ESOT

Volume 37 | Issue 01
January 2024

Transplant International

Domino Effect in Kidney Transplantation

Transplant International

frontiers | Publishing Partnerships

Transplant International

ESOT

Transplant International

Domino Effect in Kidney Transplantation

ISSN 1432-2277
ISBN 978-2-8325-5289-6
DOI 10.3389/978-2-8325-5289-6

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TRANSPLANT TRIAL WATCH
published: 22 January 2024
doi: 10.3389/ti.2024.12597

Transplant Trial Watch

John M. O'Callaghan[1,2]* *and Simon R. Knight*[2,3]*

[1]University Hospitals Coventry & Warwickshire, Coventry, United Kingdom, [2]Centre for Evidence in Transplantation, Nuffield Department of Surgical Sciences, University of Oxford, Oxford, United Kingdom, [3]Oxford Transplant Centre, Churchill Hospital, Oxford, United Kingdom

Keywords: randomised controlled trial, kideny transplantation, solid organ transplant (SOT), tacrolimus, cytomegalovirus

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

A prospective controlled, randomized clinical trial of kidney transplant recipients developed personalized tacrolimus dosing using model-based Bayesian Prediction.

by Lloberas, N., et al. Kidney International 2023 [record in progress].

Aims

The aim of this study was to evaluate the clinical applicability of a Population pharmacokinetic (PPK) model for achieving Tac Co (therapeutic trough Tac concentration) versus the manufacturer's labelling dosage.

Interventions

Participants were randomised to either the PPK group or the control group with patients receiving Tac adjustment according to the manufacturer's labeling.

Participants

96 adult renal transplant recipients.

Outcomes

The primary outcome was the percentage of patients reaching the Tac Co target (6 and 10 ng/mL) after the first steady state. The secondary outcomes were the timing needed to reach the therapeutic target, the number of dose modifications needed to reach the target, and the clinical outcome.

Follow-Up

90 days posttransplantation.

CET Conclusion

This single-centre randomised study compared initial tacrolimus dosing by body weight (control), or by Bayesian prediction (study), following renal transplantation. Patients in the study group had their tacrolimus dosing guided by a Bayesian model incorporating age, haematocrit and CYP3A genotype. The

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***Correspondence**
John M. O'Callaghan,
ocallaghan.john@gmail.com
Simon R. Knight,
simon.knight@nds.ox.ac.uk

Received: 21 December 2023
Accepted: 09 January 2024
Published: 22 January 2024

Citation:
O'Callaghan JM and Knight SR (2024) Transplant Trial Watch. Transpl Int 37:12597. doi: 10.3389/ti.2024.12597

authors demonstrate that a significantly higher proportion of patients in the study arm achieved therapeutic target, with lower interpatient variability, shorter time to target trough concentrations and fewer dose modifications. Whilst no differences in clinical outcomes were seen, there was a trend towards lower incidence and shorter duration of DGF in the study group. These results are very promising and appear to demonstrate the benefit of personalised dosing using the Bayesian model. The population in this study are from a single centre, and predominantly male and Caucasian. Future studies should confirm these findings in populations with a greater mix of ethnicity, and confirm potential clinical benefit in a larger sample.

Jadad Score
2.

Data Analysis
Per protocol analysis.

Allocation Concealment
No.

Trial Registration
EudraCT—2016-000340-34

Funding Source
Non-industry funded.

RANDOMISED CONTROLLED TRIAL 2

Immune monitoring-guided vs. fixed duration of antiviral prophylaxis against cytomegalovirus in solid-organ transplant recipients. A Multicenter, Randomized Clinical Trial.

by Manuel, O., et al. Clinical Infectious Diseases 2023 [record in progress].

Aims
The aim of this study was to compare the effect of an immune monitoring–guided approach versus the current standard for tailoring the duration of antiviral prophylaxis to measure cytomegalovirus (CMV)-specific immunity in solid-organ transplant recipients.

Interventions
Participants were randomised to receive a duration of antiviral prophylaxis according to immune–guided monitoring or a fixed duration (control).

