CACI) (10, 11) and the American Society of Anesthesiologists (ASA) classification (12) were used.

HLA compatibility was evaluated on loci A, B and DR as per our center policy, and graded from 0 to 6 depending on the number of compatible alleles.

Graft with multiple vessels were defined as kidneys with multiple arteries or veins that needed to be re-implanted either because they vascularized the lower pole and ureter or because their clamping during procurement demonstrated significant parenchymal ischemia; therefore, grafts with small accessory veins or arteries ligated during procurement or bench surgery were considered as standard anatomy grafts.

Vascular allografts were retrieved either from DBD or DCD by dedicated vascular surgeons and transferred to the tissue bank. After dissection and cleansing from the surrounding tissues, vessels were decontaminated in an antibiotic solution and stored in cryoprotective solution (RPMI added with 20% human albumin and 10% DMSO), then gradually cooled up to −140/−180°C. The vascular allografts were then preserved in nitrogen vapors, where they could be stored for a maximum time of 5 years, and shipped upon request to the operating room for bench reconstruction.

Left kidneys were procured with open retroperitoneal approach through a lumbar mini-incision (10 cm), while right kidneys were procured with transperitoneal approach through a right subcostal mini-laparotomy (10 cm) (13). Renal vessel extension was performed during bench surgery according to graft's anatomy and side, to the length of the graft's vessels and to the transplant surgeon preference. Kidney transplant was performed on the same side of the procured graft unless specific contraindications occurred (e.g., severe atheromasia of the external iliac artery, previous kidney transplant on that side or narrow surgical field due to native kidney polycystosis); all transplants in this series were performed by the same surgeon (MR). All grafts were stored in sterile ice-cold perfusion solution until implantation. Cold ischemia time (CIT) was defined as the time interval from cold *ex-vivo* flushing to the beginning of implantation. Warm ischemia time (WIT) was defined as the time interval from the beginning of implantation to reperfusion; the interval between clamping of the renal vessels in the donor and cold *ex-vivo* flushing was not accounted in the warm ischemia time as it was shorter than 2 min in all cases.

Complications were defined as any deviation from the normal postoperative course that is not inherent to the procedure and that does not imply failure to cure (14). For each patient, the postoperative complications were graded individually according to the Clavien–Dindo classification of surgical complications (14) and summarized with the Comprehensive Complication Index (CCI®) (15). Vascular complications were defined as any immediate or delayed complication derived from malposition, thrombosis, pseudoaneurysm, stenosis or technical failure of the vascular anastomosis that required surgical or angiographic correction.

Delayed graft function (DGF) was defined as post-transplant acute kidney injury that required dialysis in the first 7 days after transplant (16). Thirty-day acute rejection was defined as deterioration of graft function with histologically proven stigmata of rejection (according to the Banff classification of kidney allograft pathology) occurring up to POD 30 (17).

Graft loss was defined as either re-listing for transplantation or resumption of dialytic treatment.

Textbook outcome achievement was defined according to the definition proposed by Halpern et al. (18).

Statistical Analysis

Categorical variables were presented as number and percentages, while continuous variables were presented as mean ± standard deviation or median and interquartile range (IQR) depending on their distribution. Categorical variables were compared through χ^2 test or Fisher's exact test depending on the numerousness of the sample, and continuous variables were compared through Student's t test of Kruskall-Wallis one-way analysis of variance depending on their distribution. Variables with $p < 0.10$ and/or clinically relevant were put in a binary logistic regression model for multivariable analysis. Survival curves were plotted through the Kaplan-Meier estimators and compared through Log-Rank test. Differences of p-value <0.05 were considered significant.

All statistical analyses were performed with IBM SPSS version 26 (IBM Corporation—Armonk, NY, United States).

RESULTS

From January 2012 to December 2020, 145 recipients of LDKT were enrolled for the study. Overall, 43 (29.7%) right donor nephrectomies were performed. Fifty-four grafts (37.2%)

underwent renal vessel extension with third-party cryopreserved vascular grafts, while 91 grafts (62.8%) were implanted without vascular extension. Among the 54 extended vessel grafts, 24 (44.4%) required the use of a venous allograft, 20 (37%) required the use of an arterial allograft and 10 (18.6%) required both venous and arterial allografts. Of the 64 vascular grafts employed, 7 (10.9%) were venous patches, 21 (32.8%) arterial patches, 27 (42.2%) were venous conduits and 9 (14.1%) arterial conduits.

Demographics and Preoperative Characteristics

Recipients of kidneys that underwent renal vessel extension were comparable in terms of age, sex, and BMI to recipients of standard kidneys. Indications for LDKT varied between the two groups, with less cases of polycystic kidney disease and primary glomerulonephritis in the renal vessel extension group ($p = 0.006$ for all indications). Grafts with vascular extension were procured from older donors (57 ± 12 vs. 51 ± 10 years, $p = 0.001$), had a higher percentage of right kidneys (55.6% vs. 14.3%, $p < 0.001$) and more often had multiple vessels (79.6% vs. 24.2%, $p < 0.001$). Preoperative characteristics are summarized in **Table 1**.

Intra- and Postoperative Characteristics

As grafts with vascular extension came more often from the right side, transplantation was performed more often in the right iliac fossa (53.7% vs. 24.2%, $p < 0.001$). Also, grafts with vascular allograft extension underwent longer CIT and total ischemia times (respectively 148 ± 53 vs. 107 ± 53 min, $p < 0.001$ and 192 ± 43 vs. 156 ± 52 min, $p < 0.001$); on the other side WIT was shorter in the renal vessel extension group (43 ± 5 vs. 53 ± 11 min, $p < 0.001$). Operative time was shorter in the group without vascular extension (209 ± 44 vs. 228 ± 54 min, $p = 0.019$). Intraoperative complications occurred in three cases in the group with vascular extension (5.6% vs. 0, $p = 0.050$), all unrelated to vascular anastomoses or vessel allograft utilization (one case of bleeding from a subcapsular renal hematoma, one case of bleeding from the renal hilum, one case of bleeding from the uretero-vescical anastomosis which required its remaking), as well as one case of postoperative bleeding from the arterial anastomosis (1.9% vs. 0, $p = 0.372$). Post-operative complications, PRBC transfusions, CCI®, delayed graft function rates, creatinine at discharge, length of hospital stay, and textbook outcome achievement rates were comparable among the two groups. Intra- and postoperative characteristics are summarized in **Table 2**.

Overall and Graft Survival

Recipients were followed for a median of 45 months after transplant [IQR: 30–71 months]; overall survival was 100% for both cohorts. Two late graft losses were observed, one for each cohort and both related to biopsy-proven primary disease recurrence on the transplanted kidney (focal segmental glomerulosclerosis in one case and IgA nephropathy in the other); graft survival curves resulted comparable between recipients of graft with and without renal vessel extension ($p = 0.333$, **Table 3**; **Figure 1**).

Subset Analysis of Grafts With Multiple Vessels

Operative time, total ischemia time, CIT and WIT were compared among multiple vessel grafts with and without vascular extension (MVG-RVE and MVG), and with multiple vessel grafts with vascular extension and standard anatomy grafts without vascular extension (SAG).

Multiple vessel grafts with renal vascular extension had similar operative times compared to multiple vessel grafts without renal vascular extension (239 ± 53 vs. 225 ± 30 min, $p = 0.542$), but longer compared to standard anatomy grafts (239 ± 53 vs. 207 ± 45 min, $p = 0.001$). WIT was shorter in MVG-RVE compared to MVG and SAG (44 ± 5 vs. 77 ± 14 min, $p < 0.001$ and 44 ± 5 vs. 48 ± 8 min, $p = 0.002$, respectively), although the mean difference between MVG-RVE and SAG was only 4 minutes. CIT was longer in MVG-RVE compared both to MVG and SAG (145 ± 39 vs. 104 ± 77 min, $p = 0.041$ and 145 ± 39 vs. 107 ± 52 min, $p < 0.001$, respectively. Total ischemia time was comparable between MVG-RVE and MVG (189 ± 41 vs. 176 ± 69, $p = 0.506$), but longer in MVG-RVE compared to SAG (189 ± 41 vs. 155 ± 51 min, $p < 0.001$). These results are summarized in **Table 4**.

Subset Analysis of Right Kidney Grafts

Operative time and ischemia times were also compared among right kidney grafts with and without renal vascular extension (RKG-MVE and RKG, respectively), and with left kidney grafts without vascular extension (LKG).

Operative times were comparable between right kidney grafts with renal vascular extension and right kidney grafts without renal vascular extension and group 5, and between right kidney grafts with renal vascular extension and left kidney grafts without renal vascular extension (223 ± 63 vs. 203 ± 53 min, $p = 0.327$ and 223 ± 63 vs. 209 ± 43, $p = 0.204$, respectively). WIT was shorter in RKG-RVE compared to RKG and LKG (43 ± 5 vs. 58 ± 9 min, $p < 0.001$ and 43 ± 5 vs. 48 ± 10 min, $p = 0.014$, respectively); nevertheless, the mean difference between RKG-RVE and LKG was 5 minutes. CIT was longer in RKG-RVE compared both to RKG and LKG (154 ± 50 vs. 103 ± 71 min, $p = 0.010$ and 154 ± 50 vs. 107 ± 50 min, $p < 0.001$, respectively. Total ischemia time was comparable between RKG-RVE and RKG (198 ± 50 vs. 161 ± 72, $p = 0.064$), but longer in RKG-MVE compared to LKG (198 ± 50 vs. 156 ± 48 min, $p < 0.001$). These results are summarized in **Table 5**.

Multivariable Analysis of Factors Associated With Prolonged Warm Ischemia Time

As shown in **Table 6**, among factors possibly related to WIT (dichotomized at 45 min) only renal vessel extension resulted associated to a WIT inferior to 45 min at univariable analysis (45.7% vs. 29.3%, $p = 0.041$). Upon multivariable analysis, renal

TABLE 1 | Preoperative variables.

Variables	LDKT with renal vascular extension (*n* = 54)	LDKT without renal vascular extension (*n* = 91)	*p*
Recipient age in years, mean ± SD	45 ± 14	42 ± 12	0.208
Sex			0.666
Female, n (%)	20 (37)	37 (40.7)	
Male, n (%)	34 (63)	54 (59.3)	
BMI in kg/m^2, mean ± SD	22.9 ± 3.3	23.4 ± 3.5	0.370
Charlson Comorbidity Index, median [IQR]	0 [0–2]	0 [0–1]	0.749
Indication for KT			**0.006**
Kidney polycystic disease, n (%)	8 (14.8)	24 (26.4)	
Tubulo-interstitial nephropathy, n (%)	12 (22.2)	19 (20.9)	
Glomerulonefritis, n (%)	15 (27.8)	34 (37.4)	
Diabetic nephropathy, n (%)	1 (1.9)	3 (3.3)	
Hypertensive nephropathy, n (%)	0	3 (3.3)	
Other, n (%)	18 (33.3)	8 (8.8)	
Relationship with donor			0.197
Parent/children, n (%)	21 (38.9)	32 (35.2)	
Sibling, n (%)	10 (18.5)	28 (30.8)	
Spouse/partner, n (%)	20 (37)	30 (33)	
Other, n (%)	3 (5.6)	1 (1.1)	
Donor age in years, mean ± SD	57 ± 12	51 ± 10	**0.001**
AB0 incompatibility, n (%)	8 (14.8)	10 (11)	0.499
HLA (A/B/DR) compatibility			0.148
0, n (%)	11 (20.4)	6 (6.6)	
1, n (%)	4 (7.4)	12 (13.2)	
2, n (%)	7 (13)	12 (13.2)	
3, n (%)	22 (40.7)	33 (36.3)	
4, n (%)	6 (11.1)	12 (13.2)	
5, n (%)	2 (3.7)	6 (6.6)	
6, n (%)	2 (3.7)	10 (11)	
Pre-emptive KT, n (%)	21 (38.9)	28 (30.8)	0.318
Haemodiayisis before KT, n (%)	27 (50)	52 (57.1)	0.404
Peritoneal dialysis before KT, n (%)	9 (16.7)	21 (23.1)	0.357
Dialysis duration in years, median [IQR]	1 [0–2]	1 [0–2]	0.570
Previous KT, n (%)	5 (9.3)	6 (6.6)	0.747

Bold values highlight statistical significance.

vessel extension resulted protective from prolonged WIT (OR 0.29, $p = 0.004$) while right kidney grafts resulted predictive of prolonged WIT (OR = 2.86, $p = 0.022$).

DISCUSSION

The gap between potentially transplantable kidneys and patients with ESRD who would benefit from transplant is one of the key issues of modern time kidney transplantation and has serious consequences on the morbidity and mortality of transplant candidates (19). The progressively increasing imbalance between donors and recipients has urged to expand the donor pool, both enrolling marginal living donors and adopting techniques to recondition marginal cadaveric grafts (20, 21). LDKT is crucial for the future sustainability of kidney transplantation programs, as it both allows to relieve transplant waiting lists and grants superior outcomes for the recipients, especially in terms of reduced DGF rates (22, 23). Although the rate of living donation is constantly increasing, its proportion remains small compared to the size of waiting lists (19). Therefore, not only living donation should be promoted and given awareness, but the process of kidney procuring from living donors should be further optimized, maintaining donor safety as the key priority.

To achieve this aim, it is essential to address surgical pitfalls in living donor procurement, such as the shortness of renal vein in right donor nephrectomy and the increased WIT in grafts with anomalous vascularization. Although reports on renal vein thrombosis and arterial kinking after right LDKT are limited and often come from older studies (2–5), right donor nephrectomy still has a prevalence around 20% in most series of living donor nephrectomies and is often influenced by transplant centers expertise (13, 24, 25) Also grafts with multiple vessels still raise concern, as the necessity of multiple arterial anastomoses implies a longer WIT, whose association with delayed graft function is well established both in deceased and living donor KT (26–32). In the field of LDKT, renal vessel lengthening may represent a technical solution both for right kidney grafts with short veins and for grafts with anomalous vascularization, although most of the reports in literature are anecdotal and only few focus on technical and surgical outcomes of the procedure (7, 33, 34). Nevertheless, over the years, many different techniques have been proposed for renovascular reconstruction in LDKT (7, 8, 35). For what concerns the right renal vein, additional length might be obtained through

TABLE 2 | Intraoperative and postoperative variables.

Variables	LDKT with renal vascular extension (*n* = 54)	LDKT without renal vascular extension (*n* = 91)	*p*
Donated kidney side			**<0.001**
Left, n (%)	24 (44.4)	78 (85.7)	
Right, n (%)	30 (55.6)	13 (14.3)	
Multiple vessels, n (%)	40 (74.1)	6 (6.6)	**<0.001**
Multiple veins, n (%)	9 (16.7)	4 (4.4)	**0.013**
Multiple arteries, n (%)	30 (55.6)	1 (1.1)	**<0.001**
Both multiple veins and arteries, n (%)	1 (1.9)	1 (1.1)	1
Number of veins, median [range]	1 [1–3]	1 [1–2]	**0.012**
Number of arteries, median [range]	2 [1–3]	1 [1–2]	**<0.001**
Transplant side			**<0.001**
Left, n (%)	25 (46.3)	69 (75.8)	
Right, n (%)	29 (53.7)	22 (24.2)	
Induction therapy			0.455
Basiliximab and steroids, n (%)	41 (75.9)	75 (82.4)	
ATG and steroids, n (%)	11 (20.4)	15 (16.5)	
Other	2 (3.7)	1 (1.1)	
Total ischemia time, mean ± SD	192 ± 43	156 ± 52	**<0.001**
Cold ischemia time in minutes, mean ± SD	148 ± 43	107 ± 53	**<0.001**
Warm ischemia time in minutes, mean ± SD	43 ± 5	50 ± 11	**<0.001**
Operative time, mean ± SD	228 ± 54	209 ± 44	**0.019**
Intraoperative complications, n (%)	3 (5.6)	0	0.050
Postoperative complications, n (%)	20 (37)	39 (42.9)	0.490
CCI®, 75th percentile	20.9	8.7	0.783
Vascular complications, n (%)	1 (1.9)	0	0.372
Urinary complications, n (%)	0	0	—
ICU readmission, n (%)	1 (1.9)	5 (5.5)	0.412
DGF, n (%)	0	2 (2.2)	0.529
Creatinine at discharge in mg/dL, mean ± SD	1.29 ± 0.44	1.24 ± 0.36	0.432
30-day acute rejection, n (%)	5 (9.3)	7 (7.7)	0.762
Urinary catheter upon discharge, n (%)	0	0	—
Length of hospital stay, median [IQR]	10 [7–15]	10 [9–15]	0.163
30-day readmission, n (%)	7 (13)	20 (22)	0.178
Textbook outcome achievement, n (%)	35 (64.8)	52 (57.1)	0.362

Bold values highlight statistical significance.

TABLE 3 | Graft survival analysis between grafts with and without renal vessel extension.

Population	1-year survival rate (%)	2-year survival rate (%)	5-year survival rate (%)	*p*
Grafts with renal vessel extension	100	100	100	0.333
Grafts without renal vessel extension	100	100	98.6	

deeper dissection of hilar structures during bench surgery, or through iliac vein transposition and eventual internal iliac vein ligation. On the other hand, multiple renal vessels are commonly reconstructed during bench surgery through end-to-side or pantaloon anastomoses. The choice to perform one type of reconstruction or another is usually dependent on the spatial configuration of the vessels, but surgeon's experience and preferences have a relevant impact in the decision making. The comparation of different techniques to achieve more length or less anastomoses on the surgical field is far from the intent of this study; however, it is undoubtable that both renal vessel extension with vascular grafts (either autologous or heterologous) and deeper surgical dissection (either on the recipient or on the graft during bench surgery) carry a risk of complications (e.g., bleeding, thrombosis of the anastomoses, unnoticed lesions on the graft pelvis).

The results of this study show that renal vessel extension with cryopreserved vascular grafts allows to perform LDKT with right kidney or multiple vessel grafts granting comparable warm ischemia time to left or standard anatomy grafts, without increasing vascular complications or DGF rates in the recipients. The first obvious result from the analysis of data is the shorter warm ischemia time in the group of grafts with vessel lengthening (43 ± 5 vs. 50 ± 11 min, $p < 0.001$), although a mean difference of 7 minutes may not imply a true clinical difference. As expected, grafts with renal vessel extension have undergone a longer cold ischemia time, possibly related to a longer bench surgery, and a longer total ischemia time. For what concerns operative time, transplants of kidney grafts with vascular extension had longer operative times: this result may be consequent to a more accurate positioning of the graft after completion of the anastomoses, to avoid kinking of the longer reconstructed renal vessels.

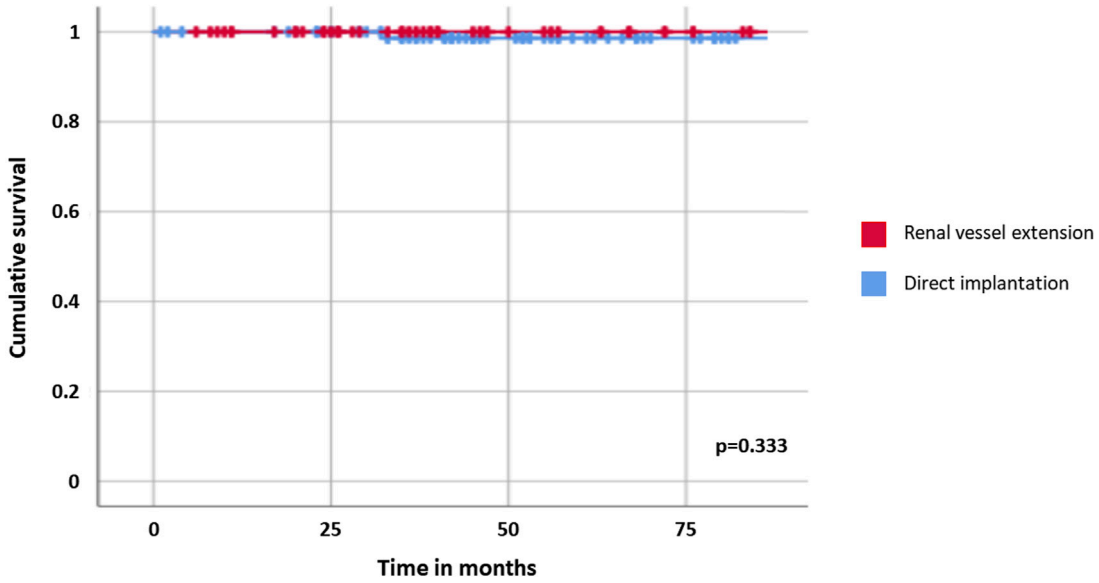


FIGURE 1 | Graft survival in kidney transplants with and without renal vessel extension.

TABLE 4 | Comparation of multiple vessel grafts with or without vascular extension and standard anatomy grafts.

Variables	Multiple vessel grafts with renal vascular extension (MVG-RVE, *n* = 40)	Multiple vessel grafts without renal vascular extension (MVG, *n* = 6)	Standard anatomy grafts (SAG, *n* = 85)	*p*	
				MVG-RVE vs. MVG	MVG-RVE vs. SAG
WIT in minutes, mean ± SD	44 ± 5	72 ± 14	48 ± 8	**<0.001**	**0.002**
CIT in minutes, mean ± SD	145 ± 39	104 ± 77	107 ± 52	**0.041**	**<0.001**
Total ischemia time in minutes, mean ± SD	189 ± 41	176 ± 69	155 ± 51	0.506	**<0.001**
Operative time in minutes, mean ± SD	239 ± 53	225 ± 30	207 ± 45	0.542	**0.001**

Bold values highlight statistical significance.

TABLE 5 | Comparation of right kidney grafts with or without vascular extension and left kidney grafts.

Variables	Right kidney grafts with renal vascular extension (RKG-MVE, *n* = 30)	Right kidney grafts without renal vascular extension (RKG, *n* = 13)	Left kidney grafts without renal vascular extension (LKG, *n* = 78)	*p*	
				RKG-MVE vs- RKG	RKG-MVE vs. LKG
WIT in minutes, mean ± SD	43 ± 5	58 ± 9	48 ± 10	**<0.001**	**0.014**
CIT in minutes, mean ± SD	154 ± 50	103 ± 71	107 ± 50	**0.010**	**<0.001**
Total ischemia time in minutes, mean ± SD	198 ± 50	161 ± 72	156 ± 48	0.064	**<0.001**
Operative time in minutes, mean ± SD	223 ± 63	203 ± 53	209 ± 43	0.327	0.204

Bold values highlight statistical significance.

Focusing on grafts with multiple vessels, the use of renal vessel extension techniques was demonstrated to be associated to a reduced WIT compared both with multiple vessels grafts without vascular lengthening (44 ± 5 vs. 72 ± 14 min) and to standard anatomy grafts without vascular lengthening (44 ± 5 vs. 48 ± 8 min), although the mean difference of 4 min with the latter may not imply a clinical significance. Nevertheless, the use of cryopreserved vascular grafts granted the possibility to

TABLE 6 | Multivariable analysis of factors associated with prolonged implantation time.

Variables	Univariable analysis			Multivariable analysis	
	WIT≤45' (*n* = 70)	WIT>45' (*n* = 75)	*p*	OR [95% C.I.]	*p*
Recipient BMI in kg/m^2, mean ± SD	23.6 ± 3.5	22.8 ± 3.4	0.172	0.91 [0.82–1.01]	0.069
Right kidney graft, n (%)	18 (25.7)	25 (33.3)	0.315	2.86 [1.17–7.00]	0.022
Multiple arteries, n (%)	17 (24.3)	14 (18.7)	0.410	2.60 [0.72–9.31]	0.143
Renal vessel extension, n (%)	32 (45.7)	22 (29.3)	**0.041**	**0.29 [0.13–0.67]**	**0.004**

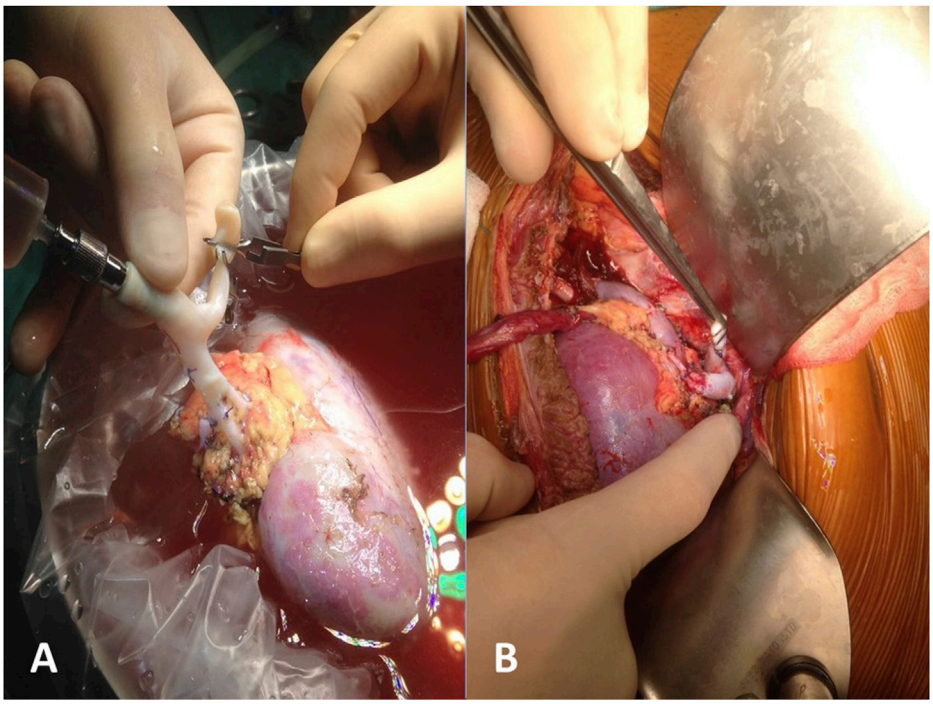


FIGURE 2 | Reconstruction of multiple arteries with a single cryopreserved vascular graft: bench surgery **(A)** and reimplantation **(B)**.

perform single vascular anastomoses, thus determining warm ischemia times comparable to those of standard anatomy grafts (**Figure 2**). This result has paramount importance given the known detrimental effects of total warm ischemia time and implantation time on graft function (26–32), and the association between multiple arteries and longer WIT [6, 32]. As expectable, renal vessel extension implied a longer CIT, both compared to multiple vessel graft without lengthening and to standard anatomy grafts; nevertheless, total ischemia time in multiple vessel grafts with and without vascular lengthening was comparable, as the time spent on bench surgery was regained through a faster implantation time. Operative time was comparable for grafts with multiple vessels regardless of vascular lengthening procedures, but shorter compared to standard anatomy grafts; this is probably due to the time spent in positioning the graft after implantation, which represents a key step of the intervention whether there are extended vessels or multiple vessels without extension.

For what concerns right kidney grafts, it is notable that in this case series the percentage of right donor nephrectomies (43/145, 29.7%) was above the majority of those previously reported in medical literature, which lays around 20% (5, 24, 25). The subanalysis of right kidney grafts showed that renal vessel extension was associated with shorter WIT, both compared to right grafts without vascular lengthening (43 ± 5 vs. 58 ± 9 min) and to left grafts (43 ± 5 vs. 48 ± 10 min); also in this case, the mean difference of 5 min between right kidney grafts with vascular extension and left kidney grafts may not imply an actual clinical significance. It is however evident that an extended renal vein allows for a bigger surgical field (**Figure 3**), and subsequently a more straightforward implantation, which by these results is comparable to that of left kidney grafts. Again, cold ischemia time was longer for right kidney grafts with vascular extension compared to standard kidney grafts and left kidney grafts; however, total ischemia time resulted comparable among right kidney grafts with and without vascular lengthening, stating that the faster implantation time in grafts with vascular extension allowed to retrieve the adjunctive time spent during bench surgery. Since the first LDKT in 1954, more than half a million donor nephrectomies have been performed, and—although selection and management of the donors have improved substantially—kidney

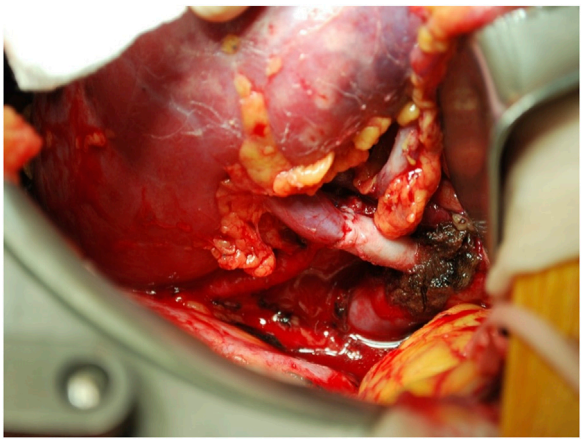


FIGURE 3 | Extension of right renal vein through cryopreserved vascular graft.

donors still carry an increased risk of end-stage renal disease (36, 37). In the process of LDKT, donor short- and long-term outcomes must be guaranteed at all costs, and—given the risk of renal failure after donation—the choice of the kidney to donate should not be affected by technical issues. As per the results of this study, renal vessel extension appears to be a useful tool to address surgical pitfalls in LDKT with right kidney grafts or grafts with anomalous vascularization, allowing to facilitate the vascular anastomoses and to reduce implantation time, making it comparable to left/standard anatomy grafts without compromising graft function and short-term outcomes. Notably, in this study only the use of right kidney grafts resulted predictive of a longer implantation time, while the use of renal vessel extension techniques was proved to be protective from a longer implantation time. Also, recipient BMI and anomalous vascularization, which have been reported as predictors of a longer WIT in a retrospective study by Hellegering et al. (32), did not result predictive of a prolonged implantation time in this case series, probably due to the small numbers and to the mitigating effect of routine use of third-party vascular allografts to lengthen the renal vessels. However, in clinical practice it is not uncommon to encounter the case of a potential donor with left kidney aberrant vascularization and a standard anatomy right kidney. In those cases, our experience suggests procuring the right kidney rather than the left, as right renal vein extension is technically simpler than reconstructing multiple vessels, and it allows similar results in terms of implantation time.

Finally, despite possible concerns on immunological sensitization due to third-party vascular allograft utilization [related to both HLA- and AB0-incompatibility)], in our series we did not observe any delayed vascular complication (i.e., arterial stenosis).

This study has some limitations. First, its prospective case–cohort design and the limited number of patients affected our ability to draw definite conclusions. Also, lack of data regarding the length of the procured renal vessels has limited the possibility to conduct a more precise analysis on variables associated with renal vessel extension procedures. Finally, although all transplants have been performed by the same surgeon (MR), the variability of techniques used for renal vessel extension may have impacted on the final results of the study.

CONCLUSION

Living donation is a fundamental tool to provide sustainability to kidney transplantation programs, and also to grant better functional and long-term results to the recipients. As of today, anomalous kidney vascularization and short right renal veins still represent an issue in the field of LDKT, being often key factors in the process of choosing which kidney to donate, together with parameters of donor kidney function. Renal vessel extension through third-party vascular allografts represents a useful mean to address surgical pitfalls of LDKT with right grafts or grafts with anomalous vascularization, allowing a shorter implantation time while maintaining adequate functional and surgical short-term outcomes in the recipients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico—Area Vasta Emilia Centro. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GF: research conception, research design, data acquisition, data analysis, manuscript draft, and critical revision. LMar: research design, data acquisition, data analysis, manuscript draft, and critical revision. CB, FC, and EP: research design, data acquisition, data analysis, and critical revision. GC, MBuz, VC, FV, FP, BP, LMau, FO, VB, FT, MBus, CZ, and MD: data acquisition and critical revision. GL and MR: research design, data analysis, manuscript draft, and critical revision.

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CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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Hypothermic Perfusion Modifies the Association Between Anti-LG3 Antibodies and Delayed Graft Function in Kidney Recipients

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We previously reported associations between autoantibodies to the LG3 fragment of perlecan, anti-LG3, and a higher risk of delayed graft function (DGF) in kidney transplant recipients. Here, we aimed to determine whether some factors that modulate ischemia-reperfusion injury (IRI) can modify this association. We performed a retrospective cohort study in kidney transplant recipients in 2 university-affiliated centers. In 687 patients, we show that high pre-transplant anti-LG3 are associated with DGF when the kidney is transported on ice (odds ratio (OR): 1.75, 95% confidence interval 1.02–3.00), but not when placed on hypothermic perfusion pump (OR: 0.78, 95% CI 0.43–1.37). In patients with DGF, high pre-transplant anti-LG3 are associated with a higher risk of graft failure (subdistribution hazard ratio (SHR): 4.07, 95% CI: 1.80, 9.22), while this was not the case in patients with immediate graft function (SHR: 0.50, 95% CI 0.19, 1.29). High anti-LG3 levels are associated with a higher risk of DGF in kidneys exposed to cold storage, but not when hypothermic pump perfusion is used. High anti-LG3 are also associated with a higher risk of graft failure in patients who experience DGF, a clinical manifestation of severe IRI.

Keywords: delayed graft function, hypothermic perfusion, kidney allograft, ischemia reperfusion injury, autoantibodies, anti-LG3

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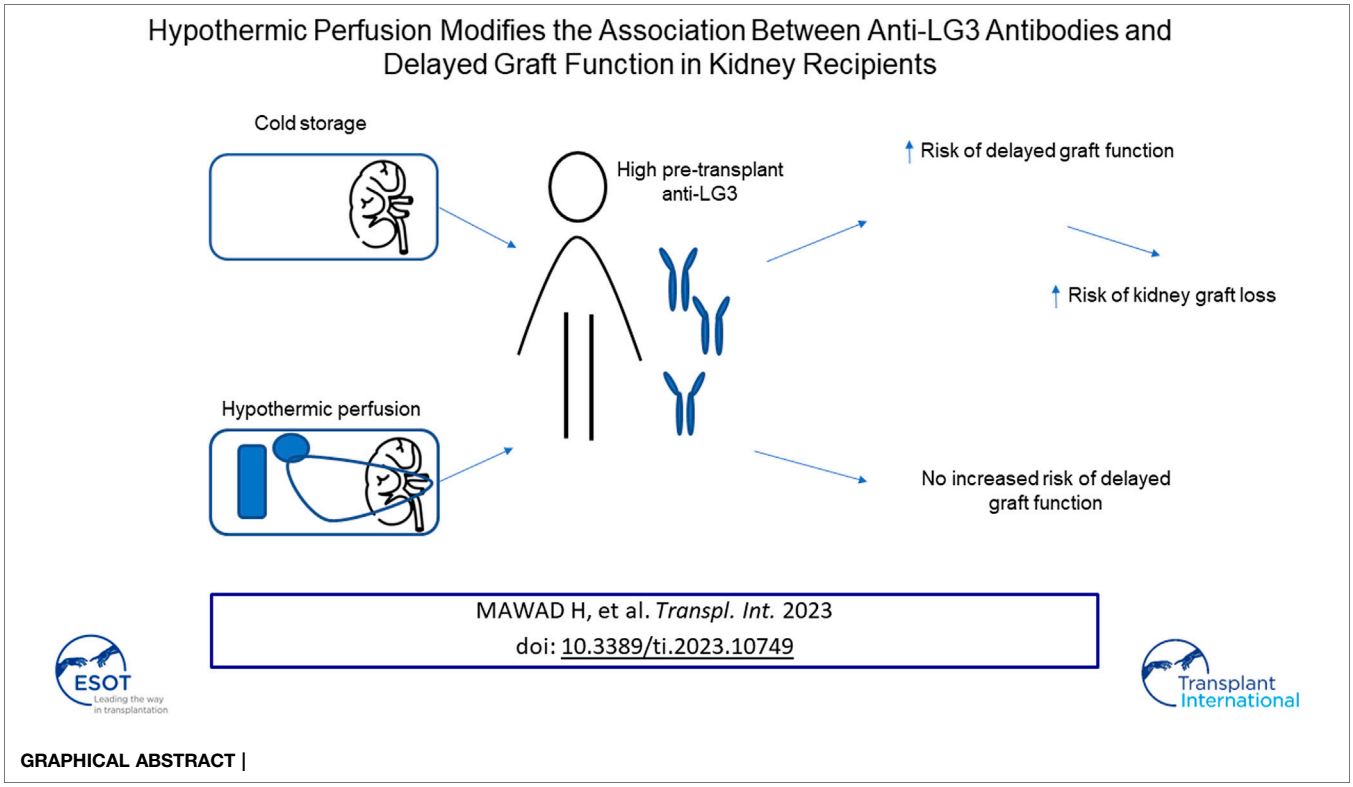
Abbreviations: DCD, Donation after cardio-circulatory death; DGF, Delayed graft function; DSA, Donor-specific antibodies; eGFR, Estimated glomerular filtration rate; HLA, Human leukocyte antigen; HR, Hazard ratio; IRI, Ischemia-reperfusion injury; IQR, Interquartile range; OR, Odds ratio; PTC, Peritubular capillary.