Participants
193 kidney and liver transplant recipients CMV-seronegative with seropositive donors or CMV-seropositive receiving antithymocyte globulins.

Outcomes
The two primary endpoints were proportion of patients with clinically significant CMV infection and reduction in days of prophylaxis. The secondary endpoints were the incidence of all CMV events including untreated CMV replication, high-level CMV-DNAemia, patient survival, graft survival and incidence of acute rejection.

Follow-Up
1 year.

CET Conclusion
This multicentre trial enrolled kidney and liver transplant recipients receiving organs from CMV-positive donors, and randomised them to either fixed-duration prophylaxis, or guided by immune monitoring. In the study group, CMV ELISpot was used to monitor, and prophylaxis stopped if positive (indicating immune reactivity). The study failed to confirm non-inferiority of the immune monitoring strategy, although the overall rates of CMV infection were similar, with earlier CMV infection seen in the study group. However, duration of prophylaxis was shorter in the study arm. The failure to demonstrate non-inferiority is due to a lack of statistical power – in reality, the infection rates were very similar between groups. The study also fails to stratify randomisation by recipient serostatus, leading to an imbalance between the two arms of the study. This is important, as the risk of CMV infection is likely different between the two subgroups. Despite these limitations, it does appear that immune monitoring-guided prophylaxis is a reasonable strategy, resulting in a shorter duration of prophylaxis and a relatively low risk of clinically relevant CMV disease.

Jadad Score
3.

Data Analysis
Per protocol analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov—NCT02538172.

Funding Source
Industry & non-industry funded.

CLINICAL IMPACT SUMMARY

This report is from a very interesting study in both liver and kidney transplantation, that could be practice changing. Monitoring for an immune response to CMV was used as a comparator to standard-duration CMV prophylaxis with valganciclovir. In the intervention arm of the study, prophylaxis was stopped if the immune monitoring showed a significant response (CMV ELISpot). The primary outcome was clinically significant CMV infection, which may be represented by

symptomatic disease or asymptomatic viraemia that required treatment.

The study was designed on a non-inferiority basis and was statistically powered as such. Approximately 31% of patients had clinically significant CMV infection, which was higher than expected. This meant that the immune monitoring approach was not shown to be statistically non-inferior, despite similar event-rates in the study and control arms. The duration of antiviral prophylaxis was however, significantly shorter with immune monitoring, by about 26 days on average. The safety of the immune monitoring approach was consistent, whether or not the recipient was CMV positive or negative. The incidence of CMV disease was very low for both groups (0 versus 2 events). As the risk of any CMV infection was higher than expected in both arms, the 95% CI for the risk difference was wide and therefore a significant inferiority could not be ruled out.

Despite the limitations of the study, it seems that the immune monitoring strategy is safe and can result in a much earlier opportunity to stop CMV prophylaxis. A cost-benefit analysis would have been interesting to see but is not formally provided in this paper.

AUTHOR CONTRIBUTIONS

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

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.

ACKNOWLEDGMENTS

Edited by Reshma Rana Magar.

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SYSTEMATIC REVIEW AND META-ANALYSIS
published: 23 January 2024
doi: 10.3389/ti.2024.12168

Unveiling the Incidence and Graft Survival Rate in Kidney Transplant Recipients With *De Novo* Thrombotic Microangiopathy: A Systematic Review and Meta-Analysis

Chien-Ya Hsiung[1,2†], *Hsin-Yu Chen*[3,4†], *Shih-Han Wang*[5] *and Ching-Ying Huang*[6*]

[1]*Division of Gastroenterology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan,* [2]*Fu Jen Catholic University, New Taipei City, Taiwan,* [3]*Division of Nephrology, Department of Internal Medicine, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan,* [4]*School of Medicine, National Yang-Ming University, Taipei, Taiwan,* [5]*Department of Pharmacy, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan,* [6]*Department of Pharmacy, Kaohsiung Veteran General Hospital, Kaohsiung, Taiwan*