GRAPHICAL ABSTRACT |

INTRODUCTION

Ischemia-reperfusion injury (IRI) is common in solid organ transplantation. In kidney transplants, severe IRI can result in delayed graft function (DGF). DGF is generally associated with poor long-term renal function and survival (1), although this is not always the case. Some clinical factors such as young donor age or donation following cardiocirculatory arrest are associated with lesser adverse impact of DGF on long-term graft outcomes (2,3), which may be due to the association these factors have with preservation of the kidney microvasculature (4). Mounting evidence indeed suggests that the intensity of peritubular capillary (PTC) injury is a major predictive factor of long-term renal dysfunction after IRI in native and transplanted kidneys (4–8).

Many animal studies have shown that IRI favors the exposure of cryptic autoantigens, which can set in motion autoantibody-dependent tissue injury (9–12). Naturally occurring autoantibodies, such as the ones targeting angiotensin II type 1 receptor (AT1R), vimentin, apoptotic cells and the perlecan fragment LG3 are likely aimed at favoring the clearance of dead cells at sites of injury (10,13–16), but can be involved in tissue injury, especially in the presence of IRI. Our group has also shown in animal models of renal IRI or aortic allogeneic transplantation, that anti-LG3 antibodies prompt complement activation and microvascular rarefaction, but again only in the presence of IRI (10,17). In kidney transplant patients, we found an association between high anti-LG3 levels at the time of transplantation and an increased risk of DGF. We also reported an association between high pre-transplant anti-LG3 and lower estimated glomerular filtration rate (eGFR) 1 year post-transplant, but only in those who experienced DGF. Taken together, these data suggest that IRI is a permissive factor for anti-LG3 to participate in allograft injury. If this is the case, one could hypothesize that factors that are associated with protection from IRI could modify the association between anti-LG3 and DGF. The use of hypothermic perfusion machine rather than ice storage has been associated with a lower risk of DGF (18) and preservation of endothelial function and renal microcirculation in solid organ transplantation (19).

Here, our aim was to assess whether the association between anti-LG3 antibodies and DGF is modified by the use of hypothermic machine perfusion and whether DGF, as a proxy for the severity of IRI, modifies the association between anti-LG3 and allograft survival.

MATERIALS AND METHODS

Patients and Setting

We performed a retrospective cohort study using the University of Montreal Renal Transplant Biobank. From 1st July 2008 to 31st December 2016, consecutive patients undergoing kidney transplantation at two Canadian, university-affiliated hospitals were entered into a clinical and biological database after providing written informed consent. ABO-incompatible

transplantations and transplantations crossing pre-transplant DSA are not performed in either center. Serum samples from participants were collected immediately prior to transplantation and banked at −80°C for subsequent analyses. Clinical information was collected prospectively and supplemented retrospectively by chart review as needed. If a patient had more than one kidney transplantation recorded in the database, only the most recent transplant was included. Patients with past or simultaneous non-renal solid organ transplants were excluded. All patients included in the present study were different from those included in our previous publication on rejection (17), and 172 patients were included in our previous work on delayed graft function (10). The project was approved by the local ethics review board of the Centre Hospitalier de l'Université de Montréal (project numbers 14.169 and 16.204).

Measurements

Exposures and Outcomes

For our first aim, the outcome was the occurrence of DGF, which was defined as the need for dialysis in the first post-transplant week, failure of serum creatinine to decrease by more than 10% within the first 3 post-operative days or serum creatinine above 250 µmol/L on post-operative day 5 with evidence of acute tubular necrosis on the allograft scintigraphy (1,10). The exposure, or main independent variable of interest, was high pre-transplant anti-LG3 antibody levels. High anti-LG3 was defined as a value in the highest quartile of the distribution (17). Anti-LG3 was measured with a locally-developed enzyme-linked immunosorbent assay (17). As potential interactions between anti-LG3 levels and ischemia/endothelial injury were suggested by our prior animal studies (17), we defined, *a priori*, use of hypothermic perfusion machine as an effect modifier to be tested. In the province of Quebec, the only pump available in the field of transplantation is hypothermic perfusion with LifePort® devices, which do not provide oxygenation. There is no protocol in place guiding the use of perfusion devices and the decision to use them is hence left to the discretion of the transplant surgeon recovering and/or transplanting the kidney.

For the second aim, the outcome was kidney allograft survival, which was defined as the time elapsed between transplantation and graft failure, either return to dialysis or retransplantation. Death with a functioning graft was taken into account as a competing risk for kidney graft failure. The exposure was high pre-transplant anti-LG3 antibodies, as defined above. The effect modifier was DGF, as defined above. Covariates for all models were selected on the basis of their previously reported associations with DGF or kidney graft survival (20–22). These included both recipient and donor characteristics. Recipient variables included age, sex, race, cause of chronic kidney disease (diabetes, vascular/hypertension, autoimmune and other), time on dialysis before transplantation, height and weight, diabetes, history of cardiovascular disease, smoking status (active, past, never smoker), cytomegalovirus serostatus, pre-transplant and peak panel reactive antibodies, previous transplantations, transfusions, pregnancies, induction (basiliximab as the standard protocol, thymoglobulin with or without intravenous immunoglobulins which are reserved for highly sensitized patients), statin and renin-angiotensin system blockers use on admission. Donor variables included age, sex, height and weight, stroke as cause of death, cytomegalovirus serostatus, history of hypertension, diabetes, vascular disease, terminal creatinine, and number of HLA mismatches with the recipient. Recipient age was the only variable with an *a priori* association with the exposure, anti-LG3 (17).

Statistical Analyses

Continuous variables are reported as means and standard deviations or medians and interquartile ranges, depending on their distribution. Categorical variables are summarized as numbers and proportions. We analyzed between-group crude differences in non-normally distributed continuous variables with Wilcoxon rank-sum (or Kruskal-Wallis) tests or with ANOVAs when normally distributed. We performed chi-square (or Fisher's exact) tests to analyze the between-group differences in categorical variables.

For the first aim, we used logistic regression models to assess the association between pre-transplant anti-LG3 level and DGF. We considered the possibility that the association between elevated anti-LG3 and DGF differed based on hypothermic machine perfusion by creating and testing an interaction term between these 2 variables. To identify confounders, we examined the association between each covariate listed above and both the exposure (high anti-LG3 antibodies) and the outcome (DGF). In the initial multivariable model, we included all the covariates with strong and moderate potential for confounding (see **Supplementary Appendix SA1**). Then, we simplified the multivariable model to avoid overfit by excluding one covariate at a time, starting with those having the highest *p*-values. Variables that were not associated with DGF in the initial multivariable model (p-value ≥0.05) and whose singular exclusion resulted in a <10% change in the point estimate for the main exposure (compared with the model including all covariates) were withdrawn from the final multivariable model. To ensure more thorough adjustment for confounders, we also fit a logistic regression model to identify the factors associated with the use of hypothermic perfusion. We then forced in the final model for DGF 2 variables that were independently associated with the use of hypothermic perfusion, while the 2 others were already included.

For the second aim, we fit a Fine and Gray proportional subdistribution hazards multivariate regression model for competing-risks to assess whether elevated pre-transplant anti-LG3 (dichotomized at the last quartile) was associated with graft failure, accounting for death as a competing risk. Patients who were lost to follow-up before the end of the study period were censored without an event at the date they were lost to follow-up. We assessed effect modification by including an interaction between anti-LG3 and DGF in the model. A backward selection approach was used to select covariables included in the final multivariable model. We performed a sensitivity analysis excluding 3 patients who had primary graft non-function. There were no missing values for the primary exposures or outcomes. Missing values for covariates were handled either by imputation

(the mean for continuous variables) or by creating a "no or unknown" category for categorical variables.

RESULTS

Pre-Transplant anti-LG3 are Associated With Delayed Graft Function When the Donor Kidney is Transported in Cold Preservation Solution, but not When the Kidney is Placed on Hypothermic Perfusion Machine

Amongst the 809 patients who underwent kidney transplantation at the 2 centers during the study period, 687 were included in the analysis for the association between pre-transplant anti-LG3, DGF and graft survival (**Figure 1**). The reason for exclusion was the absence of available pre-transplant serum to test, which occurred at the inception of our biobank in 1 center for logistical reasons. **Table 1** shows recipient, donor and procedure-related characteristics of patients who had pre-transplant sera available, stratified by anti-LG3 level. DGF occurred in 265 (39%) patients. The number of missing values for covariates are described at the bottom of **Table 1**. Patients who had high pre-transplant anti-LG3 antibodies were more likely to be of African descent, have diabetic nephropathy as a cause of CKD and have a positive pre-transplant CMV serology. Patients with high pre-transplant anti-LG3 had a slightly longer time on dialysis prior to transplant than those without pre-transplant anti-LG3. There was no association between elevated anti-LG3 antibodies and autoimmune disease as a cause of CKD, nor did we observe an association between anti-LG3 and pre-transplant anti-HLA antibodies or factors associated with their development such as transfusions, previous transplantations or pregnancies.

While the use of hypothermic perfusion during organ transportation was left to the discretion of transplant surgeons recovering or transplanting the kidney, we identified some associations between clinical characteristics of the donors/recipients and use of hypothermic pump (**Supplementary Table S1**). In a multivariable analysis, its use differed by center, was higher in recent years and in donors with diabetes and in donors after cardiocirculatory death (**Supplementary Table S2**). The use of hypothermic perfusion machine modified the association between high pre-transplant anti-LG3 levels and DGF. In multivariable analyses (**Table 2**), pre-transplant anti-LG3 values in the upper distribution quartile were independently associated with DGF (odds ratio (OR): 1.75, 95% confidence interval 1.02–3.00, $p = 0.04$) when the donor kidney was transported in static cold storage ($n = 468$). When the kidney was placed on hypothermic machine perfusion ($n = 219$), there was no association between anti-LG3 and DGF (OR: 0.78, 95% CI 0.44–1.37, $p = 0.38$). Recipient diabetes, longer time on dialysis prior to transplantation, use of thymoglobulin, older donor age, neurologically deceased or deceased after cardiocirculatory arrest versus living donor, donor serum creatinine >120 µmol/L were all associated with a higher risk of DGF. Use of renin-angiotensin system blockers at the time of transplantation and higher donor height were associated with a lower risk of DGF, while there was a center effect in the incidence of DGF. Univariable analyses are presented in **Supplementary Table S3** and the initial multivariable model (before simplification) is presented in **Supplementary Table S4**.

High Pre-transplant anti-LG3 are Associated With a Higher Risk of Graft Failure in Patients Who Experienced DGF, but not in Those With Immediate Graft Function

We had previously shown that patients in patients with DGF, high pre-transplant anti-LG3 were associated with reduced eGFR 1 year post transplant, which was not the case in patients with immediate graft function (10). To verify whether this led to long-term poor outcomes, we examined the association between pre-transplant anti-LG3 and kidney graft survival in patients with and without DGF, accounting for the competing risk of death. Over a median follow-up of 6.9 years (interquartile range (IQR) 5.2–8.9 years), 93 patients died and 51 experienced graft loss (return to hemodialysis or retransplantation). The presence of DGF modified the association between anti-LG3 and graft survival. In patients with DGF, high anti-LG3 was associated with a higher risk of graft loss (subdistribution hazard ratio (SHR): 4.07, 95% CI: 1.80, 9.22), while this was not the case in patients with immediate graft function (SHR: 0.50, 95% CI 0.19, 1.29) (**Table 3**; **Figure 2**). Other factors that were associated with a higher risk of graft loss were younger recipient age, time on dialysis pre-transplant, number of HLA mismatches, donor age, donor female sex and higher donor serum creatinine, earlier vintage, transplant center, as well as active recipient smoking at the time of transplant and induction with intravenous immunoglubulins. Univariable analyses are presented in **Supplementary Table S5**. Removing the 3 patients with primary non-function did not modify the results.

DISCUSSION

The characterization of novel biomarkers and/or potential therapeutic targets that could prevent the occurrence of DGF and its adverse impact on long-term allograft outcomes could represent significant advances in the field of kidney transplantation. Here, we found that pre-transplant anti-LG3 antibodies were associated with an increased risk of DGF, but only when the organ was placed in static cold storage during transportation. When the organ was placed on hypothermic perfusion machine, this association was no longer seen. We also examined whether DGF, as a proxy for severe IRI, was a permissive factor for the adverse impact of anti-LG3 on graft survival to occur. We hence extended findings from our previous work by showing that in patients with DGF, but not in those with immediate graft function, pre-transplant anti-LG3 levels are associated with a higher risk of graft loss.

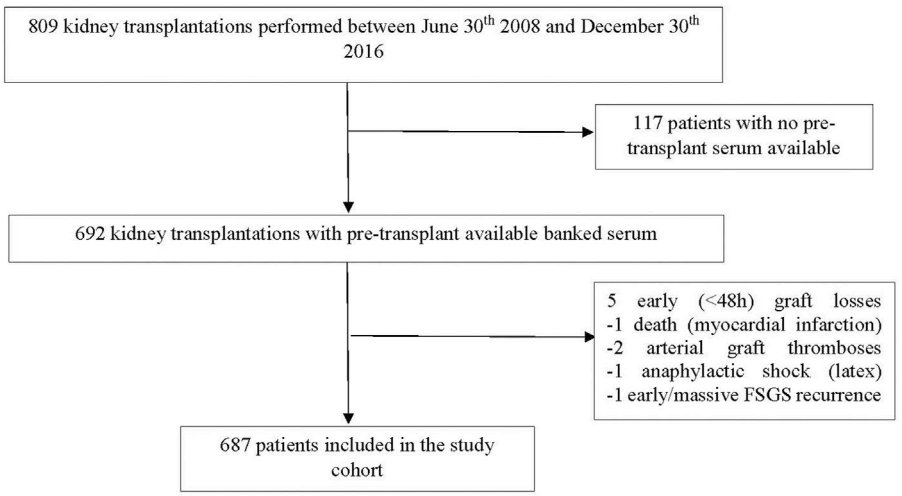


FIGURE 1 | Patient flow chart.

Taken together, our findings are consistent with our initial hypothesis, namely that IRI favors the exposure of cryptic autoantigens, such as LG3, from the graft microvasculature setting in motion autoantibody-dependent microvascular injury and rarefaction. We showed previously that renal IRI in mice leads to increased circulating levels of apoptotic exosome-like vesicles bearing LG3 (23). When the allograft is transplanted into a recipient with elevated anti-LG3 antibodies, these antibodies may bind to their antigenic targets if the vasculature is already stressed allowing for LG3 release or exposure. This is turn would favor antibody-antigen interactions and complement activation, therefore promoting microvascular damage and fibrosis leading to decreased graft survival. Indeed, injection of anti-LG3 antibodies in a murine model of renal IRI led to microvascular rarefaction, complement deposition and enhanced renal fibrosis while this was not the case when the mice had not undergone renal IRI (10). Other autoantibodies have also been reported to promote tissue inflammation and injury in the presence of IRI. Angiotensin II type 1 receptor (AT1R) antibodies alter the vascular reactivity of renal arteries, but only in the presence of ischemia or allogeneic transplantation (11). Infusion of autoantibodies against myosin lead to syngeneic heterotopic heart transplant failure in mice when administered at the time of IRI, but not when administered later on (24). Transfer of autoantibodies to K-alpha-1-tubulin and Collagen V leads to inflammation and tissue injury in a model of syngeneic orthotopic lung transplantation while sham treated mice who received these autoantibodies developed no lesion (12). Human studies cannot reproduce the level of details obtained in experimental models. Nevertheless, one can hypothesize that in the presence of hypothermic machine perfusion, a technique that reduces endothelial injury and improves microcirculatory flow in animal models of kidney transplantation (19), the exposure of cryptic autoantigens such as LG3 and potential for anti-LG3 interaction and graft injury may be diminished. Hence, the results from the present study support a role for IRI as a permissive factor for anti-LG3 to adversely affect the kidney transplant.

DGF is associated with reduced long-term kidney graft survival in most studies (1). Nevertheless, the outcome of patients with DGF is not uniformly unfavorable and can be modulated by factors such as donor type and donor age (2,3). While biomarkers such as kidney injury molecule-1 and neutrophil gelatinase-associated associated lipocalin are associated with DGF, they show no association with subsequent graft function in patients with DGF (25). These biomarkers, which evaluate tubular epithelial cell injury or inflammation, may not be reflective of microvascular injury, which plays a predominant role in predicting loss of renal function on the long-term (6,7). We recently observed in caspase-3 deficient mice that show enhanced early epithelial injury after IRI but preservation of PTC integrity, that microvascular injury, but not early tubular damage, predicts renal fibrosis and progressive loss of renal function (6). We also showed, in renal transplant patients with DGF, that loss of PTC density in the first year after transplantation is associated with eGFR 1 and 3 years post-transplantation (4). These observations are in line with multiple studies establishing a link between microvascular damage, renal fibrosis and progressive renal dysfunction (4,5,7,10,15,26–28). The identification of factors regulating microvascular injury at the time of transplantation should lead to a better capacity to predict and prevent long-term renal dysfunction.

In the present study, other factors associated with DGF and graft survival were those observed in many previous studies (20,21,29). Although RAS blockers are discontinued at the time of transplantation in our centers, we had hypothesized that the biological effect of long-acting RAS blockers could have increased the risk of DGF (30). In contrast to our *a priori* expectations, use of RAS blockers were associated with a decreased risk of DGF. Although prior studies have shown a null (31) or protective impact of perioperative RAS system blockers on

TABLE 1 | Recipient and donor characteristics in kidney transplant recipients with pre-transplant sera available stratified by anti-LG3 level (*n* = 687 unless specified otherwise).

Characteristics	High anti-LG3 (n = 172)	Low anti-LG3 (n = 515)	*p*-value
Recipient			
Mean age at transplantation in years (SD)	52 (14)	51 (13)	0.67
Male sex, n (%)	112 (65)	315 (61)	0.35
African American race, n (%)	25 (15)	29 (6)	0.01
Mean body mass index kg/m^2 (SD)	26 (5)	26 (4)	0.29
Cause of chronic kidney disease, n (%)			
Glomerular diseases	55 (32)	187 (36)	0.30
Diabetes	36 (21)	69 (13)	0.02
Hypertension/vascular	17 (10)	54 (10)	0.82
Polycystic kidney diseases	25 (15)	94 (18)	0.27
Autoimmune diseases	9 (5)	24 (5)	0.76
Other or unknown	30 (17)	87 (17)	0.71
Median time on dialysis pre-transplant in months, (IQR)	29 (10–55)	26 (1–49)	0.07
Positive CMV serology, n (%)	107 (62)	235 (46)	0.01
Pretransplant diabetes, n (%)	47 (27)	111 (22)	0.12
Coronary artery disease at transplantation, n (%)	34 (20)	85 (17)	0.33
Active smoking at transplantation, n (%)	29 (17)	69 (13)	0.26
Statin use at transplantation, n (%)	94 (55)	276 (54)	0.81
ACE inhibitor/angiotensin-2 blocker use at transplantation, n (%)	82 (48)	216 (42)	0.19
Median pre-transplant panel reactive antibodies (IQR)	0 (0–0)	0 (0–0)	0.51
Median peak historical panel reactive antibodies (IQR)	0 (0–4)	0 (0–5)	0.99
First transplantation, n (%)	157 (91)	456 (89)	0.32
HLA mismatches, n (%)			
0–2	25 (15)	114 (22)	0.03
3–4	93 (54)	255 (50)	0.30
5–6	54 (31)	146 (28)	0.45
Previous transfusions, n (%)	75 (44)	212 (41)	0.57
Previous pregnancies, n (%)	43 (25)	147 (29)	0.37
Induction[a]			
Thymoglobulin, n (%)	47 (27)	115 (22)	0.18
Intravenous immunoglobulin, n (%)	14 (8)	28 (5)	0.20
Plasma exchange, n (%)	2 (1)	1 (0.4)	0.73
Maintenance immunosuppression, n (%)			
Tacrolimus and mycophenolate mofetil	166 (97)	496 (96)	0.90
Cyclosporine and mycophenolate mofetil	4 (2)	11 (2)	0.88
Donor			
Living donor, n (%)	38 (22)	123 (24)	0.63
Deceased donor			
Neurological determination of death, n (%)	112 (65)	336 (65)	0.98
Donor after cardiocirculatory arrest, n (%)	22 (13)	56 (11)	0.49
Mean age in years, (SD)	49 (15)	49 (15)	0.85
Male sex, n (%)	92 (53)	271 (53)	0.84
Mean height in meters (SD)	1.68 (0.10)	1.68 (0.10)	0.82
Positive CMV serology, n (%)	72 (42)	190 (37)	0.25
Hypertension, n (%)	42 (24)	112 (22)	0.47
Diabetes, n (%)	13 (8)	39 (8)	1.00
Tobacco history, n (%)	88 (51)	273 (53)	0.70
Donor vascular disease, n (%)	20 (12)	41 (8)	0.14
Mean terminal serum creatinine in μmol/L (SD)	71 (37)	70 (45)	0.73
Procedure			
Median total ischemic time in hours, (IQR)	9 (5–13)	9 (5–15)	0.40
Use of hypothermic perfusion, n (%)	57 (33)	162 (31)	0.68
Center 1, n (%)	88 (51)	267 (52)	0.88
Early post-transplant course			
Delayed graft function, n (%)	72 (42)	193 (37)	0.31

[a]*One patient in the group low anti-LG3 received induction with alemtuzumab.*

Missing data: Recipient: *pre-transplant transfusion* (n = *1), pre-transplant pregnancies* (n = *2), smoking history* (n = *6),* Donor: *terminal creatinine* (n = *27), CMV serology* (n = *3), height and weight* (n = *2), hypertension* (n = *27), diabetes* (n = *33), peripheral vascular disease* (n = *41), smoking history* (n = *21);* Procedure: *total ischemic time* (n = *12), use of hypothermic pump* (n = *97).*

SD, standard deviation; IQR, interquartile range.

TABLE 2 | Associations between recipient, donor and procedure characteristics and DGF in final multivariable analyses (*n* = 687).

Recipient/Donor/Procedure characteristics	Univariable odds ratio (95% CI[a])	*p*-value	Multivariable odds ratio (95% CI[a])	*p*-value
Pre-transplant elevated anti-LG3 antibodies[b]				
In cold static storage	1.58 (1.03, 2.39)	0.04	1.75 (1.02, 3.00)	0.04
Placed on a hypothermic perfusion machine	0.65 (0.33, 1.29)	0.22	0.78 (0.44, 1.37)	0.38
Recipient age at transplant (per 10 years higher)	1.17 (1.04, 1.31)	<0.01	0.98 (0.97, 1.00)	0.06
Recipient African American race (versus Caucasian)	1.80 (1.03, 3.14)	0.04	0.84 (0.39, 1.79)	0.65
Time on dialysis pre-transplant (per 1-month higher)	1.00 (1.00, 1.20)	<0.01	1.01 (1.01, 1.02)	<0.01
Recipient diabetes	2.34 (1.63, 3.36)	<0.01	2.14 (1.35, 3.39)	<0.01
Recipient positive CMV serology	1.28 (0.94, 1.74)	0.11	1.02 (0.68, 1.54)	0.92
Recipient ACE inhibitor/angiotensin-2 blocker use at transplantation	0.57 (0.42, 0.79)	<0.01	0.54 (0.37, 0.81)	<0.01
Previous transplantations	1.69 (1.04, 2.74)	0.03	1.61 (0.84, 3.10)	0.15
Thymoglobulin induction	2.34 (1.63, 3.35)	<0.01	2.24 (1.36, 3.69)	<0.01
Donor type (References neurologically deceased)				
Living donor	0.12 (0.06, 0.21)	<0.01	0.21 (0.11, 0.41)	<0.01
Donor after cardiac arrest	4.10 (2.37, 7.11)	<0.01	4.99 (2.62, 9.50)	<0.01
Donor age (per 10-year higher)	1.30 (1.17, 1.45)	<0.01	1.48 (1.28, 1.72)	<0.01
Donor height (per 10 cm higher)	0.82 (0.71, 0.95)	<0.01	0.75 (2.34, 15.63)	<0.01
Donor peripheral vascular disease	2.00 (1.18, 3.40)	0.01	0.96 (0.50, 1.87)	0.91
Donor diabetes	1.52 (0.86, 2.69)	0.15	0.94 (0.48, 1.85)	0.59
Donor terminal serum creatinine ≥120 umol/L	4.69 (2.05, 10.69)	<0.01	6.14 (2.37, 15.92)	<0.01
Center 1	0.31 (0.22, 0.42)	<0.01	0.38 (0.23, 0.63)	<0.01
Transplant date (per 1 year higher)	1.04 (0.97, 1.11)	0.26	1.03 (0.93, 1.13)	0.86

[a]*CI, confidence interval.*
[b]*The p-value for the interaction between anti-LG3 and use of hypothermic pump is 0.02.*

TABLE 3 | Associations between recipient, donor and procedure characteristics and graft survival in final multivariable analyses (n = 687).

Recipient/Donor/Procedure characteristics	Univariable SHR (95% CI[a])	*p*-value	Multivariable SHR (95% CI[a])	*p*-value
Pre-transplant elevated anti-LG3 antibodies[b]				
In patients with immediate graft function	0.97 (0.37, 2.58)	0.92	0.50 (0.19, 1.29)	0.15
In patients with delayed graft function	2.42 (1.19, 4.95)	0.01	4.07 (1.80, 9.22)	<0.01
Recipient age at transplant (per 1 year higher)	0.97 (0.95, 0.99)	0.01	0.96 (0.93, 0.98)	<0.01
Time on dialysis pre-transplant (per 1-month higher)	1.01 (1.01, 1.02)	<0.01	1.02 (1.01, 1.02)	<0.01
Active recipient smoking at transplantation	2.68 (1.50, 4.80)	<0.01	3.66 (2.05, 6.56)	<0.01
Recipient prior history of coronary artery disease	0.40 (0.15, 1.11)	0.08	0.26 (0.08, 0.83)	0.02
Center 1	0.64 (0.37, 1.12)	0.12	0.54 (0.29, 0.99)	0.05
Transplant date (per 1 year higher)	0.91 (0.81, 1.02)	0.09	0.87 (0.76, 0.99)	0.04
Induction with intravenous immunoglobulins	1.87 (0.79, 4.44)	0.15	4.07 (1.44, 11.50)	<0.01
Donor age (per 10-year higher)	1.22 (0.97, 1.54)	0.09	1.56 (1.19, 2.04)	<0.01
Female donor sex	1.64 (0.94, 2.85)	0.08	2.04 (1.17, 3.53)	0.01
Donor serum creatinine (per 10 umol/L higher)	1.03 (1.00, 1.06)	0.10	1.07 (1.04, 1.10)	<0.01
HLA mismatches (per 1 mismatch higher)	1.19 (0.97, 1.46)	0.10	1.35 (1.04, 1.76)	0.02

[a]*SHR: subdivision hazard ratio, CI: confidence interval.*
[b]*The p-value for the interaction between anti-LG3 and DGF is 0.001.*

short-term graft function (32) and long-term allograft survival (33,34), many clinicians may fear hyperkalemia or hemodynamic deterioration in kidney function in the early post-transplant period and discontinue these medications. Intervention studies examining the benefit of these medications at the time of transplant are needed to clarify this issue. Induction with intravenous immunoglobulins was associated with a higher risk of graft failure, which is probably due to the fact that this induction was reserved to patients perceived at the highest immunological risk by their transplant physicians.

In addition to increasing length and cost of hospital stay for kidney transplantation (35), DGF is associated with reduced long-term kidney graft survival in most studies (1). Hence, strategies that decrease its occurrence should be of clinical benefit. The use of hypothermic perfusion has been shown to lower the incidence of DGF in multiple studies (36). Nevertheless, its use varies by center in our jurisdiction (37), which may be due to logistic reasons as well as to the absence of clearly demonstrated benefits to kidney graft survival (36). Measuring pre-transplant anti-LG3 may be a strategy to identify recipients who would derive the greatest benefit from hypothermic machine perfusion of the donor kidney. Other strategies such as plasma exchange could also be tested in patients with high anti-LG3 antibodies.

Our study has certain limitations. First, it was performed in 2 University-affiliated centers, with a majority of Caucasian

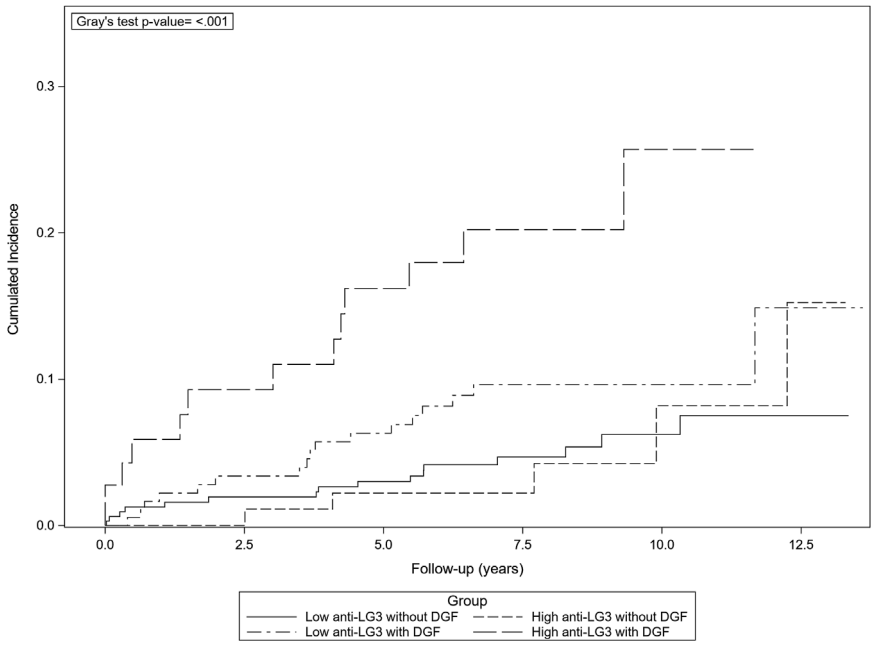


FIGURE 2 | Cumulative incidence of death-censored graft loss by delayed graft function and anti-LG3 status. Patients with both high anti-LG3 and DGF show an increased risk of death-censored graft loss taking into account the competitive risk of death ($p < 0.001$).

patients at low immunological risk, which may affect its generalizability. Also, given the observational and retrospective nature of the study, and the absence of early post-transplant biopsies, we can only speculate as to the mechanisms by which anti-LG3 adversely affect allograft outcomes and report associations which may or may not be causal. Moderate elevations in anti-LG3, for instance when anti-LG3 was stratified in tertiles, did not lead to a significant increase in the risk of DGF, but remained associated with kidney graft survival (data not shown). Hence, the levels at which anti-LG3 can be considered deleterious may vary by outcome. Last, when we were unable to retrieve information on the use of hypothermic pump from the chart, we classified this in the 'no or unknown' category. Although may have resulted in misclassification, this is likely to have been non-differential, resulting in bias towards observing no difference by machine perfusion status.

In conclusion, the present study supports the notion that IRI represents a permissive factor for anti-LG3 to exert a negative effect on the allograft. High anti-LG3 levels are associated with a higher risk of DGF in kidneys exposed to cold storage but not in kidneys place on hypothermic pump perfusion. Favoring the use of hypothermic perfusion for organs to be transplanted to candidates with high anti-LG3 levels may offer a therapeutic strategy to prevent DGF and extend renal allograft survival.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the author is willing to share the raw data only if this is allowed by the institutional Ethics Committee of Centre Hospitalier de l'Université de Montréal. Requests to access the datasets should be directed to heloise.cardinal.chum@ssss.gouv.qc.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by local ethics review board of the Centre Hospitalier de l'Université de Montréal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors agree with the content of the manuscript and have made substantial contributions to the work; HM, SC, LS, BF, M-JH, and HC participated in research design, and KH, AR, AB, and BY participated in the performance of the research. HM, LP, SM, JB, and KH participated to the clinical data collection. HM, JT, BF, M-JH, MC, and HC participated in data analysis and in the writing of the paper. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10749/full#supplementary-material

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ORIGINAL RESEARCH
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SARS-CoV-2 Vaccination-Induced Immunogenicity in Heart Transplant Recipients

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Among heart transplant (HT) recipients, a reduced immunological response to SARS-CoV-2 vaccination has been reported. We aimed to assess the humoral and T-cell response to SARS-CoV-2 vaccination in HT recipients to understand determinants of immunogenicity. HT recipients were prospectively enrolled from January 2021 until March 2022. Anti-SARS-CoV-2-Spike IgG levels were quantified after two and three doses of a SARS-CoV-2 vaccine (BNT162b2, mRNA1273, or AZD1222). Spike-specific T-cell responses were assessed using flow cytometry. Ninety-one patients were included in the study (69% male, median age 55 years, median time from HT to first vaccination 6.1 years). Seroconversion rates were 34% after two and 63% after three doses. Older patient age (p = 0.003) and shorter time since HT (p = 0.001) were associated with lower antibody concentrations after three vaccinations. There were no associations between vaccine types or immunosuppressive regimens and humoral response, except for prednisolone, which was predictive of a reduced response after two (p = 0.001), but not after three doses (p = 0.434). A T-cell response was observed in 50% after two and in 74% after three doses. Despite three vaccine doses, a large proportion of HT recipients exhibits a reduced immune response. Additional strategies are desirable to improve vaccine immunogenicity in this vulnerable group of patients.

Keywords: immunosuppression, heart transplantation, humoral response, COVID-19 vaccination, T-cell response

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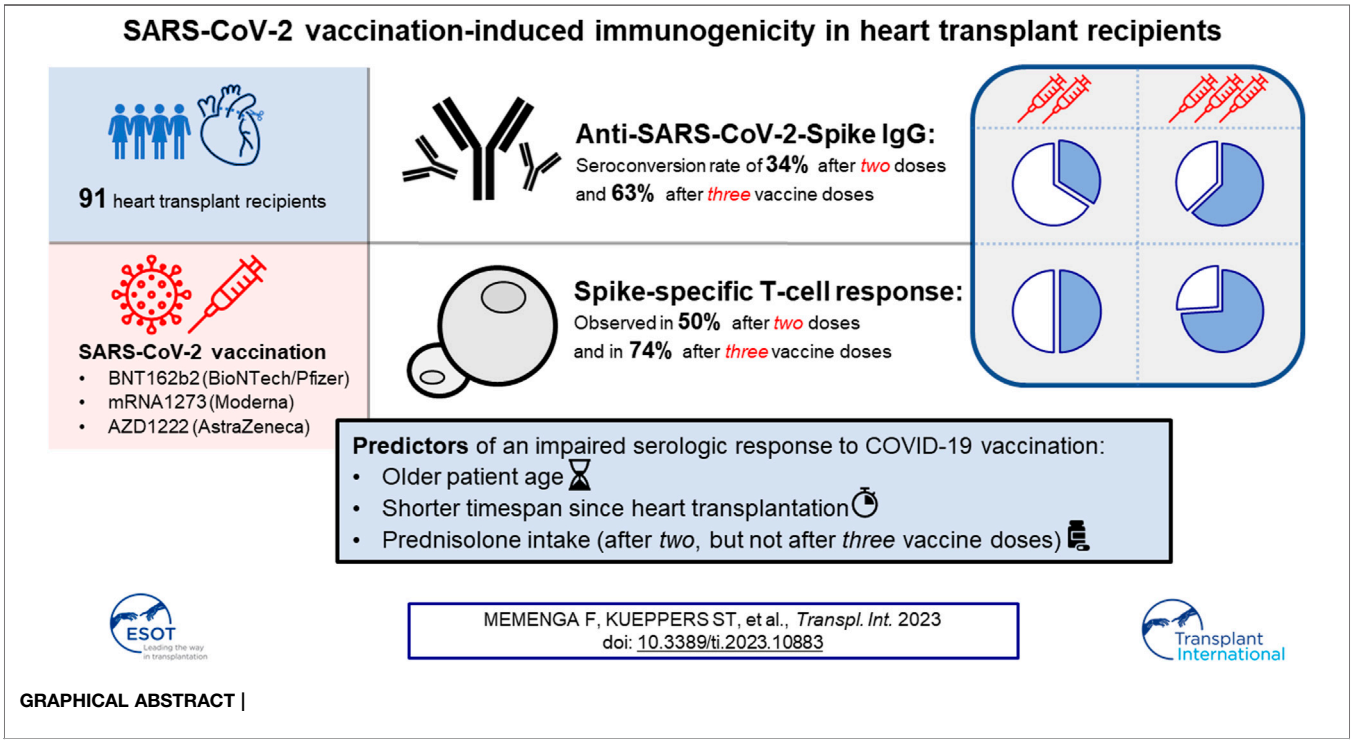
Abbreviations: AIM, Activation-induced marker assay; AZD1222, Vaxzevria (AstraZeneca, Cambridge, United Kingdom); BAU/mL, Binding antibody units per milliliter; BNT162b2, Tozinameran (Pfizer, New York City, USA; BioNTech, Mainz, Germany); CD, Cluster of differentiation; CI, Confidence interval; COVID-19, Coronavirus disease 2019; EDTA, Ethylenediaminetetraacetic acid; eGFR, Estimated glomerular filtration rate; HT, Heart transplantation; IgG, Immunoglobulin G; IQR, Interquartile range; ISHLT, International Society for Heart and Lung Transplantation; mAB, Monoclonal antibody; mRNA, Messenger ribonucleic acid; mRNA1273, Spikevax (Moderna, Cambridge, USA); OR, Odds ratio; PrEP, Pre-Exposure Prophylaxis; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2; SI, Stimulation index; SOT, Solid organ transplant; STIKO, Standing vaccination committee (Germany), "Ständige Impfkommission"; T2DM, Type 2 diabetes mellitus.