De novo thrombotic microangiopathy (TMA) is a rare and challenging condition in kidney transplant recipients, with limited research on its incidence and impact on graft survival. This study conducted a systematic review and meta-analysis of 28 cohorts/single-arm studies and 46 case series/reports from database inception to June 2022. In meta-analysis, among 14,410 kidney allograft recipients, *de novo* TMA occurred in 3.20% [95% confidence interval (CI): 1.93–4.77], with systemic and renal-limited TMA rates of 1.38% (95% CI: 06.5–2.39) and 2.80% (95% CI: 1.27–4.91), respectively. The overall graft loss rate of *de novo* TMA was 33.79% (95% CI: 26.14–41.88) in meta-analysis. This study provides valuable insights into the incidence and graft outcomes of *de novo* TMA in kidney transplant recipients.

Keywords: thrombotic microangiopathy, kidney allograft, renal function, graft survival rate, graft loss rate

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***Correspondence**
Ching-Ying Huang,
gingying0510@gmail.com

[†]*These authors share first authorship*

Received: *27 October 2023*
Accepted: *09 January 2024*
Published: *23 January 2024*

Citation:
Hsiung C-Y, Chen H-Y, Wang S-H and Huang C-Y (2024) Unveiling the Incidence and Graft Survival Rate in Kidney Transplant Recipients With De Novo Thrombotic Microangiopathy: A Systematic Review and Meta-Analysis. Transpl Int 37:12168. doi: 10.3389/ti.2024.12168

INTRODUCTION

Thrombotic microangiopathy (TMA) is a rare complication of kidney transplantation that is often associated with poor graft and patient outcomes. TMA can be diagnosed based on clinical or histopathological features. Clinical recognition of TMA requires evidence of (a) microangiopathic hemolytic anemia: fragmented red blood cells on a peripheral blood smear, decreased haptoglobin levels, elevated lactate dehydrogenase and indirect bilirubin levels, and a decline in hemoglobin levels; (b) thrombocytopenia; and (c) evidence of organ damage. The common sites are the kidneys, central nervous system, and gastrointestinal tract [1]. Allograft biopsy is the gold standard method for establishing the diagnosis. Histologically, TMA is characterized by the patchy distribution of the vessel wall and detachment of edematous endothelial cells from the basement membrane. This causes intravascular platelet aggregation with subsequent formation of platelet-rich thrombi within the microcirculation and obstruction of vessel lumina [2].

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AMR, antibody-mediated rejection; CI, confidence interval; CNIs, calcineurin inhibitors; ESRD, end-stage renal disease; PE, plasma exchange; TMA, thrombotic microangiopathy.

Post-transplant TMA is classified into recurrent TMA and *de novo* TMA. Recurrent TMA is characterized by the same disease process that manifests as TMA involving the native kidney and recurs in the case of the allograft. In contrast, *de novo* TMA develops for the first time in kidney transplant recipients who had no evidence of the disease before transplantation. A study based on the United States Renal Data System indicated that the incidence of overall TMA in kidney allograft recipients was 5.6 episodes per 1,000 person-years, with approximately 50% patient mortality at 3 years [3]. As for *de novo* TMA, the incidence had been reported with a wide range and could be incorrectly estimated due to missed diagnosis of TMA before kidney transplantation. The incidence of *de novo* TMA has been reported to range from 3% to 14%, and the allograft loss rate ranges from 10% to 57%, both with a wide range [4]. *De novo* TMA not only causes acute decline of allograft function but also different degrees of sequelae. Graft loss in the case of *de novo* TMA is up to 40% within 2 years of diagnosis [3]. Outcomes range from transient renal dysfunction with mild clinical significance to acute renal failure requiring temporary dialysis therapy, potential allograft loss, and patient mortality. The outcome depends on the histopathological severity of the TMA, the promptness of the diagnosis, and the initiation of treatment [5].