GRAPHICAL ABSTRACT |

INTRODUCTION

The clinical management of heart transplant (HT) recipients during the ongoing COVID-19 (coronavirus disease 2019) pandemic has been challenging, as these patients are at high risk of severe clinical impairment and adverse outcomes upon infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (1–3). Several vaccines with high efficacy against SARS-CoV-2 infection and good safety profiles have been approved, including the mRNA-based vaccines BNT162b2 (Tozinameran, Pfizer-BioNTech, New York City, USA/Mainz, Germany) and mRNA1273 (Spikevax, Moderna, Cambridge, USA), and the non-replicating viral vector vaccine AZD1222 (Vaxzevria, AstraZeneca, Cambridge, United Kingdom) (4–6). Vaccination of HT recipients has been recommended by the International Society for Heart and Lung Transplantation (ISHLT) (7). However, as immunocompromised individuals have been largely excluded from clinical trials, there is a paucity of data on the immunological response after vaccination of solid organ transplant (SOT) recipients.

Recent studies have reported a reduced humoral response to SARS-CoV-2 vaccination in SOT recipients (8–13), who may have an especially low probability for seroconversion after two vaccine doses compared to other immunocompromised patients (13). Seroconversion rates in HT recipients after two doses vary widely in the current literature (from 10% to 75%) (9–12,14). To improve vaccine responses, the ISHLT currently recommends three doses of an mRNA vaccine as primary series (15). Additional booster doses have been proposed and modification of immunosuppressive regimens are being investigated in ongoing trials (16,17). Impaired humoral responses have been associated with older patient age, shorter time since transplantation, and immunosuppression with anti-metabolite agents such as mycophenolate mofetil (9,10,14). In addition to circulating antibodies, T-cell activity is an important component of the immune response against SARS-CoV-2 infection (18,19). So far, only few studies have analyzed T-cell immunity in vaccinated HT recipients (14,20,21).

Here, we report quantification of the humoral and T-cell response after a second and third dose of a COVID-19 vaccine in a consecutive cohort of heart transplant patients seen at a large transplant center. We also report determinants of vaccine response in this cohort.

PATIENTS AND METHODS

Study Participants and Data Collection

From January 2021 until March 2022, we enrolled HT recipients that presented to the HT outpatient clinic of the University Heart & Vascular Center Hamburg, a large tertiary care center. Clinical variables including age, sex, date of transplantation, immunosuppressive medications, renal function *via* estimated glomerular filtration rate (eGFR) and history of diabetes were assessed at time of registration.

Participants had previously received two doses of the mRNA-based vaccines BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna), or the viral vector-based AZD1222 (AstraZeneca) vaccine. After each the second and the third vaccination, anti-SARS-CoV-2 IgG concentrations in the blood serum and, in a

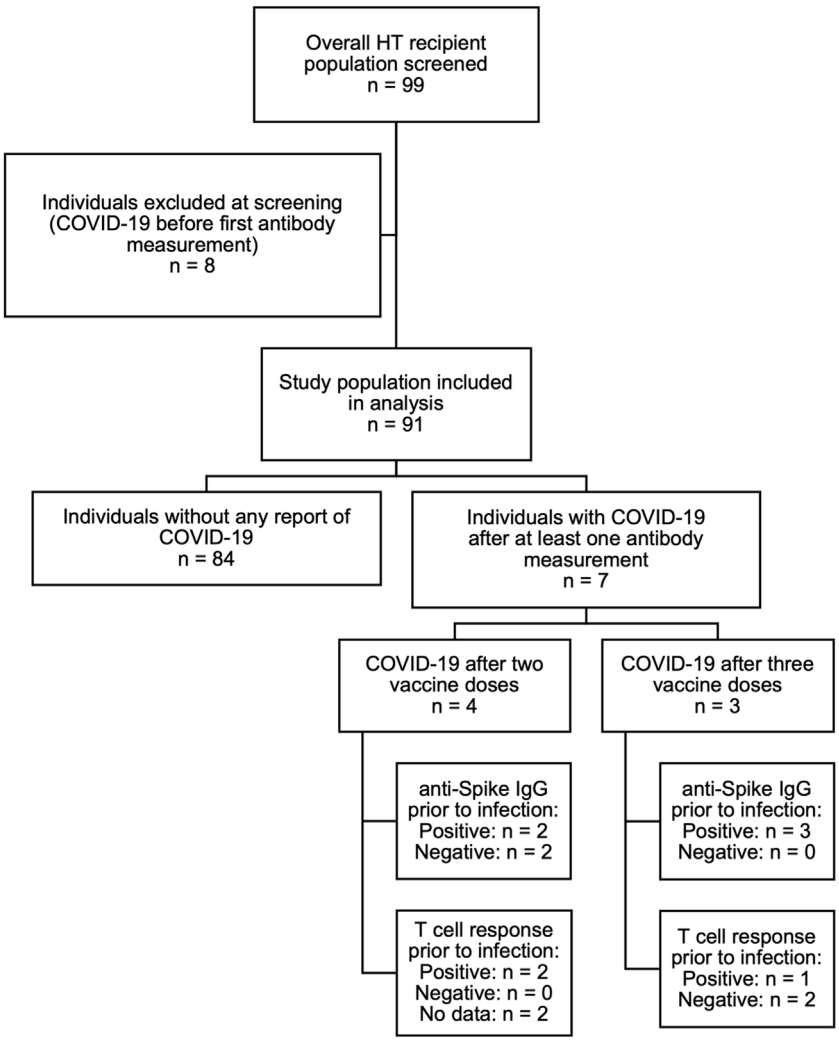


FIGURE 1 | Overview of the study population.

subset of patients, spike-specific T-cell responses were assessed during routine ambulatory follow-up visits. Vaccinations had been administered by the patients' primary care physicians or by specialized vaccination centers in accordance with the German prioritization guidelines and recommendations of the standing vaccination committee (STIKO) (22). We did not include any HT recipients with a known history of COVID-19 prior to the first sampling timepoint. Also, if a participant developed COVID-19 after the first samples were taken, all measurements obtained after infection were excluded from the analysis (**Figure 1**). Accordingly, we did not include any measurements after administration of therapeutic monoclonal antibodies against SARS-CoV-2 in our analysis. Rates of COVID-19 infection during the study period were low, with only 7 cases reported after at least one antibody measurement. The study was approved by the local ethics committee (PV 6079) and conducted in concordance with the Declaration of Helsinki. Written informed consent was provided by all participants.

Assessment of SARS-CoV-2 Vaccine-specific Humoral and T-cell Response

We assessed the vaccine-specific humoral response after a median of 42 days (interquartile range [IQR] 29.0–98.8) after the second vaccination ("pre-booster") and 39.5 days (28.0–62.0) after the third vaccination ("post-booster"). The DiaSorin LIAISON XL anti-SARS-CoV-2 TrimericS IgG ChemiLuminescent ImmunoAssay (sensitivity 99.4%, specificity 99.8%) (23) was used to quantitatively determine the anti-SARS-CoV-2-Spike IgG (anti-S Trimer) levels. As proposed by the manufacturer, positive humoral response was defined by an anti-S Trimer IgG concentration of ≥33.8 BAU/mL (23).

TABLE 1 | Baseline characteristics.

	Overall, N = 91[a]	Sex	
		Female, N = 28[a]	Male, N = 63[a]
Clinical characteristics			
Patient age at first vaccine dose [years]	55 [48.5, 61]	54 [41.8, 60]	55 [50.5, 62]
Time from HT to first dose [years]	6.1 [1.6, 13.2]	4.3 [1.7, 10.6]	7.2 [1.5, 13.2]
Time between first and second dose [days]	42.0 [35.0, 42.0]	42.0 [38.5, 42.2]	41.0 [35.0, 42.0]
Time between second dose and first antibody measurement [days]	42.0 [29.0, 98.8]	37.0 [27.0, 94.8]	44.5 [32.5, 94.2]
Time between third vaccination and second antibody measurement [days]	39.5 [28.0, 62.0]	32.5 [24.2, 75.8]	41.0 [32.8, 48.2]
History of type 2 diabetes mellitus	27 (30%)	5 (18%)	22 (35%)
Estimated glomerular filtration rate (eGFR [mL/min])	49.0 [34.0, 69.0]	46.5 [33.2, 62.0]	52.0 [34.0, 69.0]
Immunosuppressive therapy			
Everolimus use	67 (74%)	22 (79%)	45 (71%)
Cyclosporine use	15 (16%)	4 (14%)	11 (17%)
Mycophenolate mofetil use	41 (45%)	8 (29%)	33 (52%)
Prednisolone use	49 (54%)	16 (57%)	33 (52%)
Tacrolimus use	62 (68%)	23 (82%)	39 (62%)
CNI regimen			
Regimen containing CNI	77 (85%)	27 (96%)	50 (79%)
CNI-free regimen	14 (15%)	1 (3.6%)	13 (21%)
Drug combinations			
Tacrolimus + Everolimus + Prednisolone	24 (26%)	11 (39%)	13 (21%)
Tacrolimus + Everolimus	21 (23%)	8 (29%)	13 (21%)
Tacrolimus + Mycophenolate mofetil + Prednisolone	8 (8.8%)	1 (3.6%)	7 (11%)
Tacrolimus + Mycophenolate mofetil	7 (7.7%)	2 (7.1%)	5 (7.9%)
Tacrolimus + Everolimus + Mycophenolate mofetil + Prednisolone	2 (2.2%)	1 (3.6%)	1 (1.6%)
Cyclosporine A + Mycophenolate mofetil + Prednisolone	5 (5.5%)	2 (7.1%)	3 (4.8%)
Cyclosporine A + Mycophenolate mofetil	4 (4.4%)	1 (3.6%)	3 (4.8%)
Cyclosporine A + Everolimus + Prednisolone	3 (3.3%)	1 (3.6%)	2 (3.2%)
Cyclosporine A + Everolimus + Mycophenolate mofetil	1 (1.1%)	0	1 (1.6%)
Cyclosporine A + Everolimus	2 (2.2%)	0	0
Everolimus + Mycophenolate mofetil + Prednisolone	7 (7.7%)	0	7 (11%)
Everolimus + Mycophenolate mofetil	7 (7.7%)	1 (3.6%)	6 (9.5%)

[a]*Median [IQR] or Frequency with number (%); Missing data excluded.*
Continuous variables with few values and/or few different values are shown as categorical.
HT heart transplantation; CNI calcineurin inhibitor.

The spike-specific T-cell response was assessed using an activation-induced marker assay (AIM) similar to previous studies (24,25). In detail, peripheral blood mononuclear cells were isolated from EDTA-blood *via* density gradient centrifugation (Lymphocytes Separation Media, Capricorn Scientific, Ebsdorfergrund, Germany) and frozen at −80°C. After thawing, a minimum of 1×10^6 cells were stimulated with an overlapping 15-mer peptide pool derived from the full sequence of the SARS-CoV-2 spike glycoprotein (PepMix™ SARS-CoV-2 Spike Glycoprotein, JPT Peptide Technologies, Berlin, Germany) or left unstimulated for 18 h at 37°C after adding 1 µL Ultra-LEAF™ purified anti-human CD40 antibody (BioLegend, San Diego, USA). Cells were stained with antibody-mix for the detection of surface molecules (see **Supplementary Table S1** for antibodies used). All samples were analyzed on a BD FACS Canto II, and FlowJo version 10.8.0 (BD Biosciences, Franklin Lakes, USA) was used for the flow cytometric analysis (see **Supplementary Figure S1** for gating strategy).

In accordance with a previous study on T-cell immunity after SARS-CoV-2 vaccination using a similar assay (25), a positive T-cell response was defined by a stimulation index (SI) ≥2 calculated by dividing $CD154^+CD137^+CD4^+$ T-cells in the stimulated samples by the corresponding cells in the unstimulated samples. SIs below 1 were set to 1.

Statistical Analysis

Continuous variables are presented as median with interquartile range (25th percentile to 75th percentile), and categorical variables as absolute numbers (relative frequencies). Pearson's Chi-squared test, the Wilcoxon rank sum test and Fisher's exact test were used to investigate the effect of the type of vaccine, vaccination regimen, and immunosuppressive agents on the immune response.

We analysed the association of several non-modifiable characteristics with antibody levels and seroconversion rates, namely patient age at first vaccine dose, sex, the timespan between vaccination and serological measurements, and timespan from HT to the first vaccination, and performed multivariable analyses (logistic and Tobit regression analyses) to identify determinants of seroconversion. Further, we used a Tobit regression model to account for values below the limit of

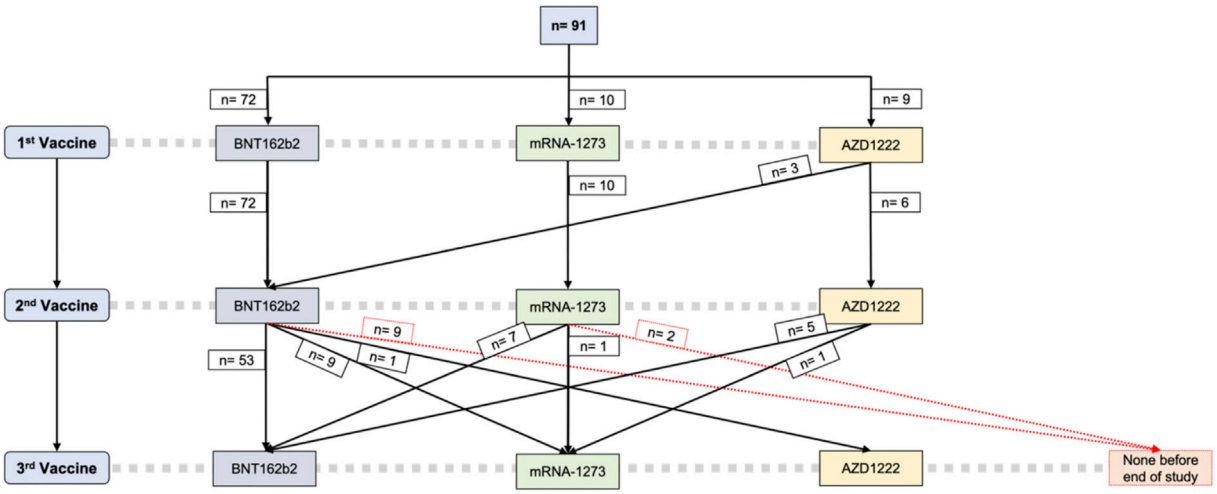


FIGURE 2 | SARS-CoV-2 vaccine regimens. BNT162b2 Tozinameran, Pfizer-BioNTech; mRNA-1273 Spikevax, Moderna; AZD1222 Vaxzevria, AstraZeneca.

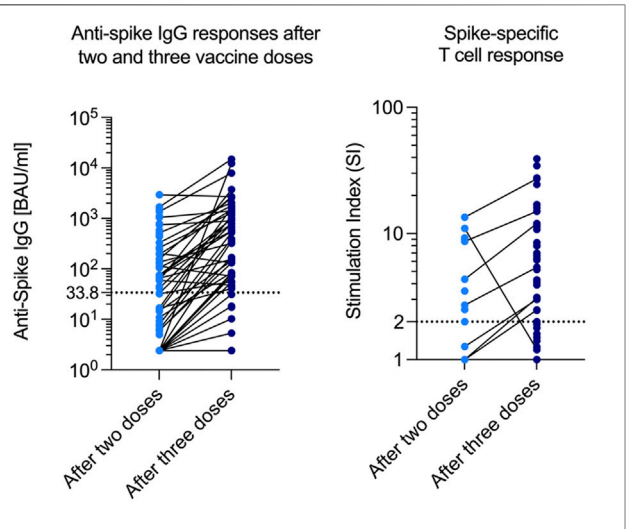


FIGURE 3 | Anti-Spike IgG and SI after two and three doses. SI Stimulation index.

detection of the assay used (<4.81 BAU/mL, which affected n = 34 after two and n = 21 after three vaccine doses). The Tobit model is a special case of the more general censored regression model and is designed to estimate linear relationships between variables when there is either left- or right-censoring in the dependent continuous variable (26,27).

Antibody concentrations were log-transformed for linear and Tobit regression analysis. For logistic regression models, we used the manufacturer's threshold for antibody positivity (≥33.8 BAU/mL) to differentiate between positive and negative antibody responses, as described above. The effect of immunosuppressive agents on seroconversion rates was assessed in multivariable logistic regression analyses adjusting for age at first vaccine dose, sex, and an interaction effect between the two. The effect of prednisolone use on IgG concentrations was also studied adjusting for the timespan from HT to vaccination (in addition to age and sex) in a Tobit linear regression model since prednisolone is often included in immunosuppressive regimens in the first years after HT.

A two-tailed p-value <0.05 was considered statistically significant. All calculations were made using statistical computing software R (Version 4.0.5.) (28).

RESULTS

Baseline Characteristics

Of 99 patients screened, 8 patients were excluded due to a SARS-CoV-2 infection prior to the first serologic assessment, resulting in a total of 91 HT recipients to be included in the study. Sixty-three patients were male (69%) and 28 female (31%). Median age was 55 years (IQR 48.5–61) and median time from HT to first vaccination was 6.1 years (1.6–13.2). Seventy-seven patients (85%) were treated with calcineurin inhibitors (62 [68%] with tacrolimus and 15 [16%] with ciclosporin), 41 (45%) with mycophenolate, 67 (74%) with everolimus and 49 (54%) with low-dose prednisolone (generally 5 mg per day). Forty-one patients (45%) were on dual, 48 (53%) on triple, and 2 (2%) on quadruple immunosuppressive therapy. All patients on a triple therapy regimen except for one received prednisolone. The most common immunosuppressive regimen was everolimus combined with tacrolimus, with or without prednisolone (24 patients [26%] and 21 patients [23%], respectively). A history of diabetes was reported in 27 patients (30%), and median eGFR was 49.0 mL/min (IQR 34.0–69.0) (**Table 1**). Antibody concentrations were available in 82 participants after two and in 70 participants after three vaccine doses.

Details of SARS-CoV-2 Vaccination

All patients screened received at least two SARS-CoV-2 vaccinations. Most participants (79%) received BNT162b2 as their first and second vaccine doses, while 11% received two doses

TABLE 2 | Humoral and spike-specific T-cell response for the whole study population.

	Overall, N = 91[a]	Sex		*p*-value[b]
		Female, N = 28[a]	Male, N = 63[a]	
Humoral response				
Anti-SARS-CoV-2 spike IgG after 2nd dose [BAU/mL][c] (*n* = 48)	74.6 [14.9, 358.0]	113.0 [45.7, 234.0]	59.2 [14.4, 473.0]	0.520
Seroconversion[d] after 2nd dose (*n* = 82)	31/82 (38%)	14/26 (54%)	17/56 (30%)	0.041
Anti-SARS-CoV-2 spike IgG after 3rd dose [BAU/mL][c] (*n* = 49)	553.0 [80.1, 1,400.0]	675.0 [131.0, 1,400.0]	456.0 [77.9, 1,332.5]	0.535
Seroconversion[d] after 3rd dose (*n* = 70)	44/70 (63%)	16/22 (73%)	28/48 (58%)	0.247
Spike-specific T-cell response				
SI after 2nd dose (n = 18)	2.2 (1.0, 7.6)	2.7 (1.1, 7.7)	2.0 (1.0, 6.1)	0.5
Positive response after 2nd dose (*n* = 18)	9/18 (50%)	4/7 (57%)	5/11 (45%)	>0.9
SI after 3rd dose (n = 39)	5 (2, 12)	6 (3, 15)	4 (2, 11)	0.3
Positive response after 3rd dose (*n* = 39)	29/39 (74%)	12/14 (86%)	17/25 (68%)	0.3

[a]*Median [IQR] or Frequency with number (%); Missing data excluded.*
[b]*Wilcoxon rank sum test; Fisher's exact test; Pearson's Chi-squared test; Wilcoxon rank sum exact test.*
[c]*Anti-SARS-CoV-2 spike IgG calculated without non-measurable patients.*
[d]*Anti-SARS-CoV-2 spike IgG ≥33.8 BAU/mL.*
Continuous variables with few values and/or few different values are shown as categorical.
SI, stimulation index.

of mRNA-1273. Of the 9 patients (9.9%) vaccinated with a first dose of AZD1222 (AstraZeneca), only 6 received a second dose of AZD1222, whereas the other 3 were switched to BNT162b2 as the second vaccine. The median time span between the two primary vaccinations was 42 days (35.0, 42.0). Regarding the third vaccination, more than two thirds (68%) received a third dose of an mRNA vaccine matching the primary vaccination (homologous vaccine regimen), while 32% were switched from BNT162b2 to mRNA-1273 or *vice versa*. Patients that had received two doses of AZD1222 received an mRNA-based vaccine as their third dose, either BNT162b2 (6.2%) or mRNA-1272 (1.2%) (**Figure 2**).

More than half of the patients (52%) reported no (solicited or unsolicited) vaccination-associated adverse event whatsoever. Systemic reactogenicity was reported by 29.2%, including fatigue (16%), fevers and chills (5.5%), headaches (4.4%) and myalgia (3.3%), and local reactogenicity in the form of pain at the injection site by 27%. There were no vaccine-related adverse events requiring professional medical attention in our cohort.

Humoral and Spike-Specific T-Cell Response

After two vaccine doses, a positive humoral response could be detected in 31 out of 82 patients (37.8%), and the median antibody concentration was 74.6 BAU/mL (14.9–358.0). After three doses, the median antibody concentration was 553.0 BAU/mL (80.1–1,400.0), and the number of participants with seroconversion rose to 44 out of 70 in which antibody concentrations were measured (62.9%). A third vaccine dose nearly doubled the probability of a positive humoral response (**Figure 3**).

The spike-specific T-cell response was measured in a subset of 49 patients: In 18 patients after two vaccine doses, and in 39 patients after three doses (data after both two and three doses were available for 8 patients). A positive T-cell response was observed in 9 out of 18 patients after two doses (50%), compared to 29 out of 39 patients (74%) after three doses. Interestingly, out of these 29 patients with a detectable T-cell response, 8 (28%) did not show a humoral response (**Table 2**).

Predictors of Immunogenicity

In multivariable logistic regression analyses, higher patient age at vaccination was identified as a predictor of lower seroconversion rates (odds ratio [OR] 0.95, 95% confidence interval [CI] 0.91–0.99, $p = 0.013$), whereas a longer timespan from HT to vaccination was a predictor of higher seroconversion rates (OR 1.1, 95% CI 1.02–1.19, $p = 0.018$) after additional adjustment for patient sex. While seropositivity was observed in 14 out of 26 (54%) female and 17 out of 56 (30%) male participants ($p = 0.041$) in which data were available after two vaccine doses, this difference was not preserved after adjusting for age and timespan from last vaccination to antibody measurement in a multivariable logistical regression model ($p = 0.085$). Here, sex alone was not an independent predictor of an impaired response to vaccination. When sex was interacting with age, however, we saw a trend that middle-aged men reached a positive antibody response more frequently than women, but this effect reversed with increasing age (see **Supplementary Figure S2**). Above the age of 55, women may have reached a positive antibody response more frequently than men. After three doses, there was no significant sex-related difference in antibody positivity overall ($p = 0.247$) and in the same regression model ($p = 0.321$). The timespan from last vaccine dose to antibody measurement was not an independent predictor of antibody response after two ($p = 0.132$) or three doses ($p = 0.756$) after adjustment for age and sex in another logistic regression model.

Using a pre-defined threshold for severe renal impairment of an eGFR <30 mL/min, we could not detect a significant influence of eGFR on log-transformed anti-Spike IgG levels after adjusting for age and timespan from HT to vaccination in a Tobit

TABLE 3 | Logistic regression results for the association between the two-dose or three-dose antibody positivity outcome and exposure to immunosuppressive drug use, adjusted for age at first vaccination and sex as an interaction term.

	Antibody positivity after two vaccine doses (n = 82)													
Predictors	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***
Age at first vaccine dose	1.04 (0.98–1.11)	0.240	1.03 (0.97–1.10)	0.303	1.04 (0.98–1.11)	0.210	1.03 (0.97–1.10)	0.322	1.01 (0.95–1.09)	0.656	1.04 (0.98–1.11)	0.172	1.03 (0.98–1.10)	0.266
Male sex	791.10 (9.48–138251.09)	0.006	749.86 (8.31–141907.54)	0.007	958.61 (10.61–185645.85)	0.005	867.15 (8.97–175602.99)	0.006	194.23 (1.61–51378.07)	0.043	1,386.33 (14.29–292694.91)	0.004	964.21 (10.69–186659.88)	0.005
Age at first vaccine dose * Male sex	0.86 (0.78–0.94)	0.001	0.87 (0.78–0.94)	0.002	0.86 (0.78–0.94)	0.001	0.86 (0.78–0.94)	0.002	0.89 (0.80–0.97)	0.012	0.86 (0.78–0.93)	0.001	0.86 (0.78–0.94)	0.002
Everolimus use			2.05 (0.62–7.60)	0.255										
Cyclosporine A use					0.67 (0.12–3.29)	0.633								
Mycophenolate use							0.37 (0.12–1.09)	0.078						
Prednisolone use									0.12 (0.03–0.38)	<0.001				
Tacrolimus use											2.35 (0.69–9.14)	0.188		
Use of any calcineurin inhibitor													3.12 (0.59–26.35)	0.224

	Antibody positivity after three vaccine doses (n = 70)													
Predictors	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***	**OR**	***p***
Age at first vaccine dose	1.00 (0.93–1.06)	0.913	1.01 (0.94–1.07)	0.817	0.99 (0.93–1.06)	0.870	1.00 (0.94–1.07)	0.883	0.99 (0.92–1.05)	0.707	0.99 (0.93–1.06)	0.867	1.00 (0.93–1.06)	0.920
Male sex	1.17 (0.01–101.76)	0.944	1.48 (0.01–138.00)	0.867	1.17 (0.01–103.15)	0.945	1.62 (0.01–155.78)	0.837	1.00 (0.01–103.15)	1.000	1.18 (0.01–103.84)	0.943	1.18 (0.01–102.10)	0.943
Age at first vaccine dose * Male sex	0.99 (0.91–1.07)	0.742	0.98 (0.91–1.07)	0.692	0.99 (0.91–1.07)	0.750	0.98 (0.91–1.07)	0.717	0.99 (0.91–1.08)	0.806	0.99 (0.91–1.07)	0.735	0.99 (0.91–1.07)	0.731
Everolimus use			3.10 (1.01–10.01)	0.051										
Cyclosporine A use					1.18 (0.28–5.52)	0.828								
Mycophenolate use							0.41 (0.13–1.19)	0.106						
Prednisolone use									0.34 (0.11–0.95)	0.046				
Tacrolimus use											0.82 (0.27–2.45)	0.730		
Use of any calcineurin inhibitor													0.87 (0.23–3.10)	0.832

OR, odds ratio.

Antibody positivity: anti-SARS-CoV-2 spike IgG concentration ≥33.8 BAU/mL.

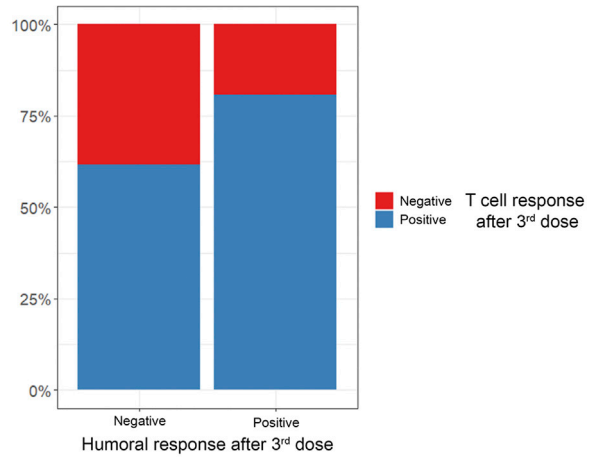


FIGURE 4 | Percentage of positive vs. negative T-cell responses in relation to antibody response after 3rd vaccine dose.

regression model. Of note, only 13 patients in our cohort had an eGFR below this threshold. While a history of type 2 diabetes mellitus (T2DM) was reported in 27 patients (30%), the median age in this group was higher compared to non-diabetic HT recipients (60 years [IQR 54.5–63] vs. 54 years [41–60], p = 0.007). T2DM was not predictive of seropositivity after three vaccine doses (p = 0.3), even when adjusting for patient age in a logistic regression analysis.

The vaccine types, and whether a homologous or heterologous scheme was used, did not influence the humoral response to the second or third vaccine dose, respectively. These results persisted in a logistic regression analysis comparing seropositivity rates after homologous and heterologous vaccine schemes, adjusting for age and sex (OR 1.24, 95% CI 0.41–3.87, p = 0.707).

In a multivariable logistic regression model adjusting for age, sex and the interaction effect between both these variables, prednisolone intake was associated with lower seroconversion rates after both two (OR 0.12, 95% CI 0.03–0.38, $p < 0.001$) and three vaccine doses (OR 0.34, 95% CI 0.11–0.95, p = 0.046, **Table 3**). Similar results were seen in a Tobit linear regression model with log-transformed anti-spike IgG concentrations as the outcome variable (see **Supplementary Table S3**). Patients on a three-drug immunosuppressive regimen exhibited a lower seropositivity rate after two doses (OR 0.09 [0.02–0.28], $p <$ 0.001 after adjusting for age and sex), while a trend towards a lower immunogenicity after the third dose remained (OR 0.37 [0.12–1.06], p = 0.071). In contrast, everolimus intake was associated with an increased antibody response after three (but not after two) doses in the univariable logistic regression analyses and the Tobit linear regression model (see **Supplementary Tables S2, S3**), but this effect did not meet statistical significance in the logistic regression model (OR 3.1 [1.01–10.01], p = 0.051, see **Table 3**). Other immunosuppressive agents, including mycophenolate, did not affect humoral responses (**Table 3**). After two vaccine doses, the predictive effect of prednisolone was preserved even after adjusting for the timespan from HT to vaccination (p = 0.001). Conversely, after three vaccine doses and adjusting for the same variables, there was no significant influence of prednisolone intake (p = 0.434).

Regarding the spike-specific T-cell responses, no association between either of the non-modifiable predictors mentioned above or the immunosuppressive regimen and the SI after three vaccine doses could be established.

DISCUSSION

This report details the humoral and cellular immune response to up to three SARS-CoV-2 vaccinations in a large, consecutive cohort of HT patients. We observed seroconversion rates of 34% and 63% and a T-cell response in 50% and 74% after two and three vaccine doses, respectively. Higher age and shorter time since transplantation were identified as predictors of seroconversion, while there was no association with vaccine type and type of immunosuppressive therapy.

While all approved SARS-CoV-2 vaccines have repeatedly and thoroughly proven to be safe in use (4–6), no serious adverse events related to vaccination were reported in our cohort. Vaccine-related effects like injection-site reactions or fever were less frequently observed compared to rates reported in these trials. Apart from safety issues, there has been ongoing debate about their immunogenicity and efficacy in immunocompromised individuals (10,11,13). The low seroconversion rates observed after two vaccine doses are in line with findings from recent studies (9,10,12), and lower than in the general population (29). Impaired vaccine responses in SOT recipients (30,31) and immunocompromised patients in general (32) have been well documented before the SARS-CoV-2 pandemic, and linked to both the primary disease-associated morbidity, the immunosuppressive medications or a combination of both. The diminished humoral response in HT recipients after SARS-CoV-2 vaccination has been shown to improve upon a third dose (20), which is strongly supported by our results. A significant subgroup of patients remains without detectable antibodies and thus in desperate need for additional strategies in terms of prevention from infection with SARS-CoV-2 and protection against severe course of the disease (13).

We identified several non-modifiable predictors for an impaired humoral response, including higher patient age, and a shorter timespan from HT to vaccination. This is consistent with previous reports (10,14). The former may be associated with a generally suboptimal antibody and T-cell response after SARS-CoV-2 vaccination among the elderly (33), while the latter may be related to a more intensive immunosuppressive therapy in the first years after HT, with most patients being on triple immunosuppressive therapy (14). In our study, prednisolone was in almost all cases used within a triple immunosuppressive regimen, so our observations regarding the association of prednisolone and 3-drug regimens with the humoral response before and after the third vaccination support the consistency of our findings, and suggest that additional booster doses may help attenuate or even overcome certain inhibitory effects of immunosuppressive agents (or triple combinations) on antibody production. Previous studies in SOT recipients reported an

TABLE 4 | T-cell response after three vaccine doses in relation to humoral response.

	Overall, *n* = 39[a]	Spike-specific T-cell response		*p*-value[b]
		Negative, *n* = 10[a]	Positive, *n* = 29[a]	
Antibody positivity				0.3
Negative	13 (33%)	5 (50%)	8 (28%)	
Positive	26 (67%)	5 (50%)	21 (72%)	

[a]*n (%).*
[b]*Fisher's exact test.*

impaired response after two vaccine doses in patients on mycophenolate mofetil (9,11), especially when higher doses were used (32), which is not supported by our data. In contrast, our findings suggest a positive effect of everolimus use on vaccine-induced immunogenicity. However, further research is warranted, especially regarding multiple booster doses, before recommendations on adjustments or withdrawal of immunosuppressive drugs can be deduced.

While antibody production represents a major mechanism of vaccine-induced immunogenicity, eliciting a T-cell response is considered important for long-term protection against severe disease after SARS-CoV-2 vaccination (34). To date, data on vaccine-induced cellular immunity in HT recipients is scarce. While overall T-cell response rates were slightly higher than antibody seroconversion rates in our cohort, 28% of patients with a positive T-cell response were seronegative after three doses (**Figure 4**; **Table 4**). This is consistent with a previous study that reported a significant proportion of HT recipients to remain seronegative after two doses although showing a positive T-cell response (21). While the clinical impact of seronegativity in light of an existing cellular response remains unclear, there is consensus on the increased vulnerability of patients without any type of response (21). SOT recipients at high risk for or suspected insufficient immunogenicity despite repeated vaccination may benefit from recently introduced pre-exposure prophylaxis (PrEP) using monoclonal antibodies (mABs) like AZD7442 (35). Further studies are needed to establish both the role of multiple additional vaccine doses in non-responders and assess the protective ability of mABs with different variants of concern currently circulating, which have not been present during phase 3 vaccine trials.

Strengths and Limitations

There are several advantages inherent to the single-center nature of the data, such as the consecutive enrollment of study participants, complete data capture and the homogeneous management regimen. On the other hand, the small sample size limits statistical power and generalizability of the findings. The small case number of COVID-19 infections of participants during the study period (see **Figure 1**) also limits statistical analysis of the available serology and/or T-cell data prior to infection and precludes evaluation of vaccine efficacy in this cohort.

In our study, the activation-induced marker assay for the assessment of the specific T-cell response was only applied in a subset of patients, which might have influenced conclusions on the interaction between cellular and humoral immunity. While rates of reported SARS-CoV-2 infection during the study period were low in our population, we did not assess antibody levels to SARS-CoV-2 nucleocapsid protein and thus cannot exclude undetected or asymptomatic infections prior to sample acquisition.

Conclusion

Despite ISHLT-recommended SARS-CoV-2 vaccination schedules, a significant proportion of HT recipients exhibit insufficient humoral and T-cell responses. Patient age and time since transplantation predict lower immunogenicity, but inhibitory effects of prednisolone (within 3-drug immunosuppressive regimens) on antibody production may be attenuated through booster vaccination. More data on the effect of immunosuppressive agents on immune response is warranted to improve management of this exceptionally vulnerable group of patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Hamburg Chamber of Physicians (Ethikkommission der Ärztekammer Hamburg). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FM and SK: Research design, data collection, analysis, and interpretation, manuscript preparation. KB: Statistical analysis, data visualization, and interpretation, critical revision of the manuscript. PK, HR, and SB: Critical revision of the manuscript. PD: Data collection, data analysis and interpretation. ML: Data collection and interpretation. MB, FB, NF, PB, CK, and AB: Critical revision of the manuscript. CM and MR: Research design, data collection, analysis, and interpretation, manuscript preparation.

CONFLICT OF INTEREST

SK received scholarship funding from the German Academic Scholarship Foundation and the German Centre for

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10883/full#supplementary-material

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Rescue Everolimus Post Lung Transplantation is Not Associated With an Increased Incidence of CLAD or CLAD-Related Mortality

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Everolimus (EVE) has been used as a calcineurin inhibitor (CNI) minimization/ elimination agent or to augment immunosuppression in lung transplant recipients (LTR) with CNI-induced nephrotoxicity or neurotoxicity. The long-term evidence for survival and progression to chronic lung allograft dysfunction (CLAD) is lacking. The primary aim was to compare survival outcomes of LTR starting EVE-based immunosuppression with those remaining on CNI-based regimens. The secondary outcomes being time to CLAD, incidence of CLAD and the emergence of obstructive (BOS) or restrictive (RAS) phenotypes. Single center retrospective study of 91 LTR starting EVE-based immunosuppression matched 1: 1 with LTR remaining on CNI-based immunosuppression. On multivariate analysis, compared to those remaining on CNI-based immunosuppression, starting EVE was not associated with poorer survival [HR 1.04, 95% CI: 0.67–1.61, $p = 0.853$], or a statistically significant faster time to CLAD [HR 1.34, 95% CI: 0.87–2.04, $p = 0.182$]. There was no difference in the emergence of CLAD (EVE, [$n = 57$, 62.6%] vs. CNI-based [$n = 52$, 57.1%], $p = 0.41$), or the incidence of BOS ($p = 0.60$) or RAS ($p = 0.16$) between the two groups. Introduction of EVE-based immunosuppression does not increase the risk of death or accelerate the progression to CLAD compared to CNI-based immunosuppression.

Keywords: lung transplantation, everolimus, bronchiolitis obliterans syndrome, restrictive allograft syndrome, calcineurin inhibitor, chronic lung allograft dysfunction

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INTRODUCTION

Chronic lung allograft dysfunction (CLAD) remains the limiting factor for long-term survival after lung transplantation (LTx), with poorer outcomes compared to other solid organ transplants (SOTs) and a median survival of 6.5 years (1). Despite the evolution of perioperative and post-operative management strategies over the last 2 decades, immunosuppressive regimens have remained relatively unchanged. Traditional regimens typically consist of a calcineurin inhibitor (CNI), such as tacrolimus or ciclosporin, an antiproliferative (mycophenolate or azathioprine) and a corticosteroid. Everolimus (EVE), a mammalian target of rapamycin (mTOR) inhibitor has only recently been considered a

Abbreviations: CMV, cytomegalovirus; eGFR, estimated glomerular filtrate rate; EVE, everolimus; LTR, lung transplant recipient; LTx, lung transplantation; RAS, restrictive allograft syndrome; SOT, solid organ transplant.

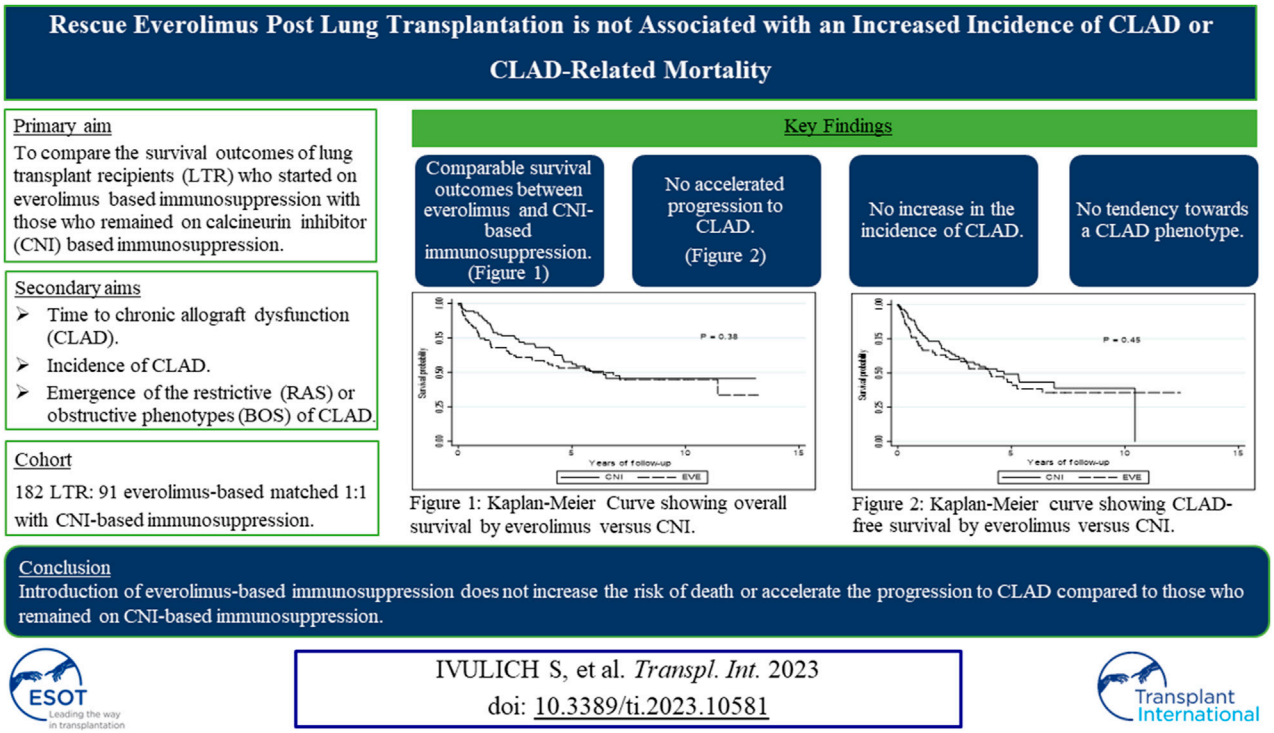

Figure 1: Kaplan-Meier Curve showing overall survival by everolimus versus CNI.

Figure 2: Kaplan-Meier curve showing CLAD-free survival by everolimus versus CNI.

GRAPHICAL ABSTRACT |

potential maintenance immunosuppressant, particularly for those with CNI induced nephrotoxicity or neurotoxicity (2–5).

EVE has unique pharmacological actions distinct from other currently available immunosuppressant classes and provides a novel potential therapeutic role in LTx (6). The use of mTOR inhibitors may reduce *Cytomegalovirus* (CMV) infection (7) and could have some anti-cancer effect due to its anti-angiogenic properties (8, 9). Further, the antifibrotic effect of EVE has been postulated to be beneficial in those with CLAD (10).

However, EVE also has potentially problematic effects in the LTx setting. In particular, its use is not recommended in the early transplant period due to the risk of wound and anastomotic dehiscence (10). In addition, EVE has infrequently been associated with pulmonary toxicity, in particular an interstitial pneumonitis, which may be difficult to distinguish from CLAD (11).

The benefits of EVE for renal preservation after LTx are well documented in several randomized trials (4, 12). Although EVE-based immunosuppression has been shown to be effective in preserving short-term renal function, the long-term benefits have not been maintained (13).

The potential of EVE to prevent CLAD is less well studied. A study by Streuber et al. investigated the impact of EVE on rejection outcomes after LTx. Freedom from bronchiolitis obliterans syndrome (BOS) was investigated in a prospective, randomized trial comparing mycophenolate to everolimus. However, the investigators were unable to prove a difference due to the high withdrawal rate.

A current trial is investigating the immunomodulatory effects of tacrolimus, everolimus and alemtuzumab on kidney function, allograft acceptance and the risk of CLAD. This study is investigating the impact of low-dose everolimus with tacrolimus and alemtuzumab in preventing CNI-driven kidney damage with the potential advantage of further reducing tacrolimus target levels and reducing CLAD (14).

Despite these recent studies, the long-term impact of its use as maintenance immunosuppression on survival and CLAD has yet to be determined. In this retrospective case-controlled study, we compare LTR who started on an EVE-based maintenance immunosuppression regimen with those who remained on a CNI-based regimen. The primary aim of the study was to assess the effect of these immunosuppression approaches on survival, with the secondary outcome being time to CLAD. In addition, we investigated whether EVE-based maintenance immunosuppression contributes to the development of the different CLAD phenotypes: BOS or restrictive allograft syndrome (RAS).

MATERIALS AND METHODS

Patient Population

Between 2005 and 2018, 1100 LTx were undertaken, with institutional recipient management and donor selection protocols described previously (15, 16). All recipients received standard triple immunosuppression with tacrolimus or ciclosporin (pre-2008), azathioprine or mycophenolate mofetil and corticosteroids. All individuals prescribed EVE were considered for inclusion. Excluded were those: lost to follow

up, early discontinuation (duration of therapy <3 months) or previous sirolimus therapy.

Recipients on EVE were matched 1:1 with those who remained on CNI-based immunosuppression based on: transplant date, procedure, age at transplant, sex, and underlying diagnosis. After matching, survival and time to CLAD outcomes were calculated from the date of EVE commencement. If a LTR who remained on CNI-based immunosuppression did not survive to the date of starting EVE, they were excluded from further analysis. The final cohort consisted of 182 LTR (91 EVE recipients with 91 CNI controls).

Definition of Rejection

Acute cellular rejection was defined as changes on transbronchial biopsy of ≥ International Society for Heart and Lung Transplantation (ISHLT) Grade 2, or in the absence of a biopsy an otherwise unexplained drop in lung function treated with intravenous corticosteroid (17, 18). Acute antibody-mediated rejection was diagnosed and managed according to Alfred Hospital protocols (19).

Spirometric Monitoring, Definition, and Treatment of CLAD

All LTR living within 300 km of our centre underwent indefinite long-term follow-up with regular spirometry. Spirometry at time of starting on EVE, 1-year following change and at time of diagnosis of CLAD was investigated for its impact on survival and time to CLAD.

CLAD was defined as an irreversible decline in forced expiratory volume in 1 s (FEV_1) to <80% of baseline (the mean of the two best post-LTx measurements, obtained at least 3 weeks apart with or without a decline in forced vital capacity [FVC]) (20). The phenotypes of CLAD were defined as either BOS (FEV_1/FVC <0.7 and FVC ≥80% predicted baseline FVC at CLAD onset) or RAS (FEV_1/FVC ≥0.7 and FVC <80% predicted baseline FVC at CLAD onset) (21, 22). Whilst total lung capacity (TLC) is not routinely undertaken and was not available on all recipients to allow its use in the definition, we utilised the spirometric definition detailed above to define RAS as detailed in the published consensus guidelines (21, 22). Declines in lung function/CLAD were treated according to the standard protocols of the time (18). For this analysis CLAD status, staging and phenotype were redefined as per ISHLT criteria (20, 23).

General Management Strategy for Renal Impairment

Induction therapy with the IL-2 receptor blocker, basiliximab, was given as a CNI sparing agent to LTR who were identified pre-transplant as being at higher risk of developing post-LTx renal dysfunction (n = 73). Subsequent strategies for LTR with renal impairment involved CNI reduction (n = 47) or elimination (n = 44); control of hypertension, diabetes, and cholesterol; and initiation of EVE (22). For LTR receiving EVE in combination with a CNI for a renal indication, further increases in serum creatinine would warrant eventual withdrawal of the CNI.

General Management Strategy for CMV

CMV prophylaxis, monitoring and treatment strategies are described elsewhere (24). Immediate post-transplant prophylaxis for all patients at risk of CMV infection received at least 7 days of intravenous ganciclovir followed by valganciclovir for a duration determined by risk category. Severe CMV infection or CMV reactivation was defined as >10,000 IU/mL in the blood or CMV >50,000 IU/mL in the bronchoalveolar lavage (BAL).

EVE Indications, Dosing, TDM and Utilization Strategy

EVE was utilized in the setting of failure of first-line immunosuppressive strategies, e.g., significant renal impairment, CNI-neurotoxicity, malignancy (25, 18). EVE was prescribed with or without a CNI, determined by the degree of CNI intolerance. As per unit protocol, EVE was typically commenced at a dose of 0.25–0.5 mg twice daily with halving of the CNI dose. If EVE was to be used in conjunction with a CNI (minimization strategy) a trough concentration of 3–5 ng/mL for EVE and 4–6 ng/mL for tacrolimus would be targeted. If CNI cessation was planned (elimination strategy), a trough concentration of 5–7 ng/mL for EVE was targeted. Whenever EVE was utilized as part of a CNI elimination strategy, the CNI was ceased when EVE trough concentration was ≥3 ng/mL.

Statistical Analyses

Continuous variables were summarized using means and standard deviations (SD) or medians and interquartile ranges (IQR) wherever appropriate. Categorical variables were expressed as counts and percentages. Overall survival was defined as the time from the date of starting EVE to the date of death or last follow-up. Time to CLAD was calculated from the date of starting EVE to the date of diagnosis of CLAD.

Univariate and multivariate analyses for overall survival and time to CLAD were performed using Cox proportional hazards regression with results reported as hazard ratios (HR) and 95% confidence intervals (95% CI). Variables with a $p < 0.05$ on univariable analyses or those deemed clinically relevant were considered for inclusion in the multivariable models.

To account for any possible imbalance between groups due to differences in baseline demographics and the evolution of treatments, propensity scores were included as an additional covariate in the regression models. Propensity score matching was also used to reduce selection bias from confounding factors between the EVE or CNI-based immunosuppression group. The individual propensities for being in the EVE group were estimated with the use of a multivariable logistic regression model that included date of transplant, age at transplant, sex, azithromycin and CMV reactivation as the predictor variables. All calculated p-values were two-tailed and a $p < 0.05$ indicated statistical significance. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, United States).

Changes in estimated glomerular filtrate rate (eGFR) over time (time of LTx, EVE commencement and 1-year post) was assessed using linear mixed models fitting main effects for time, group (EVE or CNI) and their two-way interactions.

TABLE 1 | Demographics.

	EVE (*n* = 91)	CNI (*n* = 91)	*p*-value
Characteristic			
Age (yr), mean	51.64 ± 13.87	50.81 ± 13.87	0.16
Gender: male, n (%)	49 (53.8)	49 (53.8)	1.00
Body mass index (mean ± SD)	25.00 ± 4.45	25.93 ± 5.28	0.15
Indication for transplantation, n (%)			
Chronic obstructive pulmonary disease	43 (47.2)	43 (47.2)	1.00
Cystic fibrosis	19 (20.9)	19 (20.9)	1.00
Interstitial lung disease	19 (20.9)	19 (20.9)	1.00
Pulmonary hypertension	6 (6.6)	6 (6.6)	1.00
Other	4 (4.4)	4 (4.4)	1.00
Transplantation type, n (%)			
Bilateral sequential lung	81 (89.0)	81 (89.0)	1.00
Single lung	9 (9.9)	9 (9.9)	1.00
Heart and lung	1 (1.1)	1 (1.1)	1.00
Maintenance Immunosuppression, n (%)[a]			
Tacrolimus	75 (82.4)	82 (90.1)	0.07
Ciclosporin	16 (17.6)	9 (9.9)	0.07
Mycophenolate	38 (41.7)	33 (36.3)	0.44
Azathioprine	34 (37.4)	52 (57.1)	0.009
No antimetabolite	19 (20.9)	6 (6.6)	0.014
Rejection			
Acute Rejection [b]	13 (14.3)	15 (16.5)	0.66
Diagnosis of CLAD[c]	57 (62.6)	52 (57.1)	0.41
RAS	27 (29.7)	19 (20.9)	0.16
BOS	30 (33.0)	33 (36.3)	0.60

Abbreviations: ACR, acute cellular rejection; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; EVE, everolimus; ISHLT, international society for heart and lung transplantation; RAS, restrictive allograft syndrome.

[a]*Maintenance immunosuppression at time of starting on EVE.*

[b]*Episode of ISHLT graded ≥2 ACR pre or post starting on EVE.*

[c]*Diagnosis of CLAD pre or post starting on EVE.*

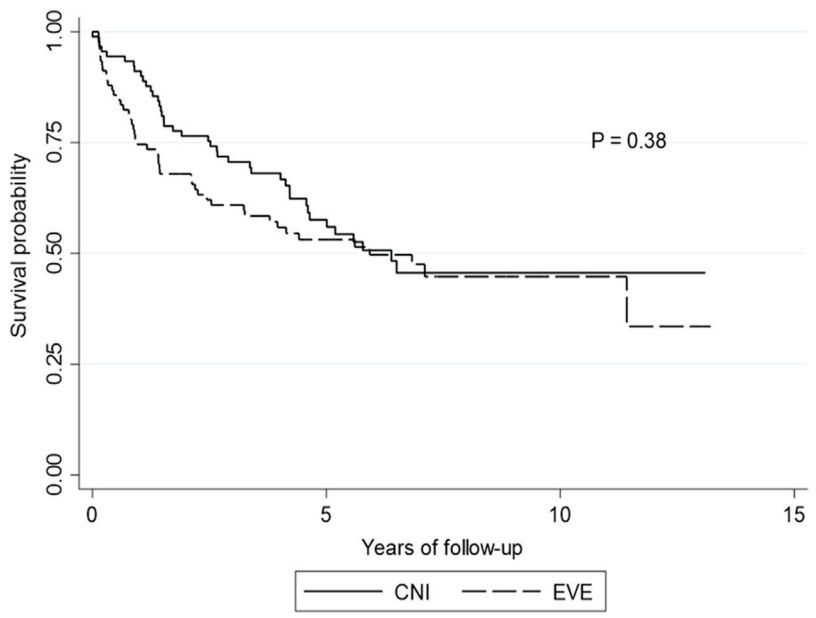

FIGURE 1 | Kaplan-Meier curve for overall survival. Kaplan-Meier curve showing overall survival by calcineurin inhibitor versus everolimus. *p*-value is calculated from a log rank test comparing the entire survival experience between the two groups (calcineurin inhibitor versus everolimus). Abbreviations: CNI, calcineurin inhibitor; EVE, everolimus.

TABLE 2 | Univariate analysis: Summary of effects of different coviarates on survival.

Summary of effects of different covariates on survival (*n* = 182)			
		Hazard ratio	***p*-value**
Demographics	Male	1.10 (0.72–1.67)	0.668
	Age	1.00 (0.99–1.02)	0.742
	Body mass index	0.98 (0.94–1.02)	0.344
	Surgical Procedure		
	BSLTx	0.98 (0.56–1.73)	0.954
	SLTx	0.84 (0.47–1.52)	0.566
Diagnosis	Cystic Fibrosis	0.81 (0.50–1.34)	0.422
	Chronic obstructive pulmonary disease	1.25 (0.83–1.90)	0.290
	Interstitial lung disease	0.87 (0.51–1.48)	0.610
	Pulmonary Hypertension	0.51 (0.22–1.18)	0.118
	Retransplant	1.49 (0.43–5.12)	0.53
	Other	2.08 (0.84–5.16)	0.113
Everolimus	Use of EVE	1.15 (0.75–1.76)	0.514
	Time of Transplant to Initiation	0.99 (0.98–0.99)	<0.0001
	Discontinuation of EVE	1.99 (1.11–3.54)	0.020
	Indication for EVE	0.91 (0.56–1.47)	0.699
	EVE Level	1.06 (0.96–1.16)	0.282
Immunosuppression[a]	Tacrolimus	1.32 (0.79–2.19)	0.290
	Ciclosporin	0.83 (0.48–1.42)	0.492
	Azathioprine	1.14 (0.75–1.72)	0.542
	Mycophenolate	0.62 (0.39–0.99)	0.047
Indication	Renal preservation	1.65 (0.87–3.12)	0.128
CLAD	Existence of CLAD[b]	3.73 (2.06–6.75)	<0.0001
	CLAD phenotype	0.59 (0.36–0.96)	0.033
	CLAD at time of starting EVE	0.84 (0.39–1.82)	0.657
	FER at diagnosis of CLAD	0.98 (0.96–0.98)	0.024
	Azithromycin prophylaxis	0.52 (0.30–0.91)	0.019
Spirometry	FEV_1: Time of starting EVE (measured)	0.70 (0.56–0.87)	0.002
	FEV_1: Time of starting EVE (percentage)	0.98 (0.97–0.99)	0.0001
	FVC: Time of starting EVE (measured)	0.79 (0.66–0.96)	0.018
	FVC: Time of starting EVE (percentage)	0.98 (0.98–0.99)	0.005
	FEV_1:1-year post starting EVE (measured)	0.49 (0.36–0.66)	<0.0001
	FEV_1:1-year post starting EVE (percentage)	0.97 (0.96–0.98)	<0.0001
	FVC: 1-year post starting EVE (measured)	0.69 (0.53–0.90)	0.006
	FVC: 1-year post starting EVE (percentage)	0.97 (0.96–0.98)	<0.0001
Cytomegalovirus	*Cytomegalovirus* reactivation[c]	1.33 (0.84–2.08)	0.219

Abbreviations: BSLTx, bilateral sequential lung transplant; CLAD, chronic lung allograft dysfunction; EVE, everolimus; FER, forced expiratory ratio; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; SLTx, single lung transplant.

[a]*Maintenance immunosuppression at time of starting EVE.*

[b]*Diagnosis of BOS or RAS pre or post starting EVE.*

[c]Cytomegalovirus *reactivation post starting EVE.*

Ethics Approval

The study was approved by the Alfred Hospital (252-12, 30 May 2012) and Monash University Ethics Committees (252-12, 19 April 2017).

RESULTS

Patient Characteristics and Indications for EVE Use

Baseline demographics are described in **Table 1**. The most common indication for starting EVE was renal impairment (79%), followed by malignancy (8%), neurotoxicity (7%), intolerance to CNI (4%) and recurrent CMV (2%). The median time from LTx to initiation of EVE was 334 days [IQR: 155-604], with the median time of follow up for all LTR included being 1881 days [IQR: 993–2970].

Overall Survival

The median survival for the entire cohort was 1881 days [IQR: 993–2970], (EVE: 1869 days [IQR: 910–3185] vs. CNI: 1944 days [IQR: 1196–2850]). One, three- and five-year survival was 80.2%, 68.1% and 59.9%, respectively. There was no difference in overall mortality between the groups (48% EVE vs. 45% CNI, $p = 0.648$) (**Figure 1**).

Univariate Analysis

On univariate analysis (**Table 2**), compared to CNI-based immunosuppression commencement of EVE based immunosuppression was not associated with a statistically significant poorer survival outcome [HR 1.15, 95% CI: 0.75–1.76, $p = 0.514$]. After adjusting for propensity score, survival outcomes were also comparable to those who remained on CNI-based immunosuppression [HR 1.12, 95% CI: 0.74–1.70, $p = 0.577$].

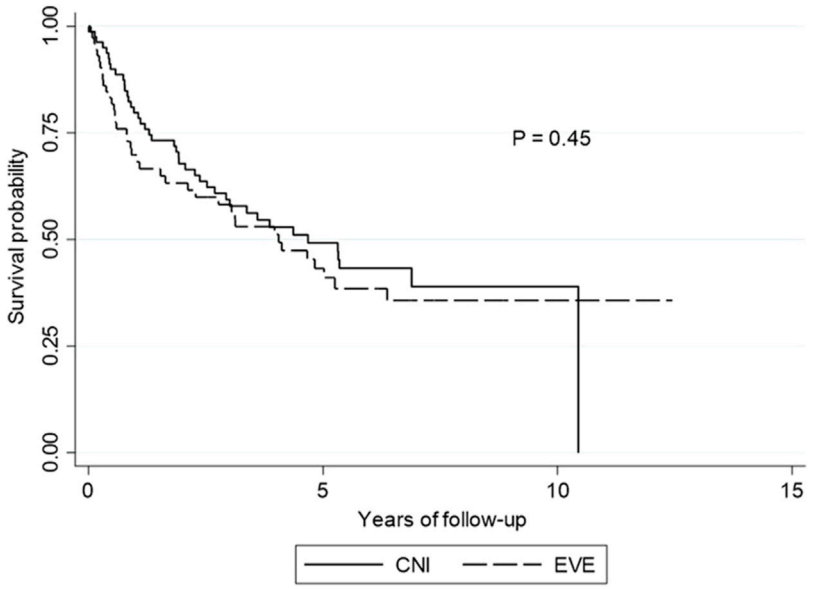

FIGURE 2 | Kaplan-Meier curve for CLAD-free survival. Kaplan-Meier curve showing CLAD-free survival by calcineurin inhibitor versus everolimus. *p*-value is calculated from a log rank test comparing the CLAD-free survival between the two groups (calcineurin inhibitor versus everolimus). Abbreviations: CM, calcineurin inhibitor; EVE, everolimus.

On univariate analysis, starting on EVE for a renal indication had no impact on survival [HR 1.65, 95% CI: 0.87–3.12, p = 0.128]. However, LTR who started EVE for renal preservation were more likely to have a faster progression to CLAD [HR 1.98, 95% CI: 1.09–3.59, p = 0.024].

The development of CLAD at any time point was associated with mortality [HR 3.73, 95% CI: 2.06–6.75, p = 0.0001]. However, CLAD diagnosed prior to starting EVE was not a predictor of death (p = 0.657). Recipients who developed RAS had a lower risk of death than those with BOS (HR 0.591 [0.365–0.959], p = 0.033).

All spirometric indices measured at time of EVE initiation and 1 year following change were significant predictors of survival (**Table 2**). The forced expiratory ratio (FEV_1/FVC) at time of CLAD diagnosis was a predictor of improved survival (HR 0.978 [0.959–0.997], p = 0.024).

Timing of EVE Commencement

The timing of starting EVE did not impact survival. LTR who started EVE prior to 1-year post LTx had similar survival outcomes to those who started after 1-year post LTx [HR 0.97, 95% CI: 0.63–1.49, p = 0.88].

Time to CLAD

Univariate Analysis

Following the univariate analysis, compared to those remaining on CNI-based immunosuppression initiation of EVE-based immunosuppression did not statistically accelerate the progression to CLAD diagnosis [HR 1.27, 95% CI: 0.87–1.86, p = 0.208] (**Figure 2**). After adjusting for propensity score, compared to CNI-based immunosuppression, EVE was not associated with a faster time to CLAD [HR 1.26, 95% CI: 0.89–1.77, p = 0.190]. All spirometric indices measured at time of starting EVE and 1 year following change were predictors of time to CLAD (**Table 3**).

Relationship of CLAD Onset and to Time to Death

The time from diagnosis of CLAD until death was longer in the EVE group compared to the CNI-based group (1127 days, [IQR: 504–2210] vs. 427 days, [IQR: 236–1229], p = 0.01).

INCIDENCE OF CLAD AND CLAD PHENOTYPES

The overall incidence of CLAD at any time point was 59.8% (109/182). In the LTR who developed CLAD, 57.8% (n = 63) developed the BOS phenotype, whereas the remainder (42.2%, n = 46) developed RAS (**Figure 3**).

There was no difference in the emergence of CLAD between the two groups (EVE, [n = 57, 62.6%] vs. CNI-based [n = 52, 57.1%], p = 0.41). There was no difference in the incidence of BOS (p = 0.60) or RAS (p = 0.16) between the EVE and the CNI groups (**Figure 3**).

Multivariate Analysis

Survival

On multivariate analysis (**Table 4**), starting on EVE was not associated with poorer survival [HR 1.10, 95% CI: 0.73–1.66, p = 0.642]. At the time of EVE introduction, FVC percentage calculated was predictive of better survival (HR 0.99 [0.98–1.00], p = 0.023) and both BOS (HR 4.30 [2.16–8.58], p=< 0.0001) and RAS (HR 2.36 [1.23–4.53], p = 0.010) phenotypes of CLAD were independently associated

TABLE 3 | Univariate analysis: Summary of effects of different covariates on time to CLAD.

Summary of effects of different covariates on time to CLAD (*n* = 182)			
		Hazard ratio	***p*-value**
Demographics	Male	1.22 (0.81–1.82)	0.342
	Age	1.01 (0.99–1.02)	0.338
	Body mass index	1.02 (0.98–1.06)	0.315
	Surgical Procedure		
	BSLTx	0.88 (0.52–1.51)	0.653
	SLTx	1.05 (0.56–1.96)	0.880
Diagnosis	Cystic Fibrosis	0.52 (0.30–0.90)	0.019
	Chronic obstructive pulmonary disease	1.46 (0.98–2.17)	0.060
	Interstitial lung disease	1.03 (0.64–1.65)	0.902
	Pulmonary hypertension	1.08 (0.52–2.22)	0.838
	Re-Transplant	1.27 (0.22–7.45)	0.789
	Other	1.01 (0.25–4.13)	0.991
EVE	Use as an Immunosuppressant	1.27 (0.87–1.86)	0.208
	Time of Transplant to Initiation	1.00 (1.00–1.00)	0.446
	Discontinuation of EVE	1.46 (0.84–2.53)	0.178
	EVE Level	1.03 (0.91–1.16)	0.677
Immunosuppression[a]	Tacrolimus	0.86 (0.51–1.46)	0.580
	Ciclosporin	1.08 (0.61–1.92)	0.782
	Azathioprine	0.99 (0.68–1.45)	0.974
	Mycophenolate	0.76 (0.51–1.13)	0.175
	Tacrolimus Level	1.04 (0.97–1.11)	0.319
	Ciclosporin Level	1.00 (1.00–1.00)	0.725
Indication	Renal preservation	1.98 (1.09–3.59)	0.024
CLAD	Azithromycin prophylaxis	1.22 (0.66–2.27)	0.522
Spirometry	FEV_1: Time of starting EVE (measured)	0.67 (0.53–0.86)	0.001
	FEV_1: Time of starting EVE (percentage)	0.98 (0.97–0.99)	0.003
	FVC: Time of starting EVE (measured)	0.84 (0.69–1.01)	0.064
	FVC: Time of starting EVE (measured)	0.99 (0.98–1.00)	0.012
	FEV_1: 1-year post starting EVE (measured)	0.57 (0.44–0.73)	<0.0001
	FEV_1: 1-year post starting EVE (percentage)	0.97 (0.97–0.98)	<0.0001
	FVC: 1-year post starting EVE (measured)	0.67 (0.54–0.83)	0.0003
	FVC: 1-year post EVE starting (percentage)	0.97 (0.96–0.98)	<0.0001
Cytomegalovirus	*Cytomegalovirus* Reactivation [b]	1.28 (0.86–1.92)	0.220

Abbreviations: BSLTx, bilateral sequential lung transplant; CLAD, chronic lung allograft dysfunction; EVE, everolimus; FER, forced expiratory ratio; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; SLTx, single lung transplant.

[a]*Maintenance immunosuppression at time of starting EVE.*

[b]Cytomegalovirus *reactivation post starting EVE.*

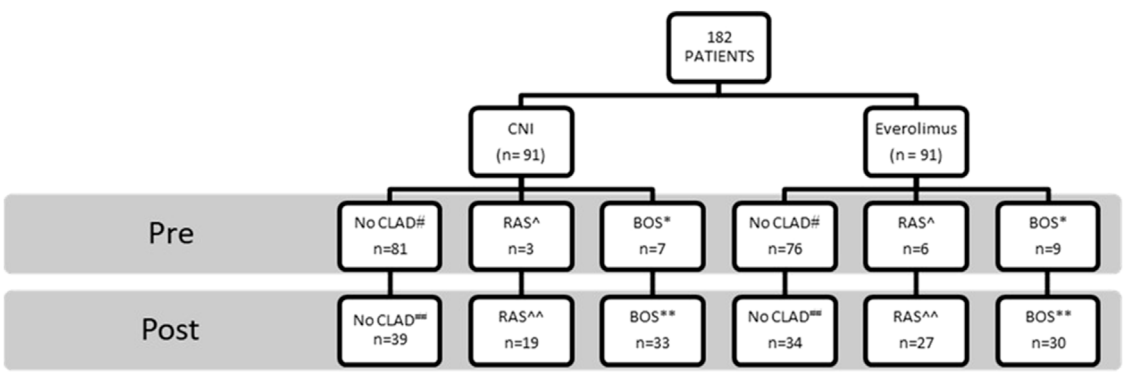

FIGURE 3 | CLAD outcomes for lung transplant cohort: Everolimus versus calcineurin inhibitor group. CLAD outcomes pre and post intervention. *Comparisons between CNI and EVE groups: Pre-switch BOS* ($p = 0.74$), post-switch BOS** ($p = 0.60$), pre-switch RAS^ ($p = 0.33$), post-switch RAS^ ($p = 0.16$), pre-switch CLAD# ($p = 021$), post-switch CLAD## ($p = 0.41$). Abbreviations: BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft syndrome; CNI, calcineurin inhibitor; RAS, restrictive allograft syndrome.

TABLE 4 | Multivariate analysis—risk factors for mortality: Propensity matched pairs post conversion to everolimus.

Variable	Hazard ratio	*p*-value
EVE	1.10 (0.73–1.66)	0.642
Estimated GFR at time of starting EVE	1.00 (0.99–1.01)	0.938
FVC % predicted at time of starting EVE	0.99 (0.98–1.00)	0.023
No CLAD	REF	
BOS[a]	4.30 (2.16–8.58)	<0.0001
RAS[a]	2.36 (1.23–4.53)	0.010
Mycophenolate	0.62 (0.40–0.98)	0.043

Abbreviations: BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; EVE, everolimus; FVC, forced vital capacity; GFR, glomerular filtrate rate; RAS, restrictive allograft syndrome.
[a]Diagnosis of BOS or RAS pre or post starting EVE.

TABLE 5 | Multivariate analysis—risk factors for time to CLAD: Propensity matched pairs post conversion to everolimus.

Variable	Hazard ratio	*p*-value
EVE	1.34 (0.88–2.06)	0.176
Estimated GFR at time of starting EVE	1.01 (1.00–1.01)	0.143
% FVC predicted at time of starting EVE	0.99 (0.98–1.00)	0.007
Cystic Fibrosis	0.49 (0.25–0.97)	0.039
ISHLT graded ≥2 ACR[a]	2.30 (1.53–3.45)	0.0001
Mycophenolate	0.67 (0.44–1.01)	0.058

Abbreviations: ACR, acute cellular rejection; CLAD, chronic lung allograft dysfunction; EVE, everolimus; FVC, forced vital capacity; GFR, glomerular filtrate rate; ISHLT, international society for heart and lung transplantation.
[a]Episode of ISHLT graded ≥2 ACR pre or post starting EVE.

with poorer survival (**Table 4**). On multivariate analysis, immunosuppressant regimens containing mycophenolate were associated with improved survival (HR 0.62 [0.40–0.98], p = 0.043).