The etiologies of kidney allograft *de novo* TMA include calcineurin inhibitors (CNIs), mammalian target of rapamycin inhibitors, ischemia-reperfusion injury, antibody-mediated rejection (AMR), viral infection, thrombotic thrombocytopenic purpura, and atypical hemolytic uremic syndrome (aHUS). CNIs, both cyclosporine and tacrolimus, are well-documented medications that cause *de novo* TMA [6–11]. Mammalian target of rapamycin inhibitors, such as sirolimus and everolimus, comprise much of the drug-related etiologies of TMA [12–15]. AMR is also a common and well-recognized cause of post-transplant TMA [16, 17]. Other less common causes, which can lead to TMA, include various viral infections such as infection of hepatitis C, cytomegalovirus, parvovirus, and BK virus [18–23]. Antiviral therapy [24], disseminated histoplasmosis [25], and thrombotic thrombocytopenic purpura are also among the reported etiologies [26–28]. aHUS is also an important cause. The presence of genetic mutations in complement systemic regulation can trigger an uncontrolled alternative complement pathway activity, resulting in endothelial injury, the pathogenetic basis of TMA [29].

Existing evidence about the incidence and outcome of *de novo* TMA is mainly based on case series and retrospective studies, comprising a wide range of data. Studies on the incidence and graft outcomes of *de novo* TMA are lacking. Therefore, this study aimed to present comprehensive data on the incidence, graft loss, and survival of kidney allografts in patients with *de novo* TMA.

MATERIALS AND METHODS

Study Design

We conducted a systematic review and meta-analysis to evaluate the incidence and survival of kidney allografts in patients with *de novo* TMA. This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses guidelines and The Cochrane Collaboration form [30, 31].

Search Strategy and Eligibility Criteria

We systematically searched PubMed, Cochrane Library, and EMBASE (until April 2022). A manual search of the reference lists of relevant studies was performed to complement our search results. Search terms included kidney transplantation, *de novo*, and thrombotic microangiopathy, including all subheadings of the Medical Subject Headings and text searches for articles that were not indexed. No language restrictions were used to reduce funnel plot asymmetry. Automatic e-mail updates were built to periodically acquire new research results from the databases. Full details of the search strategy are presented in **Supplementary Table S1**. The reference lists of the relevant reports were manually searched to identify any missing relevant research articles or strategies.

Study Selection

All randomized controlled trials, observational studies, case reports, and case series were included in this systematic review and meta-analysis if they reported the following: 1) kidney allograft recipient; 2) *de novo* TMA; 3) incidence; or 4) graft survival. The exclusion criteria were as follows: 1) studies without retrievable endpoints; 2) studies with recurrent TMA; and 3) studies with posters or editorial comments only. The titles, abstracts, and contents were screened by three authors (C-YHs, S-HW, and C-YHu) to determine whether the studies met the inclusion criteria. The full texts of potentially relevant studies were retrieved and assessed in more detail.

Data Extraction

Three reviewers (C-YHu, S-HW, and C-YHs) independently assessed the studies for eligibility and extracted the data using a standardized data extraction form. Disagreements were resolved through a discussion with a fourth author (H-YC). The following parameters were extracted from each study: general characteristics (first author, year of publication, study terms, study design, and country), patient characteristics (number of patients in each treatment arm, patient age, sex, kidney donor types, genetic variants for complement dysregulation, cause of end-stage renal disease [ESRD], anti-rejection regimen, kidney pathological features, treatment of TMA, and follow-up duration), TMA incidence, and kidney allograft survival.

Quality Assessment

All cohort studies that met the inclusion criteria were subjected to quality appraisal using the Newcastle–Ottawa Scale, which contains 8 items within 3 domains and a total maximum score of 9 for cohort studies. Scores of 7–9 indicate high quality, 4–6 indicate high risk, and 0–3 indicate a very high risk of bias. All the case reports that met the inclusion criteria were subjected to quality appraisal using the CARE checklist and were recorded as "YES," "PARTLY," or "NO," according to

information reported by the included studies. The responses were assigned scores of 1, 0.5, and 0, respectively. The overall score was the sum of the 21 sub-items and was defined as "high" (more than 15), "medium" (10.5–14.5), and "low" (less than 10) [32]. These quality assessments were judged independently by two reviewers (S-HW and C-YHs), and any conflict was discussed with the third reviewer (C-YHu).