Time to CLAD

On multivariate analysis, starting on EVE was not associated with a faster time to CLAD diagnosis [HR 1.34, 95% CI: 0.88–2.06, p = 0.176]. The variables associated with a faster time to CLAD included FVC percentage calculated at time of EVE introduction [HR 0.99, 95% CI: 0.98–1.00, p = 0.011], diagnosis of cystic fibrosis [HR 0.49, 95% CI: 0.25–0.97, p = 0.039] and a history of ISHLT grade ≥2 ACR [HR 2.37, 95% CI: 1.56–3.60, p=<0.0001] (**Table 5**).

Preservation of Renal Function

The baseline renal function between the two groups was comparable at the time of LTx (p = 0.478). Estimated GFR declined significantly from the time of LTx to the introduction of EVE for all included LTR (from 84.4 to 56.1 mL/min/1.73 m^2, $p \leq 0.0001$). At the time of commencement of EVE, there was a significant difference in estimated glomerular filtrate rate (eGFR) between the two groups (EVE, 44.5 mL/min/1.73 m^2 vs. CNI, 67.7 mL/min/1.73 m^2, $p \leq 0.0001$). At 1-year follow up, the eGFR in the EVE group was significantly lower than the CNI group (EVE, 56.2 vs. CNI, 64.1 mL/min/1.73 m^2, p = 0.03) (**Figure 4**). The changes in eGFR assessed over time between the two groups and their two-way interactions demonstrated significant interaction between group and time (p < 0.0001), suggesting that the EVE and CNI groups behaved differently over time (**Figure 4**).

Calcineurin Inhibitor Minimization Versus Elimination

Demographics of the two EVE strategies (CNI minimization and CNI elimination) are described in **Table 6**. The CNI elimination strategy was not associated with poorer survival (p = 0.158) or a faster time to CLAD (p = 0.944). When comparing the eGFR changes over time, there is no statistical difference between the two groups (p = 0.498).

Cause of Death

There was no difference between the groups with regards to cause of death (**Table 7**). In particular, no difference in death due to CLAD (31.9% EVE vs. 29.6% CNI, p = 0.74). Additionally, whilst EVE was utilized primarily as a CNI-sparing agent to preserve renal function, there was no difference in mortality from renal failure (2.2% EVE vs. 1.1% CNI, p = 1.00).

DISCUSSION

We believe this is the largest study examining the use of EVE-based maintenance immunosuppression in LTR. Compared to other SOTs, experience with EVE for maintenance immunosuppression in the LTx setting remains limited (27, 26). EVE was predominantly initiated in our cohort as a CNI-sparing agent in the setting of renal impairment. The most important findings from the study demonstrated that EVE can likely be safely utilized for second line immunosuppression with the aim of minimizing or eliminating CNIs without an increase in mortality, incidence of CLAD or time to CLAD.

Overall Survival

The primary aim of our study was to determine whether LTR who started on EVE had poorer survival outcomes compared to those that remained on CNI-based immunosuppression. Overall survival, including CLAD related mortality was similar to those who remained on CNI-based immunosuppression, suggesting a similar trajectory for both groups. Our study demonstrated that EVE is a safe and effective option when prescribed for CNI intolerance, primarily nephrotoxicity or neurotoxicity as part of maintenance immunosuppressive regimens.

There has been some reluctance in using EVE as part of immunosuppressive regimens in the perioperative period due to challenges with the risk of wound dehiscence as well as destabilizing immunosuppression. While we would recommend EVE for stable long-term LTR as part of a maintenance immunosuppression regimen in LTR with CNI-induced nephrotoxicity, we would not recommend starting on EVE in the perioperative period.

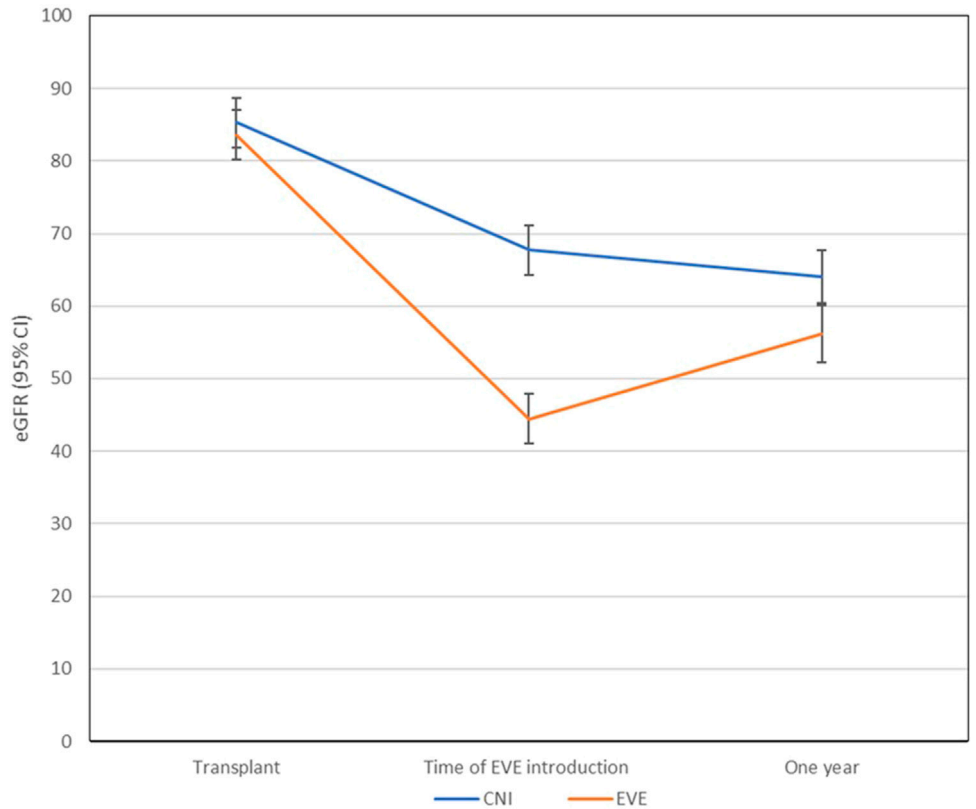

FIGURE 4 | Changes in renal function (eGFR) from time to transplant to starting EVE to 1-year post. The changes in eGFR assessed over time between the two groups and their two-way interactions demonstrated significant interaction between group and time ($p < 0.0001$), suggesting that the EVE and CNI groups behaved differently over time. Abbreviations: CNI, Calcineurin inhibitor; eGFR, estimated glomet-ular filtrate rate; EVE, everolimus.

CLAD

We found no statistically significant increase in the incidence of CLAD, an accelerated progression to CLAD or a tendency towards a specific CLAD phenotype with EVE. Not unexpectedly, the greater the ventilatory reserve at the time of starting EVE (FEV_1 and FVC), the less likely the recipient would progress to CLAD.

Traditionally outcomes in LTR with RAS tend to be worse than those with BOS with shortened survival (28). We found on multivariate analysis, patients with BOS unexpectedly had a greater risk of death compared to those with RAS. The potential benefit of EVE in LTR with RAS and the potential mechanisms for these findings warrant further investigation. The development of CLAD has been suggested to be due to chronic fibroblast activation and EVE is known to downregulate fibroblast activity (10). A potential mechanism could be the antifibrotic activity of EVE influencing the chronic fibroblast activation present in CLAD.

Other benefits of EVE may be due to its impact on angiogenesis. Angiogenesis is a complex process in the transplanted organ and involves cellular proliferation, vascular remodelling, and endothelial activation. EVE has anti-angiogenic effects *in vitro* that prevent cellular proliferation, vascular remodelling, and endothelial activation with potential benefits in chronic allograft rejection (9). EVE may indeed have promoted a stabilizing influence on pulmonary function once CLAD was established. These findings also reassure us that there was no long-term clinically significant underlying EVE lung toxicity (29).

On multi-variate analysis, as a time-dependent variable, mycophenolate containing immunosuppressant regimens were associated with improved survival. At our institution, mycophenolate is utilized as a second line agent in sensitized recipients or LTR who are commenced for the management of ACR. Azzola et al. demonstrated that EVE and mycophenolate were the two most potent antifibroproliferative drugs at concentrations achieved clinically (30). Low doses of EVE and mycophenolate can achieve at least 50% inhibition of fibroblast proliferation at therapeutic doses (30). The combination of mycophenolate and EVE may provide a synergistic benefit in LTR with CLAD due to their antifibrotic activity warranting further investigation.

Preservation of Renal Function

The most common indication for starting on EVE in our cohort was renal preservation due to CNI-related nephrotoxicity. Preservation of renal function is paramount post-LTx as nephrotoxicity is associated with significant morbidity and mortality (31). EVE provides an alternate immunosuppressive agent in the LTx setting with potentially less nephron loss over time from reduced long-term exposure to CNIs (3, 4). Other studies in LTx have

TABLE 6 | Everolimus strategies.

Demographics	CNI minimization (*n* = 55)	CNI elimination (*n* = 36)	*p*-value
Characteristic			
Age (yr), mean	51.47 ± 14.10	51.89 ± 13.75	0.89
Gender: male, n (%)	25 (45.4)	24 (66.7)	0.047
Indication for transplantation, n (%)			
Chronic obstructive pulmonary disease	24 (43.6)	19 (52.8)	0.305
Cystic fibrosis	12 (21.8)	7 (19.4)	0.785
Interstitial lung disease	12 (21.8)	7 (19.4)	0.785
Pulmonary hypertension	4 (7.3)	2 (5.6)	0.747
Other	3 (5.5)	1 (2.8)	0.416
Transplantation type, n (%)			
Bilateral sequential lung	49 (89.0)	32 (88.9)	0.83
Single lung	5 (9.2)	4 (11.1)	0.75
Heart and lung	1 (1.8)	0 (0.0)	1.00
Maintenance Immunosuppression, n (%)[#]			
Tacrolimus	47 (85.5)	26 (72.2)	0.12
Ciclosporin	8 (14.5)	6 (16.7)	0.78
Mycophenolate	19 (34.5)	19 (52.8)	0.09
Azathioprine	25 (45.5)	9 (25.0)	0.049
Rejection			
ISHLT graded ≥2 ACR[^]	8 (14.5)	5 (13.9)	0.93
Diagnosis of CLAD[*]	34 (61.8)	23 (63.9)	0.72
RAS	18 (32.7)	13 (23.6)	0.48
BOS	16 (29.1)	10 (18.2)	0.63

Abbreviations: ACR, acute cellular rejection; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; CNI, calcineurin inhibitor; EVE, everolimus; RAS, restrictive allograft syndrome.

[#]Maintenance immunosuppression at time of switch to EVE.

[^]Episode of ISHLT graded ≥ 2 ACR pre or post switch to EVE.

[*]Diagnosis of BOS or rCLAD pre or post switch to EVE.

TABLE 7 | Cause of death.

Cause of death			
Cause	**EVE (n = 44)**	**Calcineurin inhibitor (*n* = 41)**	***p*-value**
CLAD	29	27	0.74
Non-specific graft failure	5	4	0.66
Infection	4	5	0.57
Cerebrovascular accident	1	3	0.99
Malignancy	3	0	1.00
Renal Failure	2	1	1.00
Other: Non-adherence/Lost to follow-up	0	1	1.00

Abbreviations: CLAD, chronic lung allograft dysfunction; EVE, everolimus.

investigated the change in renal function parameters following the introduction of EVE (3, 4). Similarly, to these studies we found no increase in renal related mortality with the introduction of EVE as part of maintenance immunosuppressive regimens.

Although on univariate analysis, LTR who started EVE for renal preservation appeared to progress to CLAD faster than those that did not, this was not borne out in multivariate or survival analyses. A possible explanation for the faster time to CLAD is the contribution of renal impairment to lung function decline. It is known that LTR with chronic kidney disease demonstrate abnormalities in lung function including obstructive and restrictive ventilatory defects, and impaired diffusing capacity (32).

Additionally, it is likely that those started on EVE-based regimens due to renal impairment had a period of subtherapeutic CNI levels with the aim of stabilizing renal function in the short-term. These potential prolonged periods of low CNI levels may have led to the immunological risk of periods of suboptimal immunosuppression contributing to an increased risk of CLAD. We would suggest that in those with CNI intolerance, it may be prudent to consider starting EVE earlier rather than continue with subtherapeutic levels of CNI and the risk of inadequate immunosuppression.

Limitations

Our study has several limitations. Firstly, the findings are retrospective, reflect a single center experience and the EVE prescribing patterns of this center have evolved since 2005. Immunosuppression regimens and target levels would be adjusted over time according to unit protocol and modified

further according to response and tolerance. In addition, the study cohort was heterogenous, with indications for EVE use including nephrotoxicity and neurotoxicity and regimen strategies that varied from CNI minimization to elimination.

Secondly, as our center does not routinely monitor TLC this could not be utilized in the definition of RAS and therefore spirometric measures as previously detailed were incorporated into our criteria for RAS diagnosis (21, 23).

Conclusion

EVE-based maintenance immunosuppression can be successfully and safely be utilized when CNI minimization or elimination is required. Most importantly, our analyses demonstrated that starting EVE does not increase the risk of death or accelerate the progression to CLAD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Alfred Hospital (252-12) Monash University (252-12). The ethics committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

SI and MP: study design, data collection and analysis, write up. EP: data analysis. CK and MD: data analysis and write up. GS: study design, data analysis, and write up. All authors approved the final version and had full access to the data.

CONFLICT OF INTEREST

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

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Impact of Culture-Positive Preservation Fluid on Early Morbidity and Mortality After Lung Transplantation

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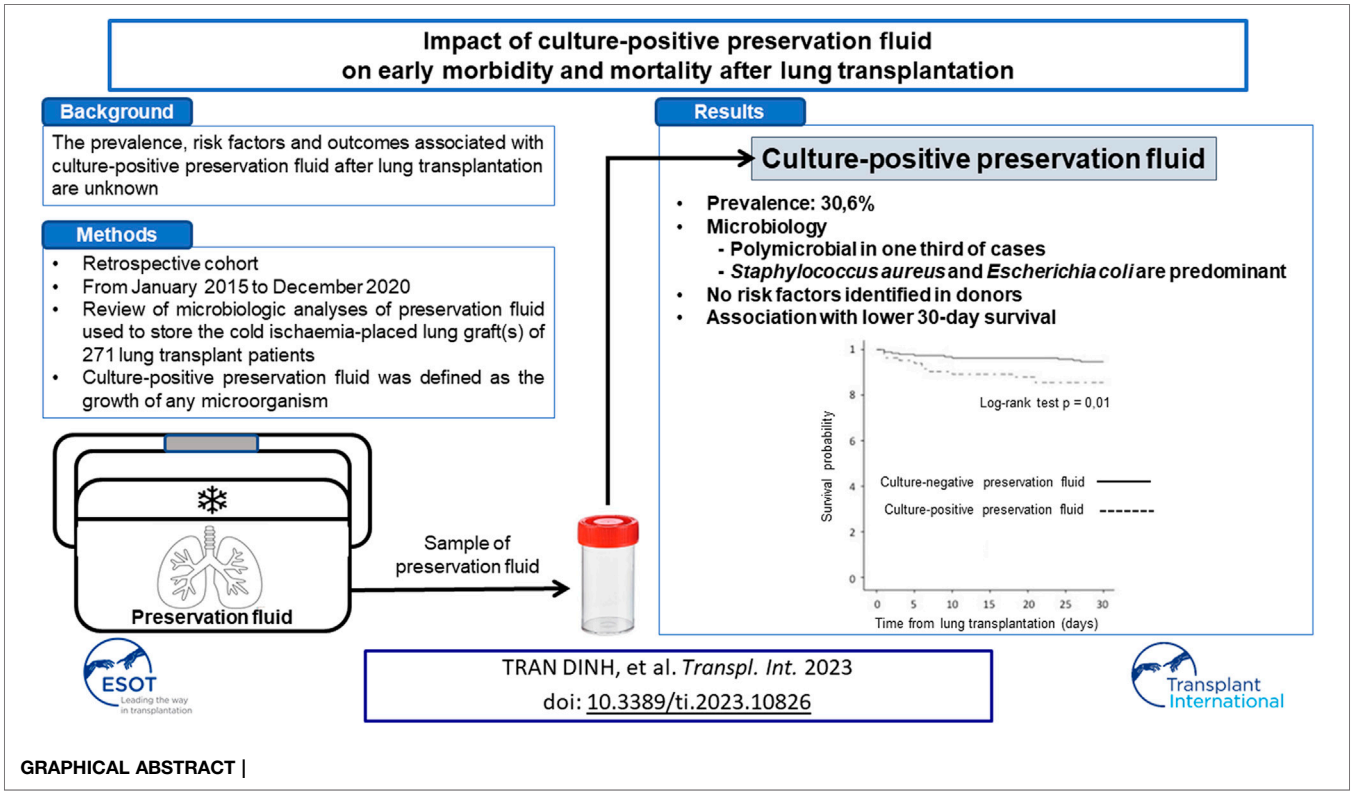
The prevalence, risk factors and outcomes associated with culture-positive preservation fluid (PF) after lung transplantation (LT) are unknown. From January 2015 to December 2020, the microbiologic analyses of PF used to store the cold ischaemia-placed lung graft(s) of 271 lung transplant patients were retrospectively studied. Culture-positive PF was defined as the growth of any microorganism. Eighty-three (30.6%) patients were transplanted with lung grafts stored in a culture-positive PF. One-third of culture-positive PF were polymicrobial. Staphylococcus aureus and Escherichia coli were the most frequently isolated microorganisms. No risk factors for culture-positive PF based on donor characteristics were identified. Forty (40/83; 48.2%) patients had postoperative pneumonia on Day 0 and 2 (2/83; 2.4%) patients had pleural empyema with at least one identical bacteria isolated in culture-positive PF. The 30-day survival rate was lower for patients with culture-positive PF compared with patients with culture-negative PF (85.5% vs. 94.7%, $p = 0.01$). Culture-positive PF has a high prevalence and may decrease lung transplant recipient survival. Further studies are required to confirm these results and improve understanding of the pathogenesis of culture-positive PF and their management.

Keywords: lung transplantation, survival, preservation fluid, antibiotic prophylaxis, ICU morbidity



GRAPHICAL ABSTRACT |

INTRODUCTION

Lung transplantation (LT) is the final resort therapy for patients with end-stage lung disease (1). Infections strongly decrease recipient survival, accounting for 17% and 33% of deaths at 30 days and 1 year, respectively (2). Among the various potential sources of posttransplant infections, donor-to-host transmission of infection in solid organ transplant is a life-threatening early complication (3–5). In a prospective study assessing 211 donors contributing to 292 transplant procedures, lung was the most likely to be performed with an infected donor (15%), although only one donor-transmitted infection occurred (6).

Investigating early lung graft infection may include peri-transplant microbiological culture of donor and recipient respiratory specimens as well as organ preservation fluid (PF) (7). Culture-positive PF may indicate graft infection, contamination during graft procurement or colonization by passage of the causative microorganisms from the organ into the storage fluid during cold ischaemic time. However, there is no recommendation for its evaluation and use to guide antibiotic therapy. A recent systematic review and meta-analysis among solid organ transplants observed an overall incidence of culture-positive PF and PF-related infections of 37% and 10%, respectively, and mortality rates among PF-related infections of 35% (8). However, specific data in LT remain very limited (5, 9).

To address this issue, we sought to describe 1) the prevalence of culture-positive PF and PF-related postoperative pneumonia and 2) risk factors and outcomes associated with culture-positive PF in LT. We also evaluated the impact of the adequacy between the peri-transplant antibiotic prophylaxis and the susceptibility of microorganisms isolated from PF on recipient outcomes.

MATERIALS AND METHODS

Study Design

We conducted a retrospective single-centre study that included all consecutive patients who underwent LT between January 2015 and December 2020. Retransplantations and *ex vivo* lung perfusion procedures were not included.

We analysed all available microbiological cultures of PF, donor respiratory specimens performed before lung procurement, and recipient respiratory specimens collected during postoperative ICU admission. The study was approved by the ethics committee CEERB Paris Nord, which waived the need for signed informed consent (Institutional Review Board -IRB 00006477- Université Paris Cité, AP-HP.Nord).

Donor lung procurement was performed identically for bilateral and single LT. Lungs were procured "*en bloc*" with the trachea immediately stapled to avoid subsequent PF contamination, stored in a bag and immersed in PF (Perfadex®, XVIVO, Goteborg, Sweden). The bag was surrounded by ice to maintain the temperature at 4°C during the cold ischaemia time for transport to our centre. Lung separation was performed on a back table upon arrival at our centre after removal from the bag containing the PF. A sample of PF was taken and sent for microbiological culture (10).

Microbiological Features and Definitions

> "PF samples were systematically collected in sterile by the surgeon during graft preparation on the back table during pneumonectomy of the native lung(s), and immediately sent to the bacteriology and mycology laboratories. Samples were inoculated all day every day onto routine agar plates (100 µL per plate), which included trypticase soy agar supplemented with 5% horse blood, Columbia sheep blood agar containing nalidixic acid and colistin and chocolate agar supplemented with PolyVitex for isolation of fastidious bacteria. The plates were incubated for 48 h at 35 ± 2°C under aerobic and anaerobic conditions. The limit of detection was 10^2 UFC/mL. All culture media were controlled weekly by the culture of ATCC strains according to applicable standards. All the different morphotypes of colonies that grew on the different plates were identified at the species level by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) Microflex LT Biotyper (Bruker Daltonics, Bremen, Germany). Bacterial susceptibility to antibiotics was determined using the disk-diffusion method according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) for all bacteria isolated. Culture-positive PF was defined as the growth of any microorganism."

High- and low-risk microorganisms were defined as described previously (11). High-risk microorganisms included Gram-negative bacilli (GNB), *Staphylococcus aureus*, *β-haemolytic Streptococcus* spp., *Streptococcus pneumoniae*, *Enterococcus* spp., *Bacteroides* spp., and *Candida* spp. Low-risk microorganisms included *coagulase-negative Staphylococcus* spp., *Corynebacterium* spp., and α-haemolytic *Streptococcus* spp. In our local policy, patients with culture-positive PF were treated for 7 days with appropriate antibiotic therapy, regardless of the results of respiratory samples on Day 0, because of the theoretical risk of pleural empyema.

Donor respiratory samples taken just prior to lung procurement by the thoracic surgeon of our centre were microbiologically analysed at the donor centre. The results were retrieved *via* the Agence de la Biomédecine, a national public agency in charge of coordination of organ, tissue and cell procurement and transplantation, as well as in the fields of human reproduction, embryology and genetics (12). There was no microbiological analysis of the donor lung just before its transplantation into the recipient.

Upon ICU admission after LT, bronchoalveolar lavage (BAL) respiratory specimens were taken from the recipient and analysed for microbiological culture. Postoperative bacterial pneumonia on Day 0 was defined as in the recommendations for cardiothoracic transplant patients (13). A diagnosis of pneumonia was established when clinical, biological, radiographic and microbiological criteria were met. Clinical, biological and radiographic criteria were fever (temperature >38°C), purulent secretions, gas exchange degradation, elevated white blood cell count, and chest imaging revealing a new or progressive alveolar or interstitial that could not be explained by any other noninfectious cause. Microbiological criteria was a positive bacterial culture at the threshold of infection of a bronchoalveolar lavage (BAL) performed at postoperative ICU admission (14, 15). Patients with pneumonia were treated for 7 days with appropriate antibiotic therapy, and were considered cured if signs of infection resolved (improvement in clinical signs, haematosis and radiological abnormalities).

Data Collection

Donor characteristics, including age, sex, smoking status, cause of death, duration of mechanical ventilation, and PaO_2/FiO_2 ratio at the time of lung procurement, were collected.

Demographic and preoperative characteristics of recipients were recorded, including age, sex, body mass index (BMI), primary diagnosis of chronic pulmonary disease, Cytomegalovirus mismatch (Donor+/Recipient-), past medical history of diabetes and ischaemic heart disease with angioplasty and/or coronary stent, high-emergency LT, extracorporeal membrane oxygenation (ECMO) as bridge to transplant and mean pulmonary arterial pressure (mPAP) measured by a right heart catheterization at listing. High emergency LT is a national prioritization system for the most severe patients with fibrosis, cystic fibrosis or pulmonary hypertension that was introduced in France in 2007 (16).

Intraoperative characteristics were collected, including type of LT (i.e., single or bilateral), maximum graft cold ischaemia time, intraoperative blood transfusion >2 packed red blood cells (PRBC) and intraoperative ECMO.

Lung graft complications were also documented, including grade 3 primary graft dysfunction (PGD) as defined by the ISHLT consensus (17), acute cellular rejection confirmed by histopathological evidence after transbronchial lung biopsies or considered and treated as if the risk of biopsy outweighed the expected benefit (18), and definite, probable or possible antibody-mediated rejection according to Levine et al. (19) with the need for plasmapheresis.

We recorded patient outcomes, including ICU stay characteristics (simplified acute physiology score II (SAPS II) and sequential organ failure assessment (SOFA) score at admission, acute kidney injury stage 3 of KDIGO (Kidney Disease: Improving Global Outcomes), renal replacement therapy, duration of mechanical ventilation, duration of norepinephrine support, ECMO in ICU, tracheostomy, ICU length of stay, chronic lung graft dysfunction and mortality rates at 30 days, 1, 3, and 5 years.

Perioperative Management

Surgical transplantation procedures and perioperative care, including postoperative management, were standardized for all patients according to our local protocol already published elsewhere (20). The immunosuppressive regimen included

mycophenolate mofetil, corticosteroids and tacrolimus. There was no induction therapy.

Perioperative antibiotic prophylaxis was defined by the antibiotic regimen started intraoperatively. It was considered appropriate towards the PF culture if it was effective against all bacteria isolated in the PF after susceptibility testing. Perioperative antibiotic prophylaxis was cefazolin, as it was recommended in "Clinical practice guidelines for antimicrobial prophylaxis in surgery" (21). Cefazolin was substituted and tailored according to the known colonisation of the donor and recipient. Perioperative antibiotic prophylaxis was systematically administered intraoperatively and continued 48 h after surgery, as recommended (22). During the immediate postoperative period, antibiotic therapy was adapted to microbiological cultures obtained from bronchoalveolar lavage (BAL) performed upon postoperative ICU admission and from PF. If BAL and PF cultures were negative without evidence of infection, antibiotic prophylaxis was stopped after 48 h.

Statistical Analysis

Baseline characteristics within each group were described with numbers and percentages for qualitative variables and medians and interquartile ranges for quantitative variables.

Thirty-day and 1-year survival rates were assessed between patients with culture-positive PF and culture-negative PF and between patients who received or did not receive an appropriate peri-operative antibiotic prophylaxis for culture-positive PF. The probability of all-cause death was estimated using the Kaplan-Meier method and compared using the log-rank test.

Donor risk factors for culture-positive PF were assessed by univariate analysis, and unadjusted odds ratios (ORs) and 95% CIs were calculated.

All reported p values were two-sided, and the level of statistical significance was specified *a priori* as less than 0.05. Statistical analysis and data management were performed using BM SPSS Statistics version 20 (IBM Corp., Armonk, NY, United States).

RESULTS

Two hundred seventy-one patients were transplanted with one or two lung grafts procured from 271 donors between January 2015 and December 2020. The median age of recipients at the time of LT was 57 [50–62] years. Primary diagnoses were mainly interstitial lung disease (48.3%) and COPD (36.2%). Double LT represented 67.9% of the procedures (**Table 1**).

Prevalence of Culture-Positive PF and Microbiological Components

Eighty-three (30.6%) patients were transplanted with lung grafts stored in a culture-positive PF. Microorganisms isolated in PF are presented in **Table 2**. Twenty-seven (27/83 = 32.5%) PFs were polymicrobial, and 73 (73/83; 88%) were positive for at least one "high-risk" microorganism. *Staphylococcus aureus* and *Escherichia coli* were the most frequently isolated microorganisms. Four (4/83; 4.8%) PF were positive for at least one fungus. None were positive for extended-spectrum beta-lactamase-producing Enterobacteriaceae or multidrug-resistant bacteria.

Antibiotic prophylaxis other than cefazolin (n = 183, 67.5%) were amoxicillin/clavulanic acid (n = 39, 14.4%), cefepime (n = 28, 10.3%), ceftazidime (n = 6, 2.2%), piperacillin/tazobactam (n = 7, 2.6%), cefotaxime (n = 5, 1.8%), carbapenem (n = 3, 1.1%) and linezolid (n = 4, 1.5%).

Risk Factors for Culture-Positive PF

We did not identify risk factors for culture-positive PF from donor characteristics or preoperative and intraoperative recipient characteristics (**Tables 1**, **3**).

Respiratory Samples From Donor Lung

Two hundred and twenty (220/272 = 81.2%) donors had a respiratory sample before lung procurement. Ninety-one (91/220 = 41.4%) had culture-positive respiratory samples. Details of the bacteria isolated from donor respiratory samples are presented in the **Supplementary Table S1**. Donors had no pneumonia or pneumonia controlled by antibiotic therapy without infiltrates on the CT scan prior to organ procurement.

Among the 83 recipients grafted with culture-positive PF, 40 donors had positive microbiological cultures of respiratory specimens, 20 donors had negative cultures, and 23 donors did not have available respiratory specimens. Twenty-eight (28/40; 70%) recipients had at least one identical microorganism documented in both the PF and the donor respiratory samples.

Postoperative Outcomes

Mortality, ICU morbidity and chronic lung graft dysfunction

The 30-day survival rate was significantly lower for patients with culture-positive PF compared with patients with culture-negative PF (85.5% vs. 94.7%, p = 0.01) (**Figure 1**).

Survival rates at 1, 3, and 5 years for patients with culture-positive PF compared with patients with culture-negative PF were 68.7% vs. 78.7% (p = −0.06), 48.5% vs. 62% (p = 0.06) and 32.4% vs. 52.7% (p = 0.04), respectively.

Deaths at 30 days (n = 22) were due to haemorrhagic shock (n = 10, 45.5%), septic shock (n = 6, 27.3%), primary graft dysfunction (n = 2, 9%) or others (n = 4, 18.2%). In the group of patients with culture-positive PF who died within 30 days (n = 12/83, 14.5%), the causes of death were haemorrhagic shock (n = 6, 50%), septic shock (n = 4, 33.3%, two of which were related to pneumonia with the same germ identified in the PF), primary graft dysfunction (n = 1, 8.3%) and other (n = 1, 8.3%). In the group of patients with culture-negative PF who died within 30 days (n = 10/188, 5.3%), the causes of death were haemorrhagic shock (n = 4, 40%), septic shock (n = 2, 20%), primary graft dysfunction (n = 1, 10%) and others (n = 3, 30%).

Patients with culture-positive PF had higher SAPS II scores on postoperative ICU admission, had more AKI and required more RRT and ECMO during their ICU stay (**Table 3**).

The occurrence of chronic lung graft dysfunction was similar between recipients with culture-positive PF and culture-negative PF (27.9% vs. 28.8%, p = 0.89).

TABLE 1 | Recipient demographics and intraoperative characteristics.

	All patients (n = 271)	Culture-positive PF (n = 83)	Culture-negative PF (n = 188)	OR [95% CI], p value
Recipient demographics and comorbidities				
Age, years	57 [50–62]	57 [50–62]	51 [56–62]	1.01 [0.99–1.03], p = 0.37
Female sex	97 (35.8)	56 (67.5)	118 (62.8)	1.23 [0.71–2.12], p = 0.49
BMI, kg/m^2	24 [20–27]	24 [20–28]	24 [20–27]	1.01 [0.94–1.07], p = 0.77
Aetiology				
COPD	98 (36.2)	33 (39.8)	65 (34.6)	1.25 [0.73–2.13], p = 0.41
ILD	131 (48.3)	35 (42.2)	96 (51.1)	0.70 [0.42–1.12], p = 0.18
Others	43 (16)	16 (19.8)	27 (14.4)	1.46 [0.74–2.89], p = 0.28
Coronary angioplasty and/or stent	11 (4.1)	1 (1.2)	10 (5.3)	0.22 [0.03–1.72], p = 0.11
Diabetes	28 (10.3)	8 (9.6)	20 (10.6)	0.90 [0.78–2.13], p = 0.80
mPAP, mmHg	25 [20–30]	25 [20–30]	25 [21–30]	0.74 [0.96–1.03], p = 0.99
CMV mismatch	56 (20.7)	16 (19.3)	40 (21.4)	0.88 [0.46–1.68], p = 0.69
ECMO as bridge-to-transplant	20 (7.4)	6 (7.2)	14 (7.4)	0.97 [0.36–2.62], p = 0.95
High-emergency LT	49 (18.1)	15 (18.1)	34 (18.1)	1.0 [0.51–1.96], p = 1
Lung transplant surgery				
Type of LT				0.97 [0.56–1.69], p = 0.92
Single LT	87 (32.1)	27 (32.5)	60 (31.9)	
Double LT	184 (67.9)	56 (67.5)	128 (68.1)	
Maximum graft ischaemic time, min	330 [270–400]	330 [270–400]	333 [270–400]	1.0 [0.99–1.0], p = 0.88
Intraoperative ECMO	190 (70.1)	59 (71.1)	131 (69.7)	1.07 [0.61–1.89], p = 0.82
Transfusion ≥3 PRBC	128 (47.6)	40 (48.2)	88 (47.3)	1.04 [0.62–1.74], p = 0.89

Quantitative variables are expressed as medians and interquartile ranges. Qualitative variables are expressed as numbers and percentages.
Abbreviations: PF, preservation fluid; BMI, body mass index; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; PAP, pulmonary arterial pressure; CMV, cytomegalovirus; ECMO, extracorporeal membrane oxygenation; LT, lung transplantation; PRBC, packed red blood cell.

Postoperative Pneumonia on Day 0 and Pleural Empyema

One hundred and twenty-one (121/271 = 44.6%) recipients had postoperative pneumonia on Day 0. The 30-day survival rate between recipients who had postoperative pneumonia on Day 0 compared to those who did not was similar (94% vs. 89.3%, p = 0.18). The overall number of pneumonia cases during the ICU stay was similar in recipients with and without culture-positive PF (**Table 4**). Bacteria isolated from pneumonia occurring during the ICU stay of patients transplanted with lung graft(s) stored in culture-positive or culture-negative PF are presented in the **Supplementary Table S2**.

Among the 83 recipients with culture-positive PF, 40 (40/83 = 48.2%) had postoperative pneumonia on Day 0. Twenty-eight (28/83 = 33.7%) recipients had postoperative pneumonia on Day 0 with at least one identical microorganism documented in both the PF and the donor respiratory sample.

The 30-day survival rate between recipients with postoperative pneumonia on Day 0 with at least one identical bacteria isolated from the PF compared to recipients without pneumonia on Day 0 or with pneumonia on Day 0 without identical bacteria isolated from PF was similar (85% vs. 86%, p = 0.90).

The 30-day survival rate between recipients with postoperative pneumonia on Day 0 with at least one identical bacteria isolated from PF compared to recipients with postoperative pneumonia on Day 0 without identical bacteria isolated from PF was similar (85% vs. 90.9%, p = 0.63).

Two (2/83; 2.4%) recipients had pleural empyema with at least one identical bacteria isolated from the PF (*Klebsiella pneumoniae* and *Corynebacterium striatum*, respectively), each occurring on day 8 post-transplant.

Impact on Recipient Outcomes of Adequacy Between Antibiotic Prophylaxis and Antibiotic Susceptibility of Microorganisms Isolated in PF

Fifty-five (55/83; 66.3%) patients with culture-positive PF were treated with appropriate antibiotic prophylaxis initiated intraoperatively. Seventy-seven (77/83; 92.8%) patients with culture-positive PF received targeted antibiotic therapy after susceptibility testing with a standard duration of 7 days. The six patients who did not receive curative antibiotic therapy had culture-positive PF with oropharyngeal flora (n = 3), coagulase-negative staphylococci (n = 2), *Streptococcus anginosus* (n = 1) and *Proteus mirabilis* (n = 1). None of them had pneumonia on Day 0.

The adequacy of antibiotic prophylaxis did not affect the 30-day survival of recipients with culture-positive PF compared to recipients without culture-positive PF (89.1% vs. 78.6%, p = 0.21, respectively) (**Figure 2**).

DISCUSSION

For the first time to the best of our knowledge, we designed a study to describe the impact of culture-positive PF on the outcomes of lung transplant patients. We reported a prevalence of 30% of recipients transplanted with grafts stored in culture-positive PF, which was associated with reduced 30-day survival. Although there is no consensual attitude to date, our results might argue for a systematic examination of the microbiological culture of the PF after LT.

TABLE 2 | Microorganisms isolated from culture-positive PF.

Microorganisms (*n* = 108)	(*n*)
High-risk pathogens (*n* = 91; 84.3%)	
Bacterial species (*n* = 86; 79.6%)	
Gram-negative bacilli (*n* = 51; 47.2%)	
Escherichia coli	13
Enterobacter cloacae	6
Klebsiella pneumoniae	5
Pseudomonas aeruginosa	4
Klebsiella aerogenes	4
Serratia marcensens	4
Klebsiella oxytoca	3
Citrobacter koseri	3
Haemophilus influenzae	3
Hafnia alvei	2
Proteus mirabilis	2
Serratia ureilytica	1
Acinetobacter pitii	1
Gram-positive cocci (*n* = 35; 32.4%)	
Staphylococcus aureus	33
Streptococcus pneumoniae	2
Fungal species (*n* = 5; 4.6%)	
Candida albicans	2
Candida glabrata	1
Candida parapsilosis	1
Candida krusei	1
Low-risk pathogens (*n* = 17; 15.7%)	
Oropharyngeal flora[a]	8
Branhamella catarrhalis	2
Streptococcus anginosus	2
Staphylococcus epidermidis	2
Streptococcus oralis	1
Corynebacterium striatum	1
Corynebacterium propinquum	1

[a]*Bacterial species composing the oropharyngeal flora are a-hemolytic streptococci excepted* Streptococcus pneumoniae, Haemophilus *spp. excepted* Haemophilus influenzae, Neisseria *spp. excepted* Neisseria meningitidis *and* Neisseria gonorrhoeae *and* Rothia mucilaginosa.

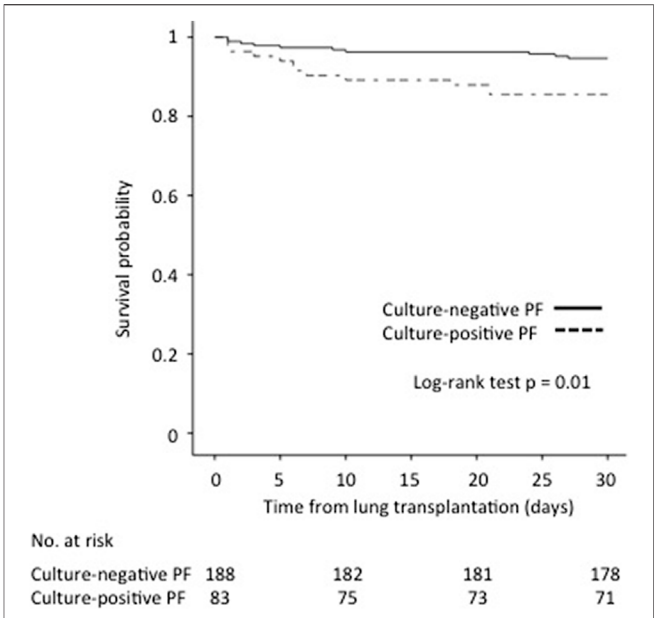


FIGURE 1 | Impact of culture-positive PF on 30-day survival after lung transplantation.

The overall prevalence of culture-positive PF in solid organ transplantation is highly variable, having recently been reported as ranging from 37% in a systematic review (8) to 62.5% in a prospective study (23). Moreover, there are disparities between each organ. Only one study assessed the prevalence of culture-positive PF in LT that was 15% of 190 procedures (5), whereas it can reach 80% in renal transplantation (24) to almost 100% in liver transplantation (25).

The mechanism(s) responsible for the contamination of the normally sterile PF are hypothetical. The PF used in our centre is Perfadex®, which is a dextran-based solution that is low in potassium, reduces interstitial oedema and maintains the integrity of epithelial cells (26). Positive microbiological culture of the PF may include 1) endogenous dissemination of microorganisms contained in the organ during the storage, which could lead to a transplant with an already infected lung graft and/or to pleural empyema; or 2) extrinsic input during graft handling prior to transplant with a risk of secondary pleural empyema. According to our results, the high proportion of approximately 50% of postoperative pneumonia on Day 0, i.e., due to the same bacteria than those isolated in the PF, suggests endogenous contamination by passage of bacteria from the lung into the PF during storage.

The possible deleterious impact of culture-positive PF on early outcome with organ failure during the postoperative ICU stay and 30-day survival is threatening, especially in the face of its high prevalence. There is unresolved debate as to why culture-positive PF is associated with such detrimental outcomes. The largest prospective multicenter study on the impact of culturing PF on solid organ transplantation also reported nearly statistical

TABLE 3 | Donor risk factors associated with culture-positive PF.

Donor characteristics	All patients (*n* = 271)	Culture-positive PF (*n* = 83)	Culture-negative PF (*n* = 188)	OR [95% CI], *p* value
Age, years	53 [41–61]	51 [39–61]	53 [42–62]	0.97 [0.98–1.01], $p = 0.68$
Female sex	121 (44.6)	34 (41)	87 (46.3)	1.24 [0.74–2.09], $p = 0.42$
Active smoking	100 (36.9)	35 (42.2)	65 (34.6)	1.38 [0.81–2.34], $p = 0.23$
Cerebral cause of death	214 (79)	62 (74.7)	152 (80.9)	0.70 [0.38–1.29], $p = 0.25$
Duration of mechanical ventilation, days				1.02 [0.94–1.10], $p = 0.65$
PaO_2/FiO_2, mmHg	398 [343–459]	383 [331–446]	400 [347–463]	0.98 [0.99–1.0], $p = 0.22$

Quantitative variables are expressed as medians and interquartile ranges. Qualitative variables are expressed as numbers and percentages.
Abbreviations: PF, preservation fluid.

TABLE 4 | Outcomes associated with culture-positive PF.

Outcomes	All patients (*n* = 271)	Culture-positive PF (*n* = 83)	Culture-negative PF (*n* = 188)	OR [95% CI], *p* value
Postoperative ICU stay				
Postoperative SAPS II	43 [38–50]	46 [38–53]	43 [38–50]	1.03 [1.01–1.05], $p = 0.01$
Postoperative SOFA score	7 [6–9]	8 [6–10]	7 [6–9]	1.07 [0.96–1.19], $p = 0.23$
Stage 3 AKI of KDIGO	39 (14.4)	19 (22.9)	20 (10.7)	2.48 [1.24–4.95], $p = 0.009$
Renal replacement therapy	31 (11.5)	16 (19.3)	15 (8.1)	2.72 [1.28–5.82], $p = 0.008$
Duration of mechanical ventilation, days	3 [1–19]	4 [1–19]	3 [1–14]	1.0 [0.99–1.01], $p = 0.59$
Duration of norepinephrine, days	2 [1–4]	2 [1–4]	2 [1–4]	1.01 [0.96–1.06], $p = 0.65$
ECMO in ICU	77 (28.5)	31 (37.3)	46 (24.6)	1.83 [1.05–3.19], $p = 0.03$
Tracheotomy	66 (24.6)	23 (28.4)	43 (23)	1.33 [0.74–2.40], $p = 0.35$
Length of ICU stay, days	17 [10–33]	16 [10–32]	17 [11–33]	1.0 [0.96–1.01], $p = 0.47$
Lung graft complications				
Grade 3 primary graft dysfunction	48 (17.8)	16 (19.5)	32 (17.1)	1.17 [0.60–2.29], $p = 0.73$
Postoperative pneumonia on Day 0	121 (44.6%)	51 (61.4)	70 (37.2)	2.68 [1.58–4.57], $p < 0.001$
Number of pneumonia cases during ICU stay	1 [0–2]	1 [0–2]	1 [0–2]	1.05 [0.86–1.28], $p = 0.64$
Acute antibody-mediated rejection	53 (19.7)	16 (19.5)	37 (19.8)	0.98 [0.51–1.89], $p = 0.98$
Acute cellular rejection	62 (23.1)	17 (21)	45 (24.1)	0.84 [0.45–1.58], $p = 0.58$

Quantitative variables are expressed as medians and interquartile ranges. Qualitative variables are expressed as numbers and percentages.
Abbreviations: PF, preservation fluid; ICU, intensive care unit; SAPS II, simplified acute physiology score II; SOFA, sequential organ failure assessment; AKI, acute kidney injury; KDIGO, Kidney Disease: Improving Global Outcomes; ECMO, extracorporeal membrane oxygenation.

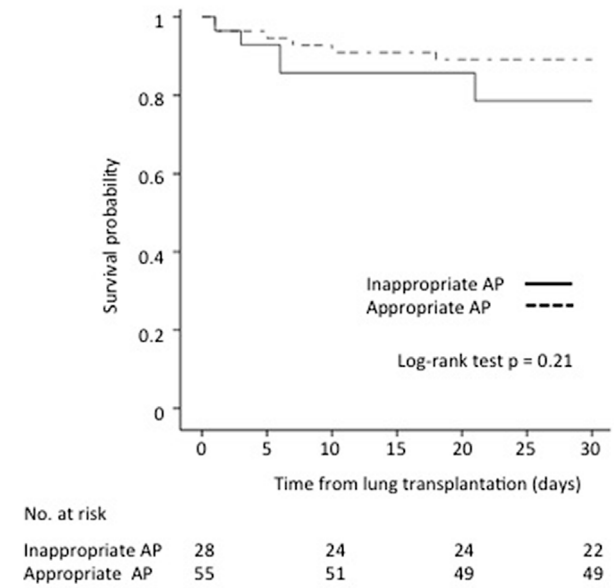


FIGURE 2 | Impact of antibiotic prophylaxis (AP) adequacy on culture-positive PF on 30-day survival after lung transplantation.

significant association of culture-positive PF with recipient mortality (23). However, the authors acknowledged that there was no established explanation for this finding and that FP culture might be considered an overall indicator of transplant quality, including the donated organ and the transplant procedure. In our study, one third of recipients with culture-positive PF had postoperative pneumonia on Day 0 with at least one identical bacteria isolated from PF. In these cases, culture-positive PF may represent an indirect marker of donor lung infection. Nevertheless, the diagnosis of postoperative pneumonia on Day 0 after LT is highly challenging. The interpretation of postoperative chest X-ray is made difficult by the almost systematic presence of infiltrates, and the patient's respiratory status is often uncertain. In addition, distinguishing with differential diagnoses such as primary graft dysfunction increases the difficulty. However, postoperative pneumonia on day 0 was diagnosed by considering international guidelines (13) and isolating bacteria at the infection threshold in bronchoalveolar lavage. Although donor-related infections have a disastrous impact on recipient outcomes (4, 25), the impact of postoperative pneumonia on Day 0 on mortality in lung transplant recipients remains unclear. We did not observe higher postoperative mortality among recipients with postoperative pneumonia on Day 0, weather or not associated to positive culture of PF.

Appropriate antibiotic prophylaxis against microorganisms isolated in the PF was administered to 60% of recipients. However, the adequacy of antibiotic prophylaxis did not influence the prognosis. One explanatory hypothesis is that 90% of transplant patients with culture-positive PF eventually received targeted antibiotic and/or antifungal therapy after identification and susceptibility testing of the microorganisms isolated from the PF within 48 h postoperatively.

Predicting and preventing the risk of culture-positive PF could help to reduce posttransplant morbidity and mortality rates. Disappointingly, we could not establish any risk factors for culture-positive PF from donor characteristics. Others identified advanced donor age as the main risk factor for culture-positive PF with high-risk microorganisms in solid organ transplants (23) and prolonged donor ICU stays (7). We showed that 70% of patients with culture-positive PF had at least one identical microorganism isolated from the donor respiratory specimen at the time of procurement. However, the time required for routine microbiological culture of the donor respiratory specimen is similar to that for PF. Given the worsening outcome when LT

is performed with a graft stored in a culture-positive PF, special attention should be given to the diagnosis and treatment of donor pneumonia. This finding may also raise the issue of routine antibiotic prophylaxis administered to the donor to prevent pneumonia and possible contamination of PF. Although the identification of risk factors for culture-positive PF does not yet appear to be applicable in clinical practice, the use of rapid multiplex polymerase chain reaction (PCR) performed on PF could represent a promising diagnostic tool. This method allows rapid detection of bacteria, viruses and antibiotic resistance genes in a few hours (27–29) and improves antibiotic stewardship (30).

This study has some limitations, which are mainly inherent in its retrospective and single-centre design. Local centre policies on candidate selection and intra- and postoperative management complicate the external validity of the results. Our cohort suffers from a particularly high mortality rate in the postoperative period. However, we reported the largest series describing the microbiological features of PF in LT.

CONCLUSION

Culture-positive PF has a high prevalence and may decrease lung transplant recipient survival. We advocate routine microbiological testing of the preservation fluid and treatment with targeted antibiotic therapy in case of positivity after lung transplantation. Further studies in LT are required to confirm these results and to improve understanding of the pathogenesis of culture-positive PF and its management.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee CEERB Paris Nord, which waived the need for signed informed consent (Institutional Review Board -IRB 00006477- Université Paris Cité, AP-HP. Nord). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AT-D, IT, and PhM: study design, data analysis, and writing of manuscript. ST, EA, BL-J, SJ-B, NZ, SB, YC, HM, PiM, IB, VB, JM, LA-L, and NG, data analysis and writing of manuscript.

CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10826/full#supplementary-material

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ORIGINAL RESEARCH
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Lung Transplantation for Primary Ciliary Dyskinesia and Kartagener Syndrome: A Multicenter Study

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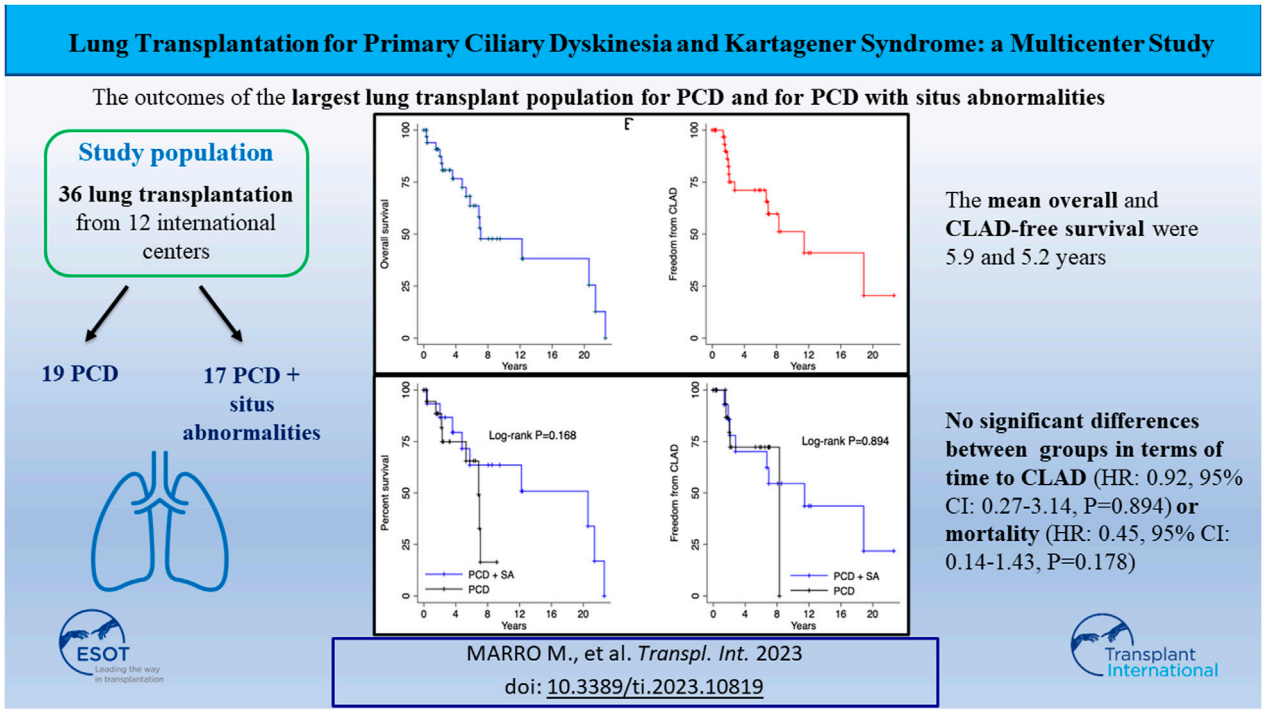
Primary ciliary dyskinesia, with or without situs abnormalities, is a rare lung disease that can lead to an irreversible lung damage that may progress to respiratory failure. Lung transplant can be considered in end-stage disease. This study describes the outcomes of the largest lung transplant population for PCD and for PCD with situs abnormalities, also identified as Kartagener's syndrome. Retrospectively collected data of 36 patients who underwent lung transplantation for PCD from 1995 to 2020 with or without SA as part of the European Society of Thoracic Surgeons Lung Transplantation Working Group on rare diseases. Primary outcomes of interest included survival and freedom from chronic lung allograft dysfunction. Secondary outcomes included primary graft dysfunction within 72 h and the rate of rejection ≥A2 within the first year. Among PCD recipients with and without SA, the mean overall and CLAD-free survival were 5.9 and 5.2 years with no significant differences between groups in terms of time to CLAD (HR: 0.92, 95% CI: 0.27–3.14, $p = 0.894$) or mortality (HR: 0.45, 95% CI: 0.14–1.43, $p = 0.178$). Postoperative rates of PGD were comparable between groups; rejection grades ≥A2 on first biopsy or within the first year was more common in patients with SA. This study provides a valuable insight on international practices of lung transplantation in patients with PCD. Lung transplantation is an acceptable treatment option in this population.

Keywords: outcomes, lung transplant, chronic lung allograft dysfunction, primary graft dysfunction, rare disease



GRAPHICAL ABSTRACT |

INTRODUCTION

Primary ciliary dyskinesia (PCD) is an autosomal recessive disorder characterized by immotile, dysmotile, or absent cilia in the surface of cells of the airway, the reproductive system, and other tissues (1). The defect in ciliary motion leads to anomalous mucociliary clearance, resulting clinically in recurrent or persistent sinorespiratory infections and infertility. The prevalence of PCD is of 1 in 15,000–20,000 individuals. Given that its symptoms overlap with other respiratory diseases, PCD it is believed to be often under diagnosed or misdiagnosed (2,3).

Since normal ciliary function is necessary to control the cardiac laterality during embryologic development, a spectrum of organ laterality defects occur with PCD including situs inversus totalis and situs ambiguus. The triad of situs inversus, chronic sinusitis, and bronchiectasis has historically been referred to as Kartagener syndrome (KS), a subgroup today identified as PCD with situs abnormalities (4,5).

Less than 50% of PCD patients present with situs inversus totalis as per a complete transposition of the thoracic and abdominal viscera, whereas situs ambiguus with a partial transposition of thoracic and or abdominal viscera occurs in at least 12% of PCD. This condition—characterized by an arrangement of internal organs somewhere between situs solitus and situs inversus, can be associated with mild (cardiac septal defects) to severe (heterotaxy) congenital heart disease. In patients with PCD, further imaging studies such as abdominal ultrasound, CT scan, or echocardiogram are pivotal to detect subtle laterality defects (e.g., intestinal malrotation, interrupted inferior vena cava, or polysplenia). PCD should be considered in patients with persistent oto-sino-pulmonary symptoms and any organ laterality or cardiac defect (6–8).

PCD leads to severely impaired mucociliary clearance with recurrent respiratory tract infections and bronchiectasis, and otitis media with hearing loss. Productive cough, shortness of breath, chronic rhinitis and pansinusitis are typical presenting symptoms afflicting young patients during their childhood (9,10).

Patients with PCD are treated with chronic suppressive antibiotics, bronchodilators and inhaled hyperosmolar agents combined with chest physiotherapy to promote airway clearance, vaccination to prevent new upper and lower respiratory infections and on-demand antibiotics for acute exacerbations (9). Lung transplantation is an option for patients with end-stage PCD resulting in respiratory insufficiency (9,11). According to the thirty-sixth registry report by the International Society for Heart and Lung Transplantation (ISHLT) (12), non-cystic-fibrosis bronchiectasis represent 0.4% of all single-lung transplants and 3.8% of all double-lung transplants since 1995. However, lung transplantation in patients with PCD with or without situs abnormalities has been described in only a few case reports (9,13–15) and series (11,16). Therefore, we sought to investigate the outcomes of patients receiving lung transplantation for PCD with or without situs abnormalities across an international multicenter effort promoted by the

European Society of Thoracic Surgeons (ESTS) Lung Transplantation Working Group on Rare Diseases.

MATERIALS AND METHODS

Patient Population and Study Design

This retrospective multicenter study was conducted by the ESTS Lung Transplantation Working Group and it was established bridging off the larger study on rare indications for lung transplantation; the study was open to non-European centers (United States, Canada, and Turkey). A total of 36 lung transplant recipients for PCD and KS from 1995–2020 were included in the study.

Data Source

Data for this study was retrospectively recorded from the participating center archives. Patient data was anonymized and collected in a dedicated database after a data transfer agreement was signed, when required. Variables collected included patient demographic characteristics, diagnoses, information on type of transplantation, induction immunosuppressive therapy and follow-up.

Data was summarized and analyzed in the primary study center (Columbia University Medical Center). PCD was defined as the presence of a genetic mutation related to ciliary motility or a structural defect in electron microscopy after cystic fibrosis was ruled out. KS was defined as the confirmed diagnosis of PCD plus any sign of situs abnormalities. Records were eligible for inclusion if the patient received his or her first lung transplantation for PCD with or without a situs abnormality. Diagnoses were established before transplantation and were attributed independently by each center. No time limits were established for the patient enrollment.

Outcomes and Study Definitions

Primary outcomes of interest included survival and freedom from chronic lung allograft dysfunction (CLAD). CLAD was defined in accordance to the ISHLT consensus statement as a persistent (lasting more than 3 months) and irreversible decline in the forced expiratory volume after 1 s (FEV_1) ≥ 20% from the post-transplant baseline. This was identified the average of the two maximal post-transplant FEV_1 values monitored at least 3 weeks apart, with absent clinical confounders (12). This definition has been retrospectively adopted for all patients. Secondary outcomes of interest included primary graft dysfunction (PGD—with the definition and grading by the report of the ISHLT in 2016) (17) within the first 24, 48, and 72 h and the rate of rejection ≥A2 within the first year and first biopsy, along with predicted FEV_1 volumes at different timepoints. We decided to consider as significant only rejections equal or more than mild; symptoms may be more frequent in patients with grade A2 or higher compared with those with grade A0 or A1 (18).

The protocol was created in adherence to the Institutional Review Board of the Columbia University Medical Center (IRB: AAAT0932).

Statistical Analysis

Continuous and categorical variables were compared for measures of central tendency and rates. Differences in continuous and categorical variables were compared using a Mann-Whitney (or a Student's t-test for normal distributions) and χ^2 test, respectively. Normality was inferred from both visual analysis of distributions and using a Shapiro-Wilk test. Time-to-event data was displayed using Kaplan-Meier plots and tested using a log rank test. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed using Stata Version 14.0 (StataCorp, College Station, TX, United States).

RESULTS

Patient Demographics

Eight European and three non-European lung transplantation centers participated in the study (**Supplementary Table S1**). Clinical records of 36 patients with end-stage severe respiratory failure were extracted and collected and baseline demographic and clinical data were analyzed. Of these, 52.8% (n = 19) had PCD and 47.2% (n = 17) had PCD + SA. Donor characteristics were similar between patients with PCD compared with those with situs abnormalities, with exception of a higher rate of donors with smoking history in the PCD group (3% vs. 1%, p = 0.031) and overall higher donor and recipient height (**Table 1**). No relevant comorbidities were reported.

Four patients, 2 (10.5%) in the PCD group and 2 (11.8%) in the PCD + SA group, presented chronic colonization by *Pseudomonas aerouginosa* and *Serratia marcescens*. All of them were treated pre-surgery by chronic antibiotic therapy with azithromycin and transplanted with no ongoing infection.

None of our patient underwent lobectomy or segmentectomy prior to LTX. As suggested by Kouis et al. (19), prevalence of lung resection in PCD is often performed before PCD diagnosis and overall is more frequent in patients with delayed diagnosis. After lung resection, lobectomised patients have poorer and continuing decline of lung function despite lung resection.

All PCD patients with situs abnormalities had situs inversus totalis. The median age of listing was 42.5 years [IQR: 32.5–53.5] with a median waiting list time of 284 days [IQR: 77–558]. The age at time of surgery ranged from 15 to 66 years (median: 43.1, IQR: 34.5–56). Preoperative hemodynamic parameters, mechanical ventilation and extra corporeal membrane oxygenator (ECMO) use were similar between groups. Patients were followed for a median of 4.23 years (range: 0.25–22.6 years) and included transplants performed between 1999 and 2020.

Perioperative Characteristics

The median cold ischemic time for the first and second lung were 281 [IQR: 181–375] and 417 [IQR: 308–499] min, respectively, and no differences were seen between subdiagnoses (**Table 2**). All the recipients transplanted with urgent priority were PCD + SA patients (0% vs. 17.6%). The majority of the patients received double lung transplant (88.9%) with a similar rate by each subdiagnosis. Two patients received single LTx due to the absence of pre-operative bacterial colonization and the center-managements strategies, while two patients underwent a heart-

TABLE 1 | Donor and recipient demographics.

	Overall (*n* = 36)		PCD (*n* = 19)		PCD + SA (*n* = 17)		*p*-value
	Median	**[IQR]**	**Median**	**[IQR]**	**Median**	**[IQR]**	
Donor demographics							
Donor age (years)	33.5	[25–49.5]	39	[25–62]	28	[20–44]	0.061
Donor height (cm)	172	[165–177.9]	169	[163–172.7]	174.5	[170–180.3]	0.02
Donor weight (kg)	75	[65.5–81]	77.8	[68.4–87.5]	73.56	[65–80]	0.421
P/F at 100%	479	[393–533]	478	[393–531]	490	[393–540]	0.597
P/F at 40%	180	[156–204]	170	[156–202]	197	[165–204]	0.516
	n	**%**	***n***	**%**	***n***	**%**	***p*-value**
Donor gender							
Male	20	55.6	11	57.9	9	52.9	0.765
Female	16	44.4	8	42.1	8	52.9	
Donor cause of death							
Anoxia	1	2.8	0	0.0	1	5.9	0.232
PE	1	2.8	1	5.3	0	0.0	
Trauma	8	22.2	3	15.8	5	29.4	
ICH/CVA	21	58.3	11	57.9	10	58.8	
Cardiac failure	1	2.8	1	5.3	0	0.0	
Donor smoker	4	11.1	3	15.79	1	5.9	0.031
	Median	**[IQR]**	**Median**	**[IQR]**	**Median**	**[IQR]**	***p*-value**
Recipient demographics							
Age at listing (years)	42.5	[32.5–53.5]	39	[32–48]	48	[34–55]	0.715
Recipient age at LTx (years)	43.1	[34.5–56]	39.9	[35–50]	48	[34–56]	0.812
Recipient height (cm)	169	[157.5–173]	163	[153–170.5]	172	[169–175]	0.009
Recipient weight (kg)	58.2	[53–65]	55	[48.2–64]	62.15	[55–71]	0.051
Waitlist time (days)	284	[77–558]	366.5	[131–626]	240	[77–415]	0.531
Pre-op systolic PAP (mm Hg)	38	[30–50]	38	[35.5–48]	36.5	[24–50]	0.552
Pre-op mean PAP (mm Hg)	24	[20–33]	23	[20–28]	29	[19–38]	0.274
Pre-op Wedge pressure (mm Hg)	10	[9–14]	9.5	[7.5–14]	13	[10–14]	0.21
Pre-op CO (L/min)	4.4	[3.2–5.5]	4.4	[3.4–5.5]	3.9	[2.9–5.4]	0.734
	n	**%**	***n***	**%**	***n***	**%**	***p*-value**
Recipient gender							
Male	17	47.2	7	36.8	10	58.8	0.187
Female	19	52.8	12	63.2	7	41.2	
Preop mechanical ventilation (days)	2	5.6	0	0.0	2	11.8	0.161
Preop ECMO (days)	1	2.8	0	0.0	1	5.9	0.306
Urgent priority	3	8.3	0	0.0	3	17.6	0.056

PCD, primary ciliary dyskinesia, SA, situs abnormalities, PAP, pulmonary arterial pressure, CO, cardiac output, PE, pulmonary embolism, ICH, intracranial hemorrhage, CVA, cerebrovascular accident, ECMO, extracorporeal membrane oxygenation, LTx, lung transplantation; P/F, ratio of artieral partial pressure of oxygen to inspired oxygen concentration.

lung transplantation at a single institution because of the presence of severe congenital heart defect. No special procedure were required during surgeries. *Ex vivo* lung perfusion technique was performed in two cases, both in the PCD group. The rates of intraoperative and postoperative ECMO were similar among patients with and without a situs abnormality. Seventeen patients (47.2%) received induction immunosuppression, which was based on anti-interleukin-2 receptor (n = 13, 36.1%) or anti-thymocyte globulin (n = 4, 11.1%) antibodies. The individual type of maintenance immunosuppression also varied by center. The majority of patients were treated by a combination of tacrolimus, mycophenolate mofetil and corticosteroids (**Table 2**). No significant differences between maintenance immunosuppressive therapies were observed between patients with or without situs abnormalities.

Postoperative Outcomes

The primary outcomes of interest were mortality and freedom from CLAD. Among patients with PCD with or without SA, the mean overall and CLAD-free survival were 5.9 and 5.2 years, respectively (p = 0.894) (**Figure 1**). There were no significant differences in the time to CLAD (PCD + SA; HR: 0.92, 95% CI: 0.27–3.14, p = 0.894) or mortality (PCD + SA; HR: 0.45, 95% CI: 0.14–1.43, p = 0.178) between PCD and KS groups (**Figure 2**). The median ICU and total length of stay after transplantation were 7 [IQR: 4–14] and 31.5 [IQR: 20–45] days, respectively. Patients with KS had longer ICU stays (5 vs. 12 days, p = 0.029) and a trend towards a longer total length of stay (26 vs. 41 days, p = 0.114) (**Table 3**). Postoperative rates of PGD within the first 72 h were comparable between groups; rejection grades ≥A2 on first biopsy or within the first year was more common in patients with KS, although no statistical significant difference was noted.

TABLE 2 | Perioperative characteristics.

	Overall (*n* = 36)		PCD (*n* = 19)		PCD + SA (*n* = 17)		*p*-value
	Median	**[IQR]**	**Median**	**[IQR]**	**Median**	**[IQR]**	
Cold ischemic time first lung (min)	281	[181–375]	271	[225–330]	291	[220.5–352]	0.812
Cold ischemic time second lung (min)	417	[308–499]	429	[335–460]	398.5	[332.5–498]	0.82
Time to extubation (hours)	36	[24–69]	36	[20–96]	33	[20–92]	0.817
	n	**%**	***n***	**%**	***n***	**%**	***p*-value**
Intraoperative CPB/ECMO	17	47.2	8	42.1	9	52.9	0.516
EVLP	2	5.6	2	10.5	0	0.0	0.169
Type of transplant							
Heart-lung	2	5.6	0	0.0	2	11.8	0.302
Double	32	88.9	18	94.7	14	82.4	
Single	2	5.6	1	5.3	1	5.9	
Postop ECMO							
VV	1	2.8	1	5.3	0	0.0	0.509
VA	3	8.3	1	5.3	2	11.8	
Induction immunosuppression[a]							
None	18	50.0	7	36.8	11	64.7	0.362
Anti-thymocyte globulin	4	11.1	3	15.8	1	5.9	
Anti IL-2r	13	36.1	8	42.1	5	29.4	
Maintenance immunosuppression							
Cyclo	3	8.3	1	5.3	2	11.8	0.415
Cyclo + AZA + CS	4	11.1	3	15.8	1	5.9	
Cyclo + MMF + CS	7	19.4	3	15.8	4	23.5	
Tacro + MMF	3	8.3	3	15.8	0	0.0	
Tacro + MMF + CS	14	38.9	6	31.6	8	47.1	
Tacro + Ever + CS	1	2.8	0	0.0	1	5.9	
Tacro + CS	3	8.3	2	10.5	1	5.9	

[a]*Data from a PCD patient is not available.*

PCD, primary ciliary dyskinesia; SA, situs abnormalities; CPB, cardiopulmonary bypass; ECMO, extracorporeal membrane oxygenation; EVLP, ex vivo lung perfusion; VA, venoarterial; VV, venovenous; AZA, azathioprine; MMF, mycophenolate mophetil; CS, corticosteroids; Anti-IL-2r, anti-interleukin-2 receptor; Ever, Everolimus; Cyclo, cycloporine; Tacro, tacrolimus.

All patients with onset of CLAD were treated with azitromicin at the beginning of respiratory function decline. Until today, none of them underwent a re-transplant.

DISCUSSION

This manuscript reports the findings of a multicenter retrospective study with the largest lung transplant population for PCD with and without situs abnormalities to date. The demographic analysis between the two groups does not highlight specific differences except for the recipient height, and the cold ischemic time for the first and second lung is longer for the PCD + SA group but with no statistically significant differences. This finding could be explained by the inverted anatomy of the PCD + SA patient resulting in increased surgical difficulty and time required by the surgeon for the transplant. No other differences have been found between the two groups in terms of peri-operative characteristics. Considering the number of the involved centers, some minor surgical technique differences are present: the bronchial anastomosis performed by a continues or interrupted suture, differences of type of sutures and/or size, and different technical approach for double lung transplant (double antero-lateral thoracotomies or Clam Shell approach).

The majority of the patients received tacrolimus, mycophenolate mofetil and corticosteroids as immunosuppressive therapy. Recipients with situs abnormalities had a longer ICU stay and total length of in-hospital stay.

Minor difference were noted in the use of extra corporal support in PCD with situs abnormalities recipients which may justify the postoperative outcome with longer ICU stays.

This multicenter report shows that approximately 70% of the total population is alive 5 years after transplantation with no difference between the two groups. In their analysis from the UNOS registry, Hayes reported a 5-year survival of 52% for PCD and 65% for KS (11). Analysis of the ISHLT registry had previously reported survival rates around 80% at 1 year and 54% at 5 years for the 63,410 adults who underwent primary lung transplantation between January 1992 and June 2017 (12). The better 5-year survival rate of our population compared with the population from the 38th ISHLT Registry could be explained by the younger age of our recipients (43.1 years vs. 54.8 years, respectively) (20). Indeed, PCD and KS patients, due to the natural course of their disease, are listed and transplanted at an earlier age compared to the global LTx population.

We report freedom from CLAD of approximately 50% at 10 years, improved from previous reports by Sato (less than 50% at 8 years from transplant) (21) and from the 38th ISHLT Registry for the overall population (57% at 5 years for the era 1996–2001, 49% for the era 2002–2007 and 47% for the era 2008–2013) (20). Moreover, this registry data show a better 5-year freedom from CLAD for recipients with cystic fibrosis

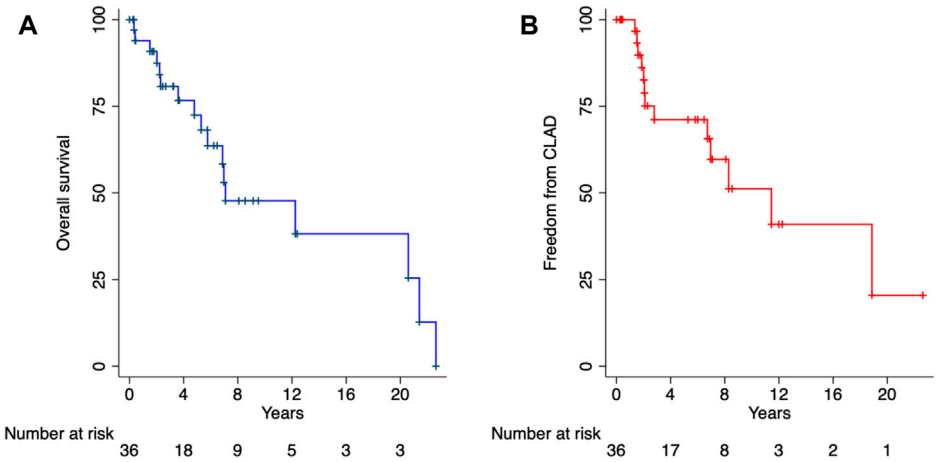


FIGURE 1 | Overall survival **(A)** and freedom from chronic lung allograft dysfunction **(B)**. CLAD, chronic lung allograft dysfunction.