Outcomes

The study outcomes were the *de novo* TMA incidence and graft survival rates. *De novo* TMA incidence was divided into systemic and renal-limited TMA. Some studies that were not classified as systemic or renal-limited TMA were classified as unknown type of TMA. Therefore, we reported the following four different TMA incidences: 1) systemic TMA, 2) renal-limited TMA, 3) total TMA, and 4) unknown type of TMA.

The graft outcomes included graft loss and graft survival. Some studies showed graft survival of 1, 2, 3, 4, 5, 8, and 10 years.

Measurements

De novo TMA incidence was reported as a percentage and event per person-year. The pooled estimated incidence of *de novo* TMA was reported with a 95% confidence interval (CI). The graft survival rate was reported as a percentage. The pooled estimated graft survival was also reported with a 95% CI.

Meta-Analyses

The effect of baseline characteristics on the incidence of *de novo* TMA was analyzed. These factors included C4d, acute AMR, acute cell-mediated rejection, and the use of tacrolimus or cyclosporine. A random effects model was used for the meta-analysis.

Statistical Analysis

We used the MedCalc statistical software version Medal 20.110 (Acacialaan 22 8400 Ostend Belgium) to conduct meta-analyses and SPSS version 23.0 (IBM Corp., Armonk, New York) for descriptive analyses. Statistical heterogeneity of studies was assessed using I^2 (inconsistency) from the fixed-effects model. All results were analyzed using a random-effects model if I^2 was greater than 50% to minimize the potential heterogeneity effect and between-study variance. For descriptive analyses, continuous data were reported as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

RESULTS

Study Selection

Overall, 229 potentially relevant articles were identified in the literature search. Based on the review of the titles and abstracts, 126 studies were excluded. Further, 103 full-text articles were assessed for their eligibility; 31 records were excluded for the reasons of posters, insufficient data, or an editorial protocol. Finally, 75 studies met the inclusion criteria. **Supplementary Figure S1** summarizes the flowchart of the search. Of the 75 included studies, 46 were case reports and case series, 21 were single-arm studies, and 8 were cohort studies. Eculizumab was approved for aHUS treatment by the Food and Drug Administration of the United States in 2011. Most of the studies published in 2012 collected data before 2012. Therefore, we categorized studies as published before 2013 and after 2013 (**Supplementary Figure S2**).

Study Characteristics of Single-Arm and Cohort Studies

Supplementary Table S4 summarizes the characteristics of single-arm and cohort studies. The percentage of males in the included studies ranged from 17% to 77.8%. The recruitment years of the studies ranged from 1980 to 2019, and 13 studies were published before 2013. The mean age was not reported in 9 studies, while the mean age reported in the other 20 studies was >23 years. The proportion of sex was not reported in 15 studies, whereas the male sex percentage was ranged from 0% to 77.7% in 14 studies. The study population was divided into kidney allograft recipients and renal biopsy recipients. The causes of ESRD included presumed chronic glomerulonephritis, presumed chronic interstitial nephritis, IgA nephropathy, focal segmental glomerulosclerosis, diabetic nephropathy, nephrosclerosis, lupus nephropathy, polycystic kidney disease, and hypertensive nephrosclerosis; however, they were not reported in 22 studies. Regarding management, CNI adjustment was reported in seven studies, two studies reported the efficacy of plasma exchange (PE), and three studies reported eculizumab therapy. Moreover, the proportion of AMR was mentioned in five studies, whereas the proportion of ABO-incompatible cases was mentioned in one study. Finally, one study reported pregnancy outcomes.