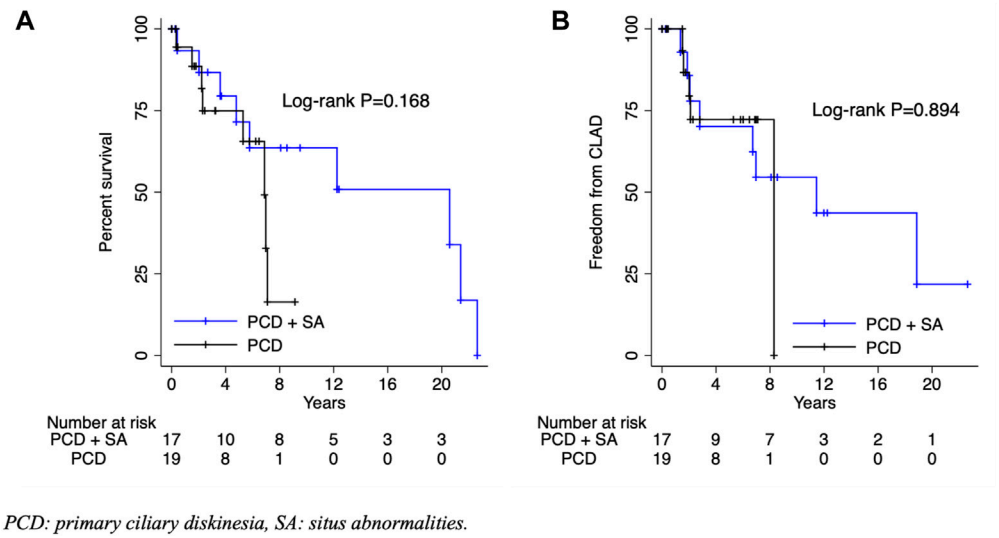


FIGURE 2 | Overall survival **(A)** and freedom from chronic lung allograft dysfunction **(B)** by presence of situs abnormalities. PCD, primary ciliary diskinesia; SA, situs abnormalities; CLAD, chronic lung allograft dysfunction.

compared with chronic obstructive pulmonary disease and idiopatic pulmonary fibrosis diagnosis at transplant. The maintenance of immunosuppression in terms of blood levels, type of therapy, prevention of cytomegalovirus replication might affect and delay the CLAD onset. Additionally, there were no significant differences in the time to CLAD or mortality in PCD with or without situs abnormalities.

In our cohort CLAD was more common in patients treated with cyclosporine than with tacrolimus (64.3% vs. 19%, but there was not statistically significant differences). This is in accordance with prior studies that demonstrated an improvement in incidence of acute rejection and long-term outcomes including a reduced risk for CLAD in patients treated with tacrolimus (22).

Since PCD + SA can be associated with spinal scoliosis and substantial distortion of the intrathoracic space, size matching is particularly important in these patients undergoing lung transplantation. A detailed preoperative study with computed tomography 3D reconstruction to better evaluate the anatomy in these patients and to precisely plan the surgery should be considered by the transplant team (23).

The main surgical transplant pitfalls in patients with situs inversus are the complete reversal of the anatomic position of both cardiac chambers and main vessels and the inverse direction and position of both lungs and main stem bronchi, leading to an anatomical mismatch between the recipient and the donor (16). However, the atrial and pulmonary anastomoses can be

TABLE 3 | Postoperative outcomes.

	Overall (*n* = 36)		PCD (*n* = 19)		PCD + SA (*n* = 17)		*p*-value
	Median	**[IQR]**	**Median**	**[IQR]**	**Median**	**[IQR]**	
ICU length of stay (days)	7	[4–14]	5	[2–11]	12	[7–26]	0.029
Total length of stay (days)	31.5	[20–45]	26	[20–42]	41	[19–55]	0.114
Percent predicted FEV1							
3 months	77	[61–86]	73.5	[61–85]	80	[64–87]	0.734
6 months	80	[63–92]	83	[57–92]	77	[68–92]	0.550
1 year	80	[57–90]	80	[57–90]	91	[62.3–90]	0.864
Latest	70	[41–88]	74	[46–97]	56	[36–76]	0.233
	n	**%**	***n***	**%**	***n***	**%**	***p*-value**
PGD at 24 h[a]							
PGD 0	17	47.2	7	36.8	10	58.8	0.407
PGD 1	11	30.6	8	42.1	3	17.6	
PGD 2	4	11.1	2	10.5	2	11.8	
PGD 3	3	8.3	2	10.5	1	5.9	
PGD at 48 h[a]							
PGD 0	17	47.2	6	31.6	11	64.7	0.067
PGD 1	14	38.9	11	57.9	3	17.6	
PGD 2	3	8.3	2	10.5	1	5.9	
PGD 3	1	2.8	0	0.0	1	5.9	
PGD at 72 h[a]							
PGD 0	18	50.0	7	36.8	11	64.7	0.371
PGD 1	11	30.6	8	42.1	3	17.6	
PGD 2	3	8.3	2	10.5	1	5.9	
PGD 3	2	5.6	1	5.3	1	5.9	
Rejection ≥A2 on first biopsy 9	25.0	3	15.8	6	35.3	0.177	
Rejection ≥A2 within first year	6	16.7	1	5.3	5	29.4	0.052
CLAD	13	36.1	5	26.3	8	47.1	0.196

[a]*Data from a PCD + SA patient is not available.*

PCD, primary ciliary dyskinesia; SA, situs abnormalities; ICU, intensive care unit; FEV1, forced expiratory volume at 1 s; PGD, primary graft dysfunction; CLAD, chronic lung allograft dysfunction.

performed without difficulties due to the midline position of the left atrium and two pulmonary arteries.

To avoid and prevent possible surgical mismatch it is important to obtain a long atrial cuff from the donor and retain maximal length of both the donor and recipient pulmonary arteries.

In patients with situs abnormalities, bronchoscopic findings are consistent with anatomic reversal of the morphology of right and left airways, which include a long right main and a short left main bronchus with early take off of the left upper lobe. Furthermore, the recipient left pulmonary artery can be located anterior to the bronchus while the donor left pulmonary artery may be located superior to the bronchus. Several groups have described methods to address this mismatch. Gauthier described a generous vascular mobilization of the recipient left pulmonary artery from a prebronchial position to an epibronchial position, tailoring the arteriotomies to facilitate an end-to-end vascular anastomosis (24). Another approach described by Mentzer facilitated the anastomosis of the left pulmonary artery by a termino-lateral anastomosis between the donor left pulmonary artery and the recipient origin of the truncus anterior artery, preventing possible distortion and obstruction of the pulmonary vessel (25). Furthermore De Castro suggested, to prevent a left pulmonary artery kinking, to leave a shorter pulmonary artery stump during the graft back table preparation (26).

Moreover, previous groups have emphasized the potential anatomical size mismatch between the donor's right lower lobe and the recipient's dextrocardia (15,25). Although in our cohort lung volume reduction surgery was not necessary, Macchiarini described right lower lobectomy during lung transplantation because of excessive volume of the right donor lung (16).

In terms of pre-operative evaluation and preparation for anesthesia, the use of a conventional right-sided double-lumen tube placed in the anatomic right main bronchus rather than a standard left sided double-lumen tube might overcome the inverted anatomy of airways, thus facilitating excision of the anatomic left lung with division of the short recipient left main bronchus (24).

Concerning the postoperative persistent lack of mucociliary clearance in the upper and central airways, all centers involved in this study used to perform pre-transplant bronchoscopy at the time of surgery. In the post-operative course, chest physiotherapy with deep breathing exercises, postural drainage combined with percussion, vibration and forced expirations, positive expiratory pressure (PEP) valves are routinely used to favor the mucous clearance.

None of the centers involved adopted prophylaptic tracheostomy to facilitate upper airway management and only two patients underwent surgical tracheostomy due to a prolonged respiratory weaning.

Limitations

There are several limitations related to the design and population of this study. First, this study is limited by the effects of small sample size typical of rare diseases. As a consequence, no reliable analysis could be performed to predict long-term outcomes and the lack of significant differences between groups could be due to a lack of power. Additionally, information on patient selection and listing process was not available. The approach to donors has changed over time, not in terms of selection, but rather due to the ongoing improvement of ICU management, of the arrangement of organ donation, and the introduction of EVLP technique have allowed for a better quality pool of grafts. Variation in individual center selection criteria could potentially affect the overall outcomes for patients suffering from this condition. Moreover, given the low number of patients, the data was collected over a long period of time which could potentially insert time effect bias. This study, however, provides a valuable scope on international practices of lung transplantation in patients with PCD despite situs abnormalities.

Conclusion

We have reported the largest multicenter study cohort of lung transplant in PCD patients with or without situs abnormalities. Our results confirm that, considering surgical pitfalls, lung transplantation is a feasible therapeutic option allowing long-term survival in patients with end-stage PCD with or without situs abnormalities.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Columbia University Medical Center (IRB: AAAT0932). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study design: MM, ML-J, FD'O, and MB. Data collection and analysis: MM, ML-J, FD'O, MB, JC, DR, LC, PM, AAK, TK, AK, JE, II, AY, EY, GB, PT, NP, CA, MS, FR, MA, FV, and SK. Writing of manuscript: MM, ML-J, and FD'O. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.10819/full#supplementary-material

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Aspergillus-Specific IgG Antibodies are Associated With Fungal-Related Complications and Chronic Lung Allograft Dysfunction After Lung Transplantation

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Fungal exposure and sensitization negatively affect outcomes in various respiratory diseases, however, the effect of fungal sensitization in lung transplant (LTx) recipients is still unknown. We performed a retrospective cohort study of prospectively collected data on circulating fungal specific IgG/IgE antibodies, and their correlation with fungal isolation, chronic lung allograft dysfunction (CLAD) and overall survival after LTx. 311 patients transplanted between 2014 and 2019 were included. Patients with elevated *Aspergillus fumigatus* or *Aspergillus flavus* IgG (10%) had more mold and *Aspergillus* species isolation ($p = 0.0068$ and $p = 0.0047$). *Aspergillus fumigatus* IgG was specifically associated with *Aspergillus fumigatus* isolation in the previous or consecutive year (AUC 0.60, $p = 0.004$ and AUC 0.63, $p = 0.022$, respectively). Elevated *Aspergillus fumigatus* or *Aspergillus flavus* IgG was associated with CLAD ($p = 0.0355$), but not with death. *Aspergillus fumigatus*, *Aspergillus flavus* or *Aspergillus niger* IgE was elevated in 19.3% of patients, but not associated with fungal isolation, CLAD or death. Mold isolation and *Aspergillus* species isolation from respiratory cultures were associated with CLAD occurrence ($p = 0.0011$ and $p = 0.0005$, respectively), and *Aspergillus* species isolation was also associated with impaired survival ($p = 0.0424$). Fungus-specific IgG could be useful in long-term follow-up post-LTx, as a non-invasive marker for fungal exposure, and thus a diagnostic tool for identifying patients at risk for fungal-related complications and CLAD.

Keywords: lung transplantation, chronic lung allograft dysfunction, fungal infection, IgG, IgE, *Aspergillus*, molds

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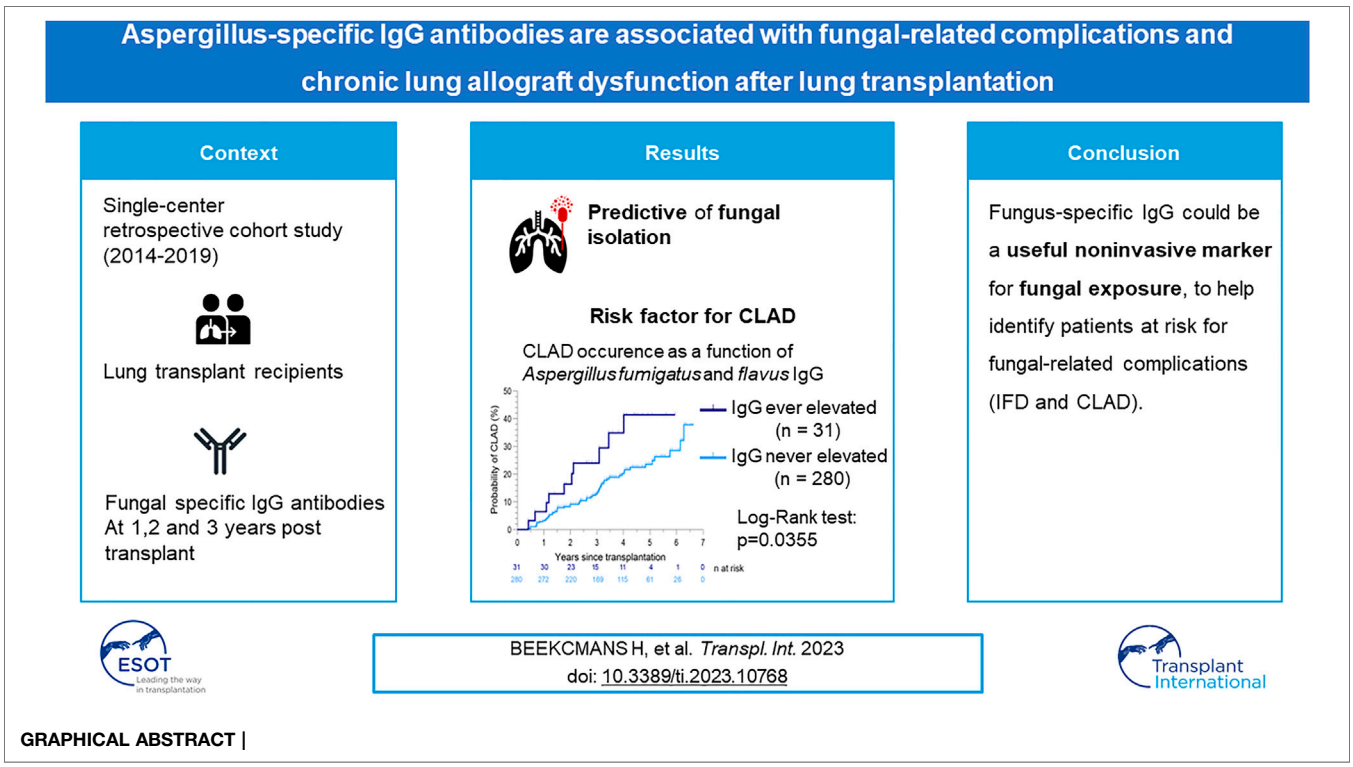

GRAPHICAL ABSTRACT |

INTRODUCTION

Fungal infections are prevalent after lung transplantation (LTx), where they are observed in 15%–35% of all patients. These numbers are notably higher than after other solid-organ transplantations (1). *Aspergillus fumigatus* is a known risk factor for chronic lung allograft dysfunction (CLAD), the leading cause of death beyond the first year after LTx, even in patients that are only colonized without signs of infection (2–5). However, respiratory samples are not always readily available, as not all patients produce sputum. Subclinical presence of *Aspergillus fumigatus* could thus remain undetected. The role of other *Aspergillus* species or non-*Aspergillus* molds in the pathogenesis of CLAD is currently unclear (2, 3, 5).

Serum *Aspergillus* IgG (or precipitins) and *Aspergillus* IgE are currently mostly used in the diagnosis of chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis (ABPA) (6, 7). However, the value of fungal-specific IgG or IgE in the absence of clinical symptoms is less clear. Fungal sensitization is defined as an immune-mediated response to a fungus without signs of inflammation or tissue damage, as opposed to ABPA (8). Recent studies in other chronic respiratory diseases like asthma, chronic obstructive lung disease, and cystic fibrosis (CF) have suggested an adverse effect of fungal sensitization, even without signs of infection (8–13). Particularly in asthma, the importance of fungal sensitization on respiratory disease control is well documented, and it is generally accepted that exposure to allergens, like fungal proteins, can trigger and exacerbate asthma symptoms (8–11).

Fungal sensitization can occur through a normal and an allergic pathway. "Normal" fungal sensitization occurs through activation of the immune system, where TH1- and TH17-cells help to build protective immunity against fungal pathogens *via* release of cytokines, which then stimulate phagocytes as well as B-cells to produce specific IgG antibodies (delayed response). On the other hand, patients may experience allergic fungal sensitization, in which exposure to fungal allergens causes an allergic reaction *via* a type 2 immune response, with activation of TH2-cells in regional lymph nodes. These immune cells secrete cytokines and stimulate B-cells to produce specific antifungal IgE-antibodies (8). Allergic fungal sensitization thus is the result of immune reactions in the airways, leading to hyperinflammation, causing deterioration of respiratory symptoms. As all LTx recipients are immunosuppressed to avoid graft rejection and therefore have a decreased immune activity, occurrence of fungal sensitization in transplanted patients could mean that their immune system might not be as suppressed as one would expect. Allergic fungal sensitization may be a sign of attenuated immunosuppression, possibly contributing to later onset of chronic rejection. Detection of fungus-specific IgG antibodies on the other hand, may reflect past fungal presence in the airways (10), and could thus potentially be used as a measure for fungal exposure, independent of airway sampling, identifying patients at a higher risk of (invasive) fungal infections and CLAD.

In this retrospective study, we therefore aimed to explore the prevalence of fungal sensitization after LTx, and its possible effects on occurrence of (invasive) fungal infections, CLAD and survival.

MATERIALS AND METHODS

Study Population

Patients who underwent LTx at UZ Leuven between September 2014 and December 2019 were evaluated for inclusion. Inclusion criteria were: 1) survival to 1 year post-LTx and 2) availability of ≥1 blood sample for specific IgG/IgE antibodies to *Aspergillus fumigatus*, *Aspergillus flavus* and specific IgE antibodies to Aspergillus niger, obtained during annual check-up at 1, 2 or 3 years post-LTx. Data regarding fungal isolation from respiratory samples, clinical phenotype of the fungus, CLAD onset and survival were obtained from the electronic patient files. The study was approved by the local Ethics Committee (University Hospitals Leuven, Belgium—S63978), and all patients provided written informed consent.

Data Collection: Blood Samples

Blood samples with IgG/IgE measurement were collected during the pre-transplant work-up prior to listing for transplant, and at every annual post-transplant check-up, taking place between September 2017 and January 2021. In these samples total protein level, specific IgG's, total IgE, and specific IgE's were measured using ImmunoCAP fluoroenzyme immunoassay, with a Phadia™ 250 instrument for IgG measurements and a Phadia™ 1000 instrument for IgE measurements (ThermoFisher, Waltham, Massachusetts, United States), as per institutional standard operating procedures.

Specific IgG's against *Aspergillus fumigatus* (Gm3) and *Aspergillus flavus* (Gm228) were determined. For the specific IgE's, the following were assessed: *Aspergillus fumigatus* (m3), *Aspergillus flavus* (m228) and *Aspergillus niger* (m207).

For specific IgG's the lower limit of detection was 2.0 mg/L, values >50.0 mg/L for *Aspergillus fumigatus* and *Aspergillus flavus* were considered positive per assay protocol. The lower limit of detection for the specific IgE's was 0.10 kU/L, and every detectable value was considered positive.

Data Collection: Airway Samples

Airway samples were collected at fixed post-transplant check-ups on day 1, 30, 90, 180, 365, 540, and 720. They were also additionally obtained when patients exhibited respiratory symptoms with or without a fall in FEV1, or presented with an abnormal chest x-ray or computed tomography during routine post-LTx follow-up.

Respiratory specimens, acquired *via* sputum or bronchoalveolar lavage, were cultured using Sabouraud dextrose agar and CHROMagar *Candida* growth mediums per institutional protocol, and considered positive if fungi were detected, with subsequent species-identification.

Positive fungal cultures were further categorized as *Aspergillus* species (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*) or molds (exclusion of yeasts, included molds in our cohort were: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *F. fomentarius*, *Fusarium* species, *P. nariotii*, *Penicillium* species, *Polyporales* species, *P. lilacinum*, *R. argilacea*, *R. microsporus*, *S. apiospermum*, *Scopulariopsis* species, *Talaromyces* species).

For further analysis regarding CLAD and survival, fungal cultures in the first postoperative month were excluded, as these were expected to be confounded by flora from the donor lung.

CLAD Definition

CLAD was defined according to the latest ISHLT consensus paper (14). Bronchiolitis obliterans syndrome (BOS) was defined as a FEV1 decline of ≥20% with an obstructive PFT pattern, in absence of persistent radiologic opacities or TLC decline. Restrictive allograft syndrome (RAS) was defined as a FEV1 decline of ≥20% accompanied with a restrictive PFT pattern (TLC decline of ≥10% compared to baseline) and persistent opacities on chest x-ray or computed tomography (CT).

Antifungal Prophylaxis and Treatment

All patients received standard prophylaxis with nebulized amphotericin B lipid complex for 1 month after LTx (patients with bronchial anastomosis necrosis at 1 month after LTx received prolonged targeted prophylaxis up to 3 months after LTx). No systemic antifungal prophylaxis with azoles was given in our cohort. Antifungal treatment with systemic voriconazole, posaconazole, isavuconazole, or amphotericin B was initiated at the treating clinician's decision, in case of fungal disease.

Fungal Disease

Clinical phenotyping (invasive fungal disease (IFD) or non-IFD) of fungi isolated from cultures was performed according to the latest EORTC-MSGERC and ISHLT consensus paper (15, 16). Patients who fulfilled ISHTL criteria for fungal pneumonia, tracheobronchitis or anastomotic infection were deemed probable or proven IFD according to EORTC-MSGERC criteria (depending on histological criteria). Patients who had clinical symptoms without radiological or endobronchial criteria, as well as patients who fulfilled ISHLT criteria for fungal colonization (i.e., no clinical symptoms and no radiological or endobronchial criteria) were categorized as non-IFD fungal isolation.

Statistics

Outcome analysis was performed on 1st June 2021 (6 months after the last measurement of IgE/IgG). Calculations were performed in GraphPad Prism 9.3.1. and R. Normality was tested by the Shapiro–Wilk test; none of the continuous variables were normally distributed. Friedman test was used to evaluate IgG levels over time, and Spearman correlation to evaluate the relationship between IgG pre- and post-LTx. Log-rank test and receiver operating characteristic analysis were used to compare mortality, occurrence of CLAD and occurrence of fungal isolation. Univariate comparisons between groups were

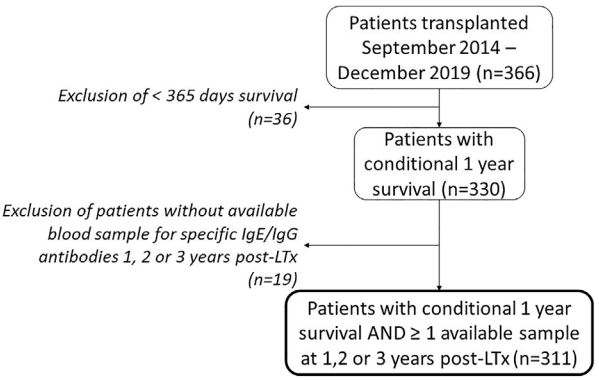

FIGURE 1 | Patient inclusion flowchart. Abbreviations: LTx, lung transplantation.

TABLE 1 | Patient characteristics.

Male gender, n (%)	154 (49.5%)
Age, median [IQR] (years)	58 [46–62]
Underlying lung disease	
Chronic obstructive pulmonary disease, n (%)	165 (53.0%)
Interstitial lung disease, n (%)	56 (18.0%)
CF and non-CF bronchiectasis, n (%)	55 (17.7%)
Pulmonary hypertension, n (%)	7 (2.3%)
Other, n (%)	28 (9.0%)
Type of LTx	
Bilateral SSL, n (%)	300 (96.5%)
Combined liver-lungs, n (%)	7 (2.3%)
Combined heart-lungs, n (%)	3 (1.0%)
Combined kidney-lungs, n (%)	1 (0.3%)
CLAD diagnosis, n (%)	67 (21.5%)
Type BOS, n (%)	48 (71.6%)
Type RAS, n (%)	19 (28.4%)
BOS to RAS phenotype switch, n (%)	4 (6.0%)
Available blood samples per patient, median	2
Patients with 1 sample, n (%)	119 (38.3%)
Patients with 2 samples, n (%)	107 (34.4%)
Patients with 3 samples, n (%)	85 (27.3%)

CF, cystic fibrosis; SSL, sequential single-lung; CLAD, chronic lung allograft dysfunction; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome.

performed by Wilcoxon rank-sum test and presented as median and interquartile range. For comparison of discrete variables, Chi-squared test and Fisher's exact test were used when appropriate, and presented as absolute numbers and percentages. A p-value <0.05 was considered significant.

RESULTS

Patient Demographics

Of the 366 consecutive patients who underwent LTx at our center in the aforementioned time interval, a total of 311 patients were included (**Figure 1**), of which *Aspergillus fumigatus* IgG/IgE antibody data were available in 201 (64%), 192 (62%) and 191 (61%) patients at respectively 1, 2 or 3 years post-LTx. The number of available samples per patient was 1 in 119 (38.3%) patients, 2 in 107 (34.4%) patients and 3 in 85 (27.3%) patients. Patient characteristics are listed in **Table 1**. IgG/IgE blood samples are presented in **Table 2**.

Fungal IgG/IgE—Fungal Isolation

Elevated *Aspergillus fumigatus* or *Aspergillus flavus* IgG at 1, 2 or 3 years post-LTx was detected in 31/311 (10%) patients. Characteristics of patients with or without elevated Aspergillus species IgG are depicted in **Table 3**. Detectable *Aspergillus fumigatus* IgG levels at 1, 2 or 3 years post-LTx were not significantly different, but there was a clear reduction in *Aspergillus fumigatus* IgG levels pre-vs. post-LTx ($p < 0.0001$). There was a correlation between *Aspergillus fumigatus* IgG levels pre- and post-LTx ($r_s = 0.6055$; $p < 0.0001$).

Elevated *Aspergillus fumigatus* IgG was associated with *Aspergillus fumigatus* isolation from respiratory samples in the following year (AUC 0.63, $p = 0.022$) and the previous year (AUC 0.60, $p = 0.004$). Elevated *Aspergillus flavus* IgG was not significantly associated with *Aspergillus flavus* isolation in the previous or consecutive year (although our study was probably underpowered for this analysis, with only 16 patients with elevated *Aspergillus flavus* IgG, and only 12 isolations from respiratory cultures). During the overall study period (up till 6 months after last IgG measurement) patients that ever had an increased *Aspergillus fumigatus* or *flavus* IgG demonstrated more mold and *Aspergillus* species isolation ($p = 0.0068$ and $p = 0.0047$, respectively, **Table 3**).

Elevated *Aspergillus fumigatus, flavus,* or *niger* IgE at 1, 2 or 3 years post-LTx was detected in 60/311 (19.3%) of patients, but was not associated with occurrence of *Aspergillus* species isolation in the consecutive or previous year.

Fungal IgG/IgE—CLAD/Survival

Elevated *Aspergillus fumigatus* or *flavus* IgG was associated with a significantly higher CLAD occurrence ($p = 0.0355$, **Figure 2**). AUC in receiver operating characteristic analysis was 0.58 ($p = 0.0490$). In patients with elevated *Aspergillus fumigatus* or *flavus* IgG, RAS as well as transition from BOS to RAS occurred more frequently (19.4% vs. 6.1%, **Table 3**).

Elevated *Aspergillus fumigatus, flavus* or *niger* IgE at 1, 2 or 3 years post-LTx was detected in 60/311 (19.3%) of patients, but was not associated with occurrence of *Aspergillus* species isolation in the consecutive or previous year, CLAD or death (data not shown).

Fungal Isolation - CLAD/Survival

A total of 268 positive cultures for fungi were obtained in 47.6% of patients ($n = 148$), with a median of 1 [1–2] positive sample per patient. Time to first positive fungal airway culture was 326 days post-transplantation on average (note: survival to 1 year post-LTx was an inclusion criterium for this cohort and cultures during the first month post-LTx were excluded). Mold isolation after the first postoperative month was significantly associated with CLAD occurrence ($p = 0.0011$) and showed a predictive trend towards death ($p = 0.0529$).

Mold isolation after the first year post-LTx also remained associated with later CLAD occurrence ($p = 0.0011$). There was no relationship between positive donor mold cultures ($n = 17$) and recipient positive mold cultures ($n = 12$) during the first postoperative month (odds ratio 1.602, $p = 0.49$).

TABLE 2 | IgG and IgE levels.

Available samples, n	Pre-LTx	Year 1 post-LTx	Year 2 post-LTx	Year 3 post-LTx
	308	201	192	192
Samples with positive IgG values				
A. fumigatus, n (%)	98 (31.8%)	18 (9.0%)	13 (6.8%)	13 (6.8%)
Median value in positive samples (IQR), mg/L	74.75 (58.18–112.80)	63.95 (55.78–82.75)	67.4 (53.10–78.75)	84.7 (61.70–123.0)
A. flavus, n (%)	NA	6 (3.0%)	9 (4.7%)	10 (5.2%)
Median value in positive samples (IQR), mg/L		69.5 (63.43–94.0)	63.7 (58.40–71.75)	78.9 (63.08–113.3)
Samples with positive IgE values				
A. fumigatus, n (%)	NA	29 (14.4%)	31 (16.1%)	40 (20.8%)
Median value in positive samples (IQR), mg/L		0.6 (0.27–2.09)	1.22 (0.36–3.10)	0.48 (0.20–2.29)
A. flavus, n (%)	NA	5 (2.5%)	12 (6.3%)	10 (5.2%)
Median value in positive samples (IQR), mg/L		0.58 (0.19–9.97)	0.2 (0.14–0.67)	0.53 (0.15–2.10)
A. niger, n (%)	NA	8 (4.0%)	9 (4.7%)	8 (4.2%)
Median value in positive samples (IQR), mg/L		0.26 (0.12–0.73)	0.18 (0.14–1.10)	1.28 (0.28–2.40)

LTx, lung transplantation; A., Aspergillus; *NA, not available.*

TABLE 3 | Patient characteristics in patients with ever vs. never elevated *Aspergillus fumigatus* or *flavus* IgG after LTx.

	***A. fumigatus* or *A. flavus* IgG ever elevated at 1, 2 or 3 years post-LTx**	***A. fumigatus* or *A. flavus* IgG never elevated at 1, 2 and 3 years post-LTx**	***p*-value**
Patients (%)	31 (9.97%)	280 (90.03%)	
Men (%)	15 (48.3%)	139 (49.6%)	
Age (years)	58 (44–61)	58 (46–62)	0.9611
LTx indication (%)			0.1261
BRECT	1 (3.2%)	5 (1.8%)	
CLAD	2 (6.5%)	12 (4.3%)	
CF	8 (25.8%)	44 (15.7%)	
Emphysema	17 (54.8%)	149 (53.2%)	
Pulmonary GVHD	1 (3.2%)	3 (1.1%)	
ILD	1 (3.2%)	54 (19.3%)	
PH	0 (0%)	5 (1.8%)	
Other	1 (3.2%)	8 (2.9%)	
Clinical phenotype (%)			**0.0282**
BOS	4 (12.9%)	41 (14.6%)	
RAS	4 (12.9%)	15 (5.4%)	
BOS to RAS	2 (6.5%)	2 (0.7%)	
Stable	21 (67.7%)	223 (79.6%)	
Ever mold isolation after POD 30 (%)	21 (67.7%)	115 (41.1%)	**0.0068**
Ever *Aspergillus* species isolation after POD 30 (%)	18 (58.1%)	88 (31.4%)	**0.0047**
Deceased (%)	4 (12.9%)	22 (7.9%)	0.3096

LTx, lung transplantation; A., Aspergillus*; BRECT, bronchiectasis; CLAD, chronic lung allograft dysfunction; CF, cystic fibrosis; GVHD, graft-versus-host-disease; ILD, interstitial lung disease; PH, pulmonary hypertension; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; POD, postoperative day.*
Bold represent significant p*-values.*

Aspergillus species were cultured 110 times (74.3%), of which 92 were identified as *Aspergillus fumigatus* (62.1%), 11 as *Aspergillus flavus* (7.4%), and 4 as *Aspergillus niger* (2.7%). *Aspergillus* species isolation after the first postoperative month was significantly associated with both CLAD and death (p = 0.0005 and 0.0424, **Figures 3A,B**), and *Aspergillus* species isolation after the first year post-LTx remained associated with later CLAD occurrence (p = 0.0004, **Figure 3D**). In both IFD and non-IFD, *Aspergillus* species were associated with CLAD (p = 0.0001, **Figure 3C**) compared to patients in whom no *Aspergillus* species were isolated. Interestingly, there was no significant difference in CLAD occurrence between IFD and non-IFD *Aspergillus* isolation (p = 0.2970).

Most *Aspergillus* species were treated (66.0%), with either voriconazole, posaconazole, isavuconazole, or amphotericin B (54.8%, 34.4%, 6.5% and 4.3%, respectively). Distribution of the clinical phenotypes (stable, CLAD: BOS, RAS, BOS to RAS) in patients with or without fungal isolation is shown in **Table 4**.

DISCUSSION

This retrospective study of prospectively collected data in a large cohort of LTx recipients demonstrated for the first time

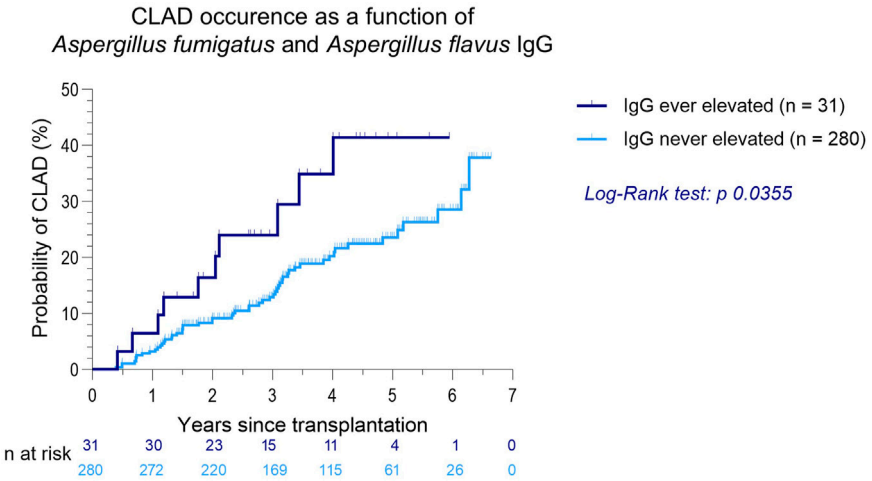

FIGURE 2 | CLAD occurrence as a function of *Aspergillus fumigatus* and *Aspergillus flavus* IgG after LTx.