Study Characteristics of Case Reports and Case Series

Supplementary Table S2 and **Supplementary Figure S3** summarize the characteristics of the case reports and case series. A total of 46 case reports and case series of 62 kidney allograft *de novo* TMA recipients were identified. A total of 42 (68%) recipients were tacrolimus users, 15 (24%) were cyclosporine users, and 5 (8%) were sirolimus users. The gene mutation data were limited. Of the 42 tacrolimus users, 9 possessed a complement factor H mutation, 2 possessed a complement factor I mutation, 1 possessed a factor II mutation, and 1 had a factor V mutation. Further, 5 of the 62 patients had a history of kidney transplants, 20 were living donor recipients, and 38 were deceased donor recipients. The onset timings (mean ± SD) of TMA were 11.26 ± 37.38, 16.68 ± 32.99, and 1.71 ± 2.96 months among tacrolimus, cyclosporine, and sirolimus users, respectively.

Six patients had AMR, six had cell-mediated rejection, and six had C4d+ on kidney pathology. Five patients were ABO-incompatible. The management of TMA included tapering the CNI and sirolimus dose, and then shifting to other immunosuppressive agents, eculizumab therapy, PE or infusion therapy, or belatacept therapy. A total of 34 (55%)

TABLE 1 | Thrombotic microangiopathy incidence in included cohort and single-arm studies.

Study	Study design	Study population	Total TMA incidence	Systemic TMA incidence	Renal-limited TMA incidence	Study follow-up time (month)	C4d+	C4d–	Tacrolimus base regimen	Cyclosporine base regimen	Acute antibody-mediated rejection	Acute cell-mediated rejection	ABO incompatible
Baid, 1999 [33]	Single-arm	KAR	3.2% (12/379)		3.2% (12/379)	84							
Braet, 2016 [34]	Single-arm	KAR	2.20%		N/A	428							
Caires, 2012 [35]	Single-arm	KAR	1.1% (17/1,549)		1.1% (17/1,549)	132							
Dessaix, 2019 [36]	Single-arm	KAB	4.80%			6.6							
Doradla, 2020 [37]	Single-arm	KAR	0.85% (17/2,000)	0.2% (4/2,000)	0.65% (13/2,000)								
Fortin, 2004 [38]	Cohort	KAR	3.53% (13/368)		3.53% (13/368)				1.29% (3/233)	3.70% (2/54)			
Franco, 2003 [39]	Single-arm	KAR	0.26% (10/3,862)	0.26% (10/3,862)		24							
Futamura, 2020 [40]	Single-arm	KAR	5.16% (69/1,336)			211							
Gumber, 2014 [41]	Single-arm	KAR	2.89% (34/1,175)			72							
Kocak, 2015 [42]	Single-arm	KAR	2.72% (13/477)	2.72% (13/477)		36			2.72% (13/477)				
Langer, 2001 [43]	Single-arm	KAR	1.5% (10/672)	1.5% (10/672)		212				1.5% (10/672)			
Nava, 2014 [44]	Cohort	KAR	7.3% (36/496)			180				N/A			
Oyen, 2006 [45]	Single-arm	KAR	0.82% (7/850)			48				0.82% (7/850)			
Ozedemir, 2018 [46]	Single-arm	KAR									33.33% (30/90)	17.6% (9/51)	
Reynolds, 2003 [3]	Cohort	KAR	4.9/1,000 PY										
Santos, 2003 [47]	Single-arm	KAR	5% (6/115)	5% (6/115)									
Satoskar, 2010 [16]	Single-arm	KAB					13.6% (33/243)	23.6% (6/715)					
Schwimmer, 2003 [4]	Single-arm	KAR	3% (21/742)	1.07% (8/742)	1.75% (13/742)				52% (11/21)	48% (10/21)			
Tasaki, 2019 [17]	Cohort	KAR	7.5% (15/201)	7.5% (15/201)		214							17.2% (15/87)
Zarifian, 1999 [48]	Single-arm	KAR	13.8% (26/188)	1.06% (2/188)	12.7% (24/188)								

KAR, kidney allograft recipients; KAB, kidney allograft biopsies; PY, person-years; TMA, thrombotic microangiopathy.