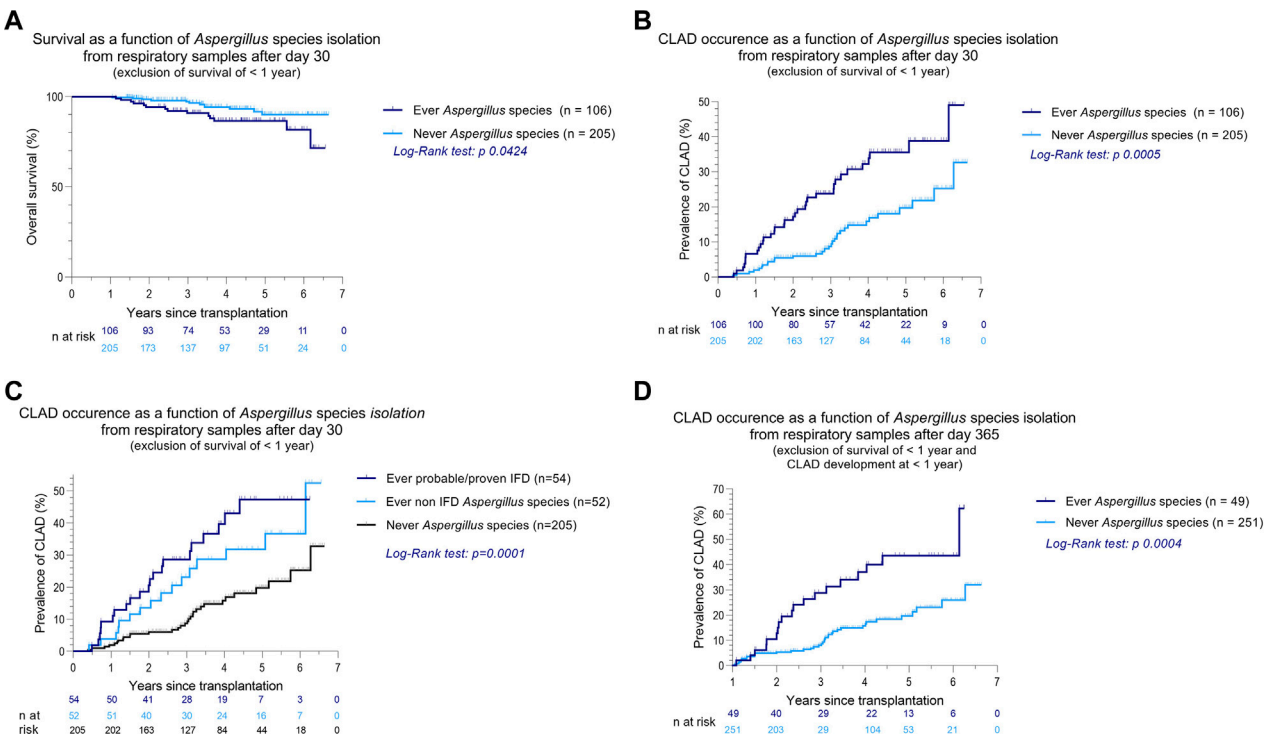

FIGURE 3 | Kaplan-Meier curves of overall survival and CLAD occurrence as a function of *Aspergillus* species isolation from respiratory samples after LTx **(A–D)**. In all analyses, only patients with survival >1 year after LTx were included. **(C)** When a culture was positive for *Aspergillus* species, further clinical phenotyping was performed according to the latest EORTC-MSGERC and ISHLT consensus (15, 16). **(D)** Patients who developed CLAD in the first postoperative year were excluded. Abbreviations: LTx, lung transplantation; A., *Aspergillus*; CLAD, chronic lung allograft dysfunction.

that *Aspergillus* species specific IgG was associated with later fungal isolation and fungal-related complications (IFD,CLAD). This study also confirmed previously published findings that mold isolation, especially *Aspergillus* species, was associated with CLAD development and impaired survival (3–5).

CLAD is the leading cause of death beyond the first post-transplant year. Respiratory infections are among the many elements reported to contribute to CLAD (4, 5, 17–19). Indeed, presence of molds within the airways after transplantation may result in local innate inflammation and epithelial injury followed by dysregulation of repair

TABLE 4 | CLAD phenotype comparison in patients with or without fungal isolation from respiratory samples after LTx.

	Ever *Aspergillus* species (n = 106)	Never *Aspergillus* species (n = 205)	Ever molds (n = 136)	Never molds (n = 175)
BOS	20 (18.9%)	24 (11.7%)	24 (17.3%)	20 (11.4%)
RAS	13 (12.3%)	6 (2.9%)	14 (10.2%)	5 (2.9%)
BOS to RAS	3 (2.8%)	1 (0.5%)	3 (2.2%)	1 (0.6%)
Stable	70 (66.0%)	174 (84.9%)	95 (69.9%)	149 (85.1%)
	$p = 0.0002$		$p = 0.0034$	

mechanisms, ultimately responsible for chronic fibroproliferation and progressive graft dysfunction (4, 5, 20–22).

However, as mentioned previously, respiratory samples are not always available, as not all patients produce sputum and bronchoscopy is not always performed or possible. Subclinical presence of fungi could thus remain undetected. Fungal-specific IgG could therefore represent a new way of identifying lung transplant patients at risk for fungal-related complications, besides sampling of the airways.

Aspergillus fumigatus IgG levels were lower after LTx compared to pre-transplant, which is probably due to our immunosuppressive regimen as well as replacement of the diseased (and often, especially in CF patients, *Aspergillus* species colonized) native lungs with (non-colonized) donor lungs. *Aspergillus fumigatus* IgG levels pre- and post-LTx were correlated, which could possibly mean that these patients are inherently more prone to develop IgG (and thus have higher immune activity), or there is an ongoing environmental exposure in these patients. *Aspergillus* IgG levels were not significantly different at 1, 2 and 3 years post-LTx. *Aspergillus fumigatus* IgG was associated with *Aspergillus fumigatus* isolation in the previous or consecutive year, and patients with elevated *Aspergillus fumigatus* or *flavus* IgG has more mold and *Aspergillus* species isolation during the study period. This could be explained by the fact that specific IgG is a measure of fungal exposure, even when fungi are not always captured by routine follow-up respiratory samples, especially when frequency of follow-up decreases over time post-LTx. Fungus-specific IgG has been used in the diagnosis of (invasive) fungal infections such as chronic pulmonary aspergillosis and ABPA, but also in determining risk factors for interstitial lung diseases, such as hypersensitivity pneumonitis (6, 7, 23, 24). The clinical advantage of IgG measurement is that it is not dependent on sampling of the airways and can therefore always be measured. Elevated *Aspergillus fumigatus* or *flavus* IgG was associated with higher CLAD prevalence, which is not surprising, as fungal infections are a risk factor for CLAD. Interestingly, RAS as well as transition from BOS to RAS occurred more frequently in the group with elevated IgG compared to non-elevated IgG (**Table 3**), indicating that elevated IgG could be a marker for fungal-related complications predisposing more to RAS than BOS.

On the other hand, positive specific IgE for *Aspergillus fumigatus* was not significantly associated with CLAD, survival or fungal isolation in our cohort**.** This is probably due to the shorter half-life of IgE in the serum (only 2–3 days) and the fact that the majority of IgE in the body is cell-bound, and only a small fraction can be measured in the circulation. This is in contrast to IgG, which has a much longer half-life of about 3 weeks and is the most prevalent antibody molecule in the serum (25, 26).

Invasive fungal infections are a known risk factor for CLAD, and even without signs of infection *Aspergillus* species isolation from airway sampling is considered a risk factor for CLAD. In our study, *Aspergillus* species were cultured in 74.3% of the respiratory samples demonstrating molds, and *Aspergillus* species isolation was significantly associated with CLAD and impaired survival, confirming findings from earlier studies (2–5). There was no significant difference in CLAD occurrence in patients with *Aspergillus* IFD vs. non-IFD *Aspergillus* isolation. Median time from *Aspergillus* isolation (without signs of infection) to CLAD in earlier studies was reported to be 633 days (IQR 575-675) (20) or 261 days (21), while in our cohort, in the group that developed CLAD, the median time from *Aspergillus* species isolation (exclusion of first postoperative month) to CLAD diagnosis was 384 days, however *Aspergillus* species infections were also included in our cohort. Interestingly, patients with *Aspergillus* species isolation showed a higher tendency to develop RAS or transition from BOS to RAS phenotype (15.1% vs. 3.4% in patients without *Aspergillus* species isolation, **Table 4**). This may again indicate that fungal infections may predispose to RAS rather than to BOS, which requires validation in other cohorts.

Overall, elevated *Aspergillus*-specific IgG should therefore increase vigilance for *Aspergillus*-related complications.

There are some limitations to our study, such as its single center set-up, the low detection rate of elevated IgG levels and the lack of skin prick tests, as there is a potential discordance between *in vitro* tests and skin prick tests. However, the value of skin prick tests in LTx patients (who are on maintenance corticosteroids) is questionable. Also, only IgG specific for *Aspergillus fumigatus* and *flavus* was measured, and as these are the most prevalent *Aspergillus* species, other *Aspergillus* species were uncommon in respiratory cultures (only 4.8% of respiratory cultures). These IgGs thus covered the majority of isolated *Aspergillus* species. Also, there is a lack of assessment of local immune activation (i.e., BAL or blood lymphocytic subtypes) or immunosuppressive treatment, which were outside the scope of the study.

In conclusion, fungus-specific IgG could be a useful non-invasive marker of fungal exposure in long-term follow up after LTx, to help identify patients at risk for fungal-related complications, CLAD, and inferior outcome.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

HB: Data collection, data curation, visualisation, preliminary and final draft preparation, review and editing. ER: Data collection, data curation, preliminary draft preparation. AS: Data collection, final draft review. VG: Data collection, final draft review. AV: Data collection, final draft review. LW: Data collection, final draft review. XJ: data collection, final draft review. SB: Final draft review. JS: Data collection, final draft review. MO: Data collection, final draft review. BV: Final draft review. LC: final draft review. DR: Final draft review. AN: Final draft review. LG: Final draft review. LD: Final draft review. GV: Final draft review. RV: Conceptualization, methodology, data collection, data curation, visualization, final draft preparation, review and editing.

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CONFLICT OF INTEREST

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

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BRIEF RESEARCH REPORT
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Economic Impact of European Liver and Intestine Transplantation Association (ELITA) Recommendations for Hepatitis B Prophylaxis After Liver Transplantation

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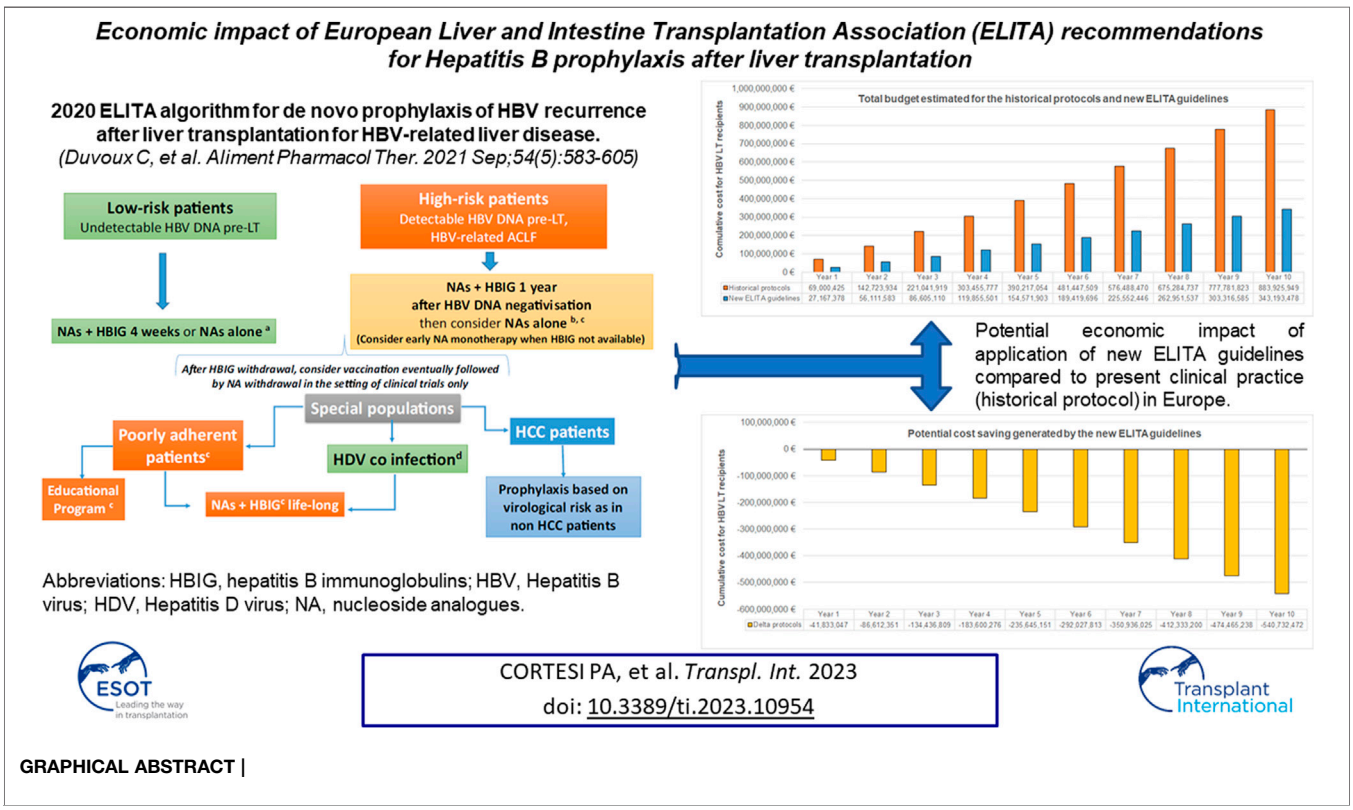
The European Liver and Intestine Transplant Association, ELITA, promoted a Consensus Conference involving 20 experts across the world which generated updated guidelines on HBV prophylaxis in liver transplant candidates and recipients. This study explores the economic impact associated with the implementation of the new ELITA guidelines. To this aim, a condition-specific cohort simulation model has been developed to compare new and historical prophylaxis, including only pharmaceutical cost and using the European perspective. The target population simulated in the model included both prevalent and incident cases, and consisted of 6,133 patients after the first year, that increased to 7,442 and 8,743 patents after 5 and 10 years from its implementation. The ELITA protocols allowed a cost saving of around € 235.65 million after 5 years and €

540.73 million after 10 years; which was mainly due to early HIBG withdrawal either after the first 4 weeks or after the first year post Liver Transplantation (LT) depending on the virological risk at transplantation. Results were confirmed by sensitivity analyses. The money saved by the implementation of the ELITA guidelines would allow healthcare decision makers and budget holders to understand where costs could be reduced and resources re-allocated to different needs.

Keywords: prophylaxis, hepatitis B, immunoglobulin (IgG), liver transplant, economics



GRAPHICAL ABSTRACT |

INTRODUCTION

Prophylaxis for HBV recurrence is of critical importance post liver transplantation (LT). Despite the efficacy of new prophylactic regimens based on short term Hepatitis B Immunoglobulins (HBIG) use [1,2], most European LT centers persist with a conservative approach, combining hepatitis B immunoglobulin (HBIG) long term with nucleos(t) ides analogues (NA). The recently published ELITA guidelines provide updated new evidence that prophylactic strategies based on a personalized use of HBIG is possible; its duration dependent on the virological risk profile at the time of LT [3].

Based on this new approach there is the potential for substantial cost-savings to the healthcare budget, which could be reinvested in other areas to improve patient management and outcomes. To better understand the possible economic impact of these new strategies we performed a budget impact analysis (BIA) using the European perspective. This analysis aims at understanding the possible cost savings associated with the implementation of the new ELITA guidelines compared to current clinical practice.

MATERIALS AND METHODS

BIAs are increasingly required by budget holders and Healthcare Authorities to understand the economic impact of adopting a new healthcare intervention/treatment protocol in a specific population. BIA addresses the expected changes in the expenditure of a healthcare system after the adoption of a new intervention proving valuable information for budget or resource planning [4]. The computing framework for a BIA can be a simple cost calculator programmed on a spreadsheet or a condition-specific cohort or individual simulation model [4].

TABLE 1 | Model data input.

Parameters			Value (range)	References
Epidemiological data				
New yearly (incident) cases of HBV LT patients, N			506 (405–607)	Adam 2018
Number of prevalent HBV LT patients, N			5,627 (4,501–6,752)	Adam 2018
Clinical data				
New LT patient				
HBV liver transplant mortality probability at 1 year, %			13.78%	Adam 2018
HBV liver transplant mortality probability at 5 years, %			12.23%	Adam 2018
HBV liver transplant mortality probability at 10 years, %			18.57%	Adam 2018
Prevalent LT patient				
HBV liver transplant mortality probability at 5 years, %			6.34%	Adam 2018
HBV liver transplant mortality probability at 10 years, %			14.51%	Adam 2018
Distribution of patients category included in ELITA protocol				
Low risk			82.0%	Adam 2018
High risk			10.0%	Fraser 2013
Special patients			8.0%	Ladin 2018
Treatment cost				
Entecavir, € per mg			11.9 € (7.3–16.2)	Duvoux 2021
Tenofovir, € per 245 mg			8.4 € (4.4–13.6)	
HBIG IV, € per 5000 IU			1,589.6 € (1,029–2,772.7)	
Historical protocol		1st treatment year	24,366 €	Estimated
		≥2nd treatment year	13,239 €	
New protocol	Low risk[a]	1st treatment year	14,828 €	
		≥2nd treatment year	3,701 €	
	High risk[b]	1st treatment year	24,366 €	
		≥2nd treatment year	3,701 €	
	Special population[c]	1st treatment year	24,366 €	
		≥2nd treatment year	13,239 €	

[a]*Low virological risk patients: patients with undetectable HBV DNA, pre-LT, irrespective of Lt indication (cirrhosis or fulminant hepatitis).*
[b]*High virological risk patients: Patients with detectable HBV DNA, at LT, Patients with HBV, reactivation resulting in HBV-related acute on chronic liver failure.*
[c]*Special populations: Patients with HDV, co-infection, at low virological risk but deserving full prophylaxis, HCC, patients, at higher virological risk in case of HCC, recurrence but not requiring, patients at risk of poor adherence to antiviral therapy post-LT.*

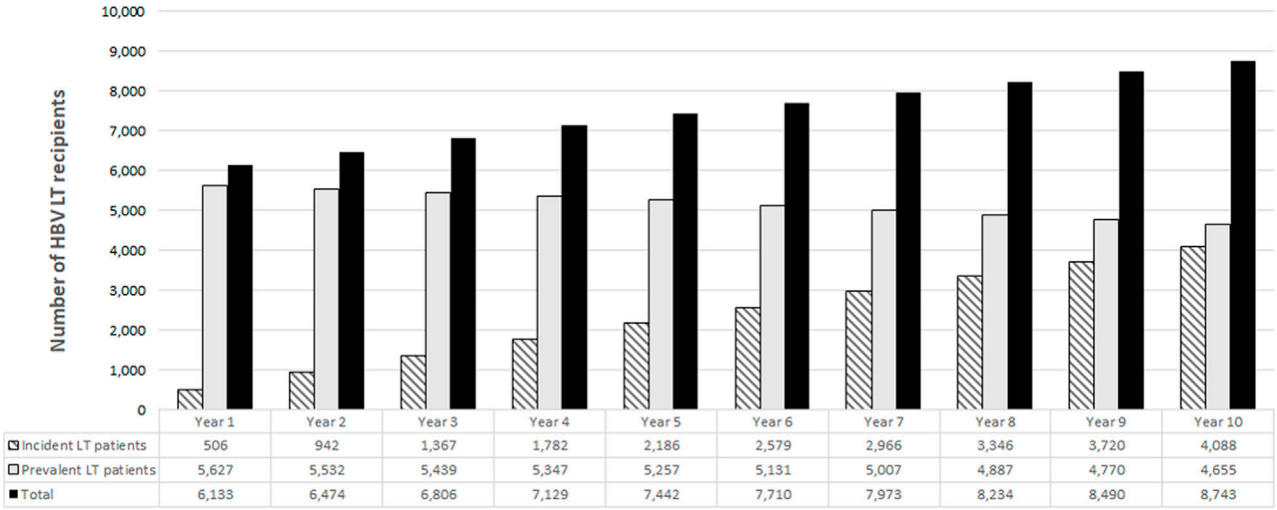
In this study, a condition-specific cohort simulation model was developed to compare new and historical treatment protocols, including only pharmaceutical cost and using the European perspective. The model estimates the cost of two different scenarios: Historical scenario, based on long term use of HBIG, and ELITA scenario, based on individualized use of HBIG according to the ELITA Clinical Practice Guidelines [3]. The model assumed that all patients in the historical scenario received long term HBIG, while patients in the ELITA scenario were treated according to the individualized virological risk at LT. The analysis was conducted using a 10-year time horizon.

The treatment protocols included in the two scenarios were.

1. *Historical protocol*
   HBIG 5.000 IU/day intravenous IV) for 7 days +5.000 IU IV every 2 months life-long + NA lifelong.
2. *ELITA protocol (ELITA guidelines)*
   Low risk populations (HBV DNA negative at LT).
   HBIG 5.000 IU/d IV for 7 days + NA lifelong.
   High risk population (HBV DNA positive at LT).
   HBIG 5.000 IU/day IV for 7 days + 5.000 IU IV every 2 months for 1year + NA life-long.
   Special population (poorly adherent and HBV/HDV):
   HBIG 5.000 IU/day IV for 7 days +5.000 IU IV every 2 months life-long + NA lifelong.

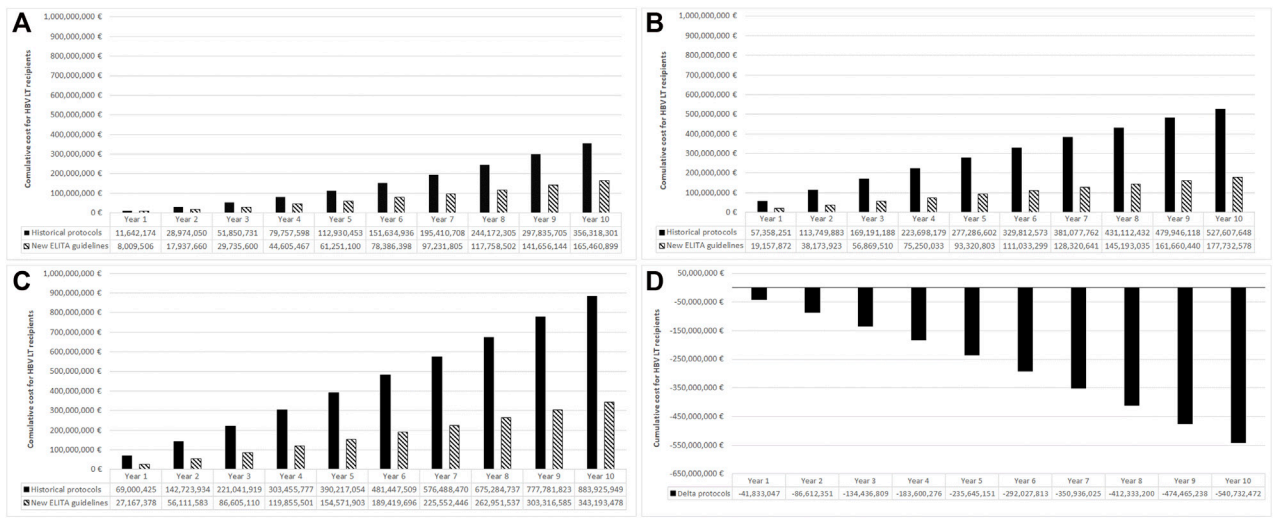
Notably, patients transplanted, with hepatocellular carcinoma (HCC), were not considered as a special population but received a prophylactic regimen based on their virological risk, as patients with decompensated cirrhosis. Further, the dose of HIBG in the 7 days post-transplant theraphy was set at 5,000 IU/kg instead of 10,000 IU/Kg as reported in ELITA guidelines. This change in treatment protocol was based on the actual treatment performed in the majority of European LT centers. However, this change has no effect on the overall budget impact because the first 7 days treatment with HBIG post-transplant is the same in both historical and ELITA protocol (HBIG 5.000 IU/day intravenous IV) for 7 days).

The population simulated for this analysis consisted of all HBV transplanted patients performed in Europe over a 10-year time period. Patients were stratified into two groups: 1. Incident LT patients, all HBV patients forecast to receive a LT in the next 10 years, and 2. Prevalent LT patients, all alive patients transplanted in the last 15 years. The clinical and epidemiological data used in the model is reported in **Table 1** and was based on the European Liver Transplant Registry (ELTR)



	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
Incident LT patients	506	942	1,367	1,782	2,186	2,579	2,966	3,346	3,720	4,088
Prevalent LT patients	5,627	5,532	5,439	5,347	5,257	5,131	5,007	4,887	4,770	4,655
Total	6,133	6,474	6,806	7,129	7,442	7,710	7,973	8,234	8,490	8,743

FIGURE 1 | Target population estimated by the model.



	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
Historical protocols	11,642,174	28,974,050	51,850,731	79,757,598	112,930,453	151,634,936	195,410,708	244,172,305	297,835,705	356,318,301
New ELITA guidelines	8,009,506	17,937,660	29,735,600	44,605,467	61,251,100	78,386,398	97,231,805	117,758,502	141,656,144	165,460,899

	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
Historical protocols	57,358,251	113,749,883	169,191,188	223,698,179	277,286,602	329,812,573	381,077,762	431,112,432	479,946,118	527,607,648
New ELITA guidelines	19,157,872	38,173,923	56,869,510	75,250,033	93,320,803	111,033,299	128,320,641	145,193,035	161,660,440	177,732,578

	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
Historical protocols	69,000,425	142,723,934	221,041,919	303,455,777	390,217,054	481,447,509	576,488,470	675,284,737	777,781,823	883,925,949
New ELITA guidelines	27,167,378	56,111,583	86,605,110	119,855,501	154,571,903	189,419,696	225,552,446	262,951,537	303,316,585	343,193,478

	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7	Year 8	Year 9	Year 10
Delta protocols	-41,833,047	-86,612,351	-134,436,809	-183,600,276	-235,645,151	-292,027,813	-350,936,025	-412,333,200	-474,465,238	-540,732,472

FIGURE 2 | Total budget for the historical protocols and new ELITA treatment guidelines in incident (new) cases **(A)**, prevalent cases **(B)**, all cases **(C)**, and the associated budget impact **(D)**. Results from the European point of view and using 10 years time horizon.

data [5]. The number of incident cases, that is new HBV LT patients, were estimated assuming 506 cases per year, and a survival probability of 86.22% after 1 year, 77.77% after 5 years, and 71.43% after 10 years [5]. The number of prevalent cases, that is historical LT patients, were assumed to be 5,627, based on 7,593 HBV LT reported by Adams et al. over the last 15 years, with the associated survival probability [5].

The predictive model assumed a survival probability of prevalent LT patients equal to 93.56% after 5 years and 85.49% after 10 years [5]. Furthermore, to define the number of patients associated to each category of the ELITA treatment protocol, the model had 82% prevalence of low-risk patients, 10% of high risk and 8% of special patients [5,6,7].

The pharmaceutical costs for HBV drugs were the only costs included in the analysis. The unit price applied to each drug was the average of drugs price reported in Spain, Italy, France, Austria, Belgium and Poland [3]. NA cost applied in the analysis was the average price of ENT (11.9 € per mg) and TDF (8.4 € per 245 mg). The price applied to HIBG was the one associated to the IV formulation (**Table 1**).

Based on these drug costs, the model estimated the total annual cost per patient per single treatment. The treatment cost per patient for the first year and the subsequent years are reported in **Table 1**. The estimated annual costs were combined with the epidemiological data to estimate the 10-year cumulative cost of the two scenarios. The difference between these two

TABLE 2 | Sensitivity analysis results.

Parameters	Value - base case	Budget impact—Base case	Value—sensitivity analysis	Budget impact—sensitivity analysis	Budget impact difference
New yearly (incident) cases of HBV LT patients, N	506	–540,732,472 €[a]	405	–502,636,429 €	–38,096,043 €
			607	–578,828,154 €	38,096,043 €
Number of prevalent HBV LT patients, N	5,627		4,501	–470,739,495	–69,992,976 €
			6,752	–610,711,633 €	69,979,161 €
HBIG IV, € per 5000 IU	1,589.6 €		1,029.0 €	–348,487,657 €	–192,244,815 €
Type and dose of HBIG	5000 IU IV every 2 months		1000 IU IM or SC HBIG every 2 weeks	–551,414,186 €	10,681,714 €
			1000 IU IM or SC HBIG every 4 weeks	–252,313,764 €	–288,418,708 €
Distribution of patients category included in ELITA protocol	82.0% (low risk) 10.0% (high risk) 8.0% (special patients)		70.0% (low risk) 16.5% (high risk) 13.5% (special patients)	–504,881,105 €	–35,851,367 €

[a]*Saving at 10 years in LT, récipients (incident + prevalent) using new ELITA, guidelines (overall cost € 343, 193, 478) instead of historical protocol (overall cost € 883, 925, 949).*

indicate the potential budget impact associated with the application of the new ELITA guidelines in Europe, over the next 10 years, considering all HBV transplanted patients.

Additional analyses were performed to assess the following: 1. The impact of HBIG price to the budget impact results, applying the lowest and highest price reported within the 6 European countries used to estimate the HBIG price, 2. The variations in the number of incident LT patient, 3. Variations in the number of prevalent LT patient (±20% of base case), 4. The use of 1000 IU intramuscular (IM) or subcutaneous (SC) HBIG (327.6 Euro) every 2 weeks instead of 5000 IU IV HBIG (1,589.6 Euro) every 2 months, and 5. The use of 1000 IU IM or SC HBIG every 4 weeks instead of 5000 IU IV every 2 months. Further, to test the impact of a lower percentage of low-risk patients, an alternative scenario was tested assuming 70.0% low risk patients, 16.5% high-risk, and 13.5% special patients.

No human studies are presented in this manuscript; ethics approval or specific consent procedures were not required.

RESULTS

The target population in the prediction model included both prevalent and incident cases, and consisted of 6,133 patients in the first year (**Figure 1**) that increased to 7,442 and 8,743 patients after 5 and 10 years from its implementation.

According to the historical protocol the cumulative costs were the following.

- For incidental patients: 11.64 Million Euro after 1 year and 356.32 million Euro after 10 years (**Figure 2A**, black bars).
- For prevalent patients: 57.36 million Euro after 1 year and 527.61 million Euro after 10 years (**Figure 2B**, black bars).
- For prevalent + incident patients: 69.00 million Euro after the first year that increased to 883.93 million Euro after 10 years (**Figure 2C**, black bars)

The costs of the adoption of the ELITA guidelines, the dashed bars in **Figure 2** panel A, B and C, was associated with a significant budget reduction and a cost saving of almost 41.83 million Euro at 1 year, 235.65 million Euro at 5 years, and 540.73 million Euro at 10 years (**Figure 2D**). The saving associated with new HBV post-LT prophylactic regimen was due to HIBG withdrawal after the first 4 weeks post LT in low and high risk populations, and to the increasing number of patients over time treated according to the ELITA guidelines.

The results of sensitivity analysis are reported in **Table 2**, and confirmed the significant cost saving associated to the adoption of the new ELITA guidelines. The use of 1000 IU IM or SC HBIG (327.6 Euro) every 4 weeks instead of 5000 IU IV HBIG (1,589.6 Euro) every 2 months was the parameter with the highest impact on the budget difference between the two scenarios, followed by HBIG price. The use of 1000 IU IM or SC HBIG every 4 weeks instead of 5000 IU IV HBIG every 2 months resulted in a cost saving of 252.31 million Euro at 10 years instead of 540.73 million Euro. Applying a price of 1,029.0 Euro per 5000 IU instead of 1,589.6 Euro, the model predicted a budget cut of 348.49 million Euro.

DISCUSSION

New guidelines or international society recommendations provide up-to-date clinical evidence for improving clinical outcomes and managing patients. Unfortunately, economic impact analysis associated with the implementation of new guidelines or recommendations is rarely performed. Our study provides the budget impact analysis of the new strategies provided by ELITA for the management of liver-transplanted patients with Hepatitis B assuming that all prevalent cases (patients already transplanted) and incident cases (new patients undergoing LT) would be treated accordingly. Costs derived from the new ELITA guidelines were compared with those associated with historical protocols.

Based on our analysis, the implementation of the ELITA guidelines, resulted in a substantial cost saving which was possible thanks to an individualized short-term use of HBIG

depending on the virological risk at the time of liver transplantation. In particular, according to the ELITA guidelines patients are considered at low or high risk if HBVDNA is undetectable (low risk) or positive at LT (high risk). Patients with low risk profile account for the vast majority of cases and are indicated to receive HBIG for the first 7 days after LT while high risk patients should be treated with HBIG for 1 year. Both low and high-risk patients are continued on NA alone after HBIG withdrawal.

Considering both prevalent and incident cases throughout Europe, the cost savings favored by the implementation of the ELITA clinical practice guidelines, was estimated at 235.65 million Euro at 5 years and 540.73 million Euro after 10 years. The cost saving is mainly associated to the reduction of HBIG use which produced a cost reduction of €9,538 per patient year both in low and high risk patients after the first year post LT. Assigning both incident and prevalent patients to new ELITA treatment guidelines provided the largest savings. The implementation of the ELITA guidelines to solely incident patients would also lead to significant cost savings, however the impact would become more significant after years due to the increasing number of patients treated with this new treatment protocol. Even the use of very low dose of HIBG, 1000 IU IM or SC HBIG every 4 weeks long term, was associated to a substantial saving (252.31 million Euro). In this scenario, the cost saving was €4,328 per patient year both in low and high risk patients after the first year post LT.

The results of this study are in accordance with the preliminary results reported in the original ELITA paper, however the current model which now includes prevalent cases, allows a more specific and accurate assessment of the potential overall economic impact. In fact, the estimated saving is higher to what assessed in previous analysis [3] as patients with HCC were not considered as a special population but received a prophylactic regimen based on their virological risk, similarly to decompensated cirrhotics.

An alternative prophylactic strategy, based on the use of NA without HBIG, has also been proposed by Fung and colleagues from Asia [2]. This treatment protocol would allow a greater reduction in treatment costs of 286.09 million Euro after 5 years and 647.73 million Euro after 10 years, compared to the historical treatment regimen. Furthermore, the approach proposed by Fung and colleagues compared favorably even with the new ELITA protocol, with around €50 million and €100 million savings at 5 and 10 years. Although such approach proved to be very effective and safe in Asia, new studies generated in western countries are needed before being accepted in European guidelines.

The study has some limitations. First, the analysis does not consider the clinical efficacy of the different scenarios (incidence of expected HBV recurrence). However, the available evidence suggests that the historical and new treatment protocols are associated with a similar efficacy that is an average 5% incidence of treatment failures [3]. Second, the analysis does not consider the adverse events associated with the historical protocols and ELITA guidelines. This approach is conservative considering the better safety profile associated to the lower use of HBIG in the new ELITA protocol. Third, the analysis assumed no treatment discontinuation in order to estimate the maximum economic impact associated with the new ELITA protocol. A different treatment adherence could in fact be observed when following historical protocols or new ELITA guidelines. In this case, a different clinical efficacy should be considered and a cost-effectiveness analysis should be conducted instead of a budget impact analysis. Fourth, the cost of HBIG IV infusion was not included in the analysis due to the high variability of cost and setting throughout Europe. However, this approach can be considered conservative due to the higher use of HBIG in the Historical scenario and the related higher cost associated to the infusions. Finally, the analysis assumed the use of Tenofovir Viread but not that of the more expensive Vemlidy. Since, the use of NA is the same in both historical protocols and ELITA guidelines; using a more or less expensive NA has no impact on the budget impact.

In conclusion, the new ELITA recommendations provide an individualized treatment prophylaxis of HBV patients based on virological risk profile at LT that would allow a significant cost reduction in Europe. The money saved would give the possibility to the healthcare system to invest in other technologies in order to improve the health of the population. New studies focusing on economic impact of ELITA guidelines in each European country could be of interest to provide specific information for local healthcare authorities and patients.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PC, CD, and LB provide substantial contributions to the conception and design of the work, analysis and interpretation of data for the work, drafting the work. RV, SC, IL, RV, SM, MA, JF, MB, AC, FD, CF, PL, FN, WP, MR, FZ, GP, MB, and LM provide substantial contributions to the interpretation of data for the wok and revising it critically for important intellectual content. All authros provide approval for publication of the content and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

PC has served as a speaker for Novartis and Roche. CD has served as a speaker, a consultant and an advisory board member for Astellas, Biotest, Chiesi, Novartis and Sandoz and has received

research funding from Novartis and Sandoz. MA has served as a speaker, a consultant and an advisory board member for Abbvie and Gilead and has received research funding from Gilead and MSD. MB1 has served as a speaker, a consultant and an advisory board member for Abbvie, Astellas, Deep-Genomic, Gilead, Intercept, Orphalan, Novartis, and has received research funding from Gilead. MB2 has served as a speaker and an advisory board member for Gilead and Janseen and has received funding from Abbvie and Gilead. AC has served as a speaker, a consultant and an advisory board member for Astellas, Novartis, Sandoz, Intercept, Gilead and has received research funding from Intercept. FD has served as a consultant for Biotest. CF has served as a speaker, a consultant and an advisory board member for Astellas, Corza Medical and Medtronic, and has received research funding from Guangdong Shunde Innovative Design Institute, Guangdong, China. PL has reported grants (research funding) from Biotest France SAS and non-financial support (financial and logistic participation for Liver Congress) from Biotest France SAS and Gilead. LM reported receiving grants from Bayer, Daiiki-Sankyo, and Boehringer Ingelheim outside the submitted work and speaker fees from Pfizer and Bayer.

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

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