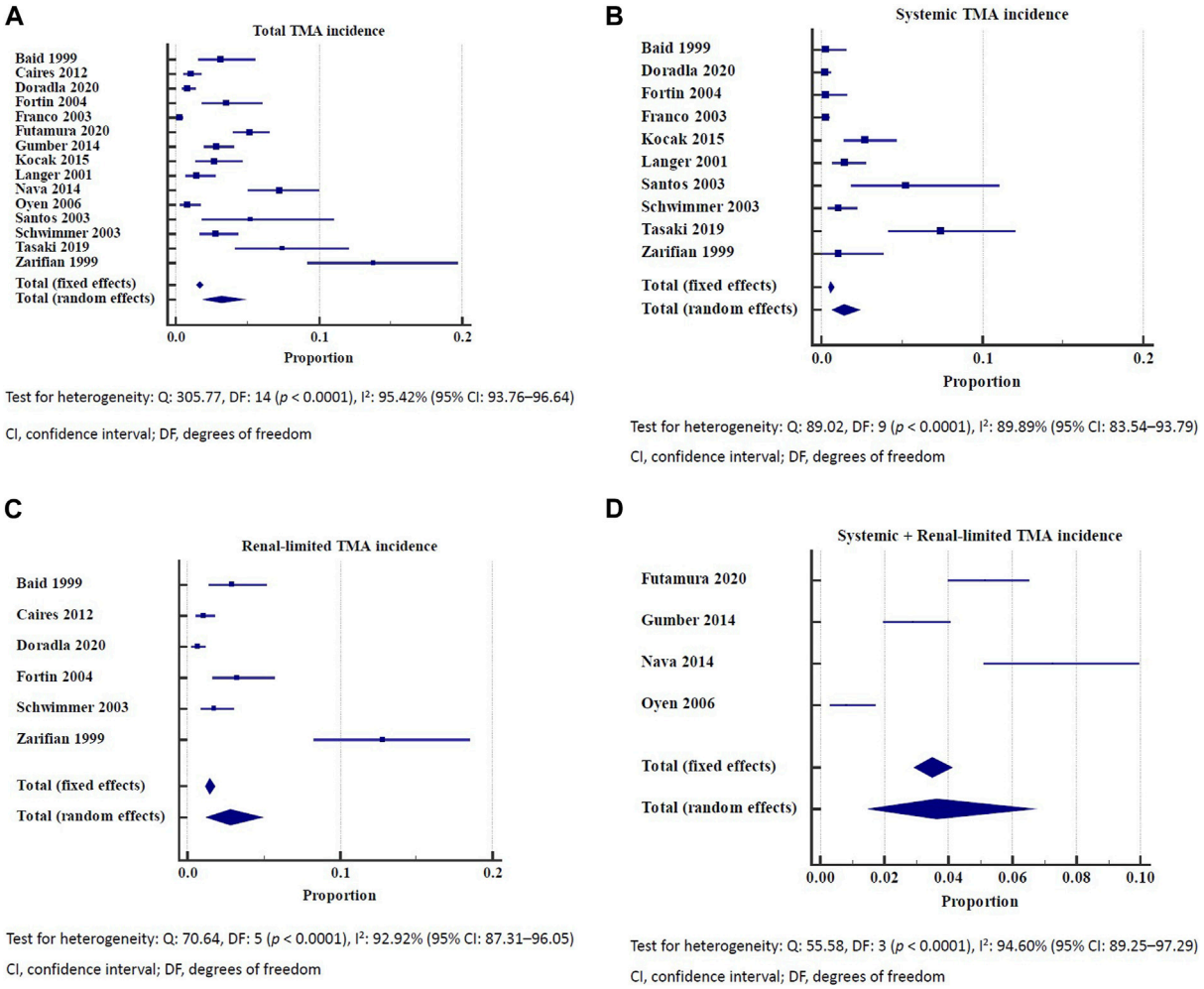

FIGURE 1 | (A) Incidence of total *de novo* thrombotic microangiopathy. Test for heterogeneity: Q: 305.77, DF: 14 ($p < 0.0001$), I^2: 95.42% (95% CI: 93.76–96.64). CI, confidence interval; DF, degrees of freedom. **(B)** Incidence of systemic thrombotic microangiopathy. Test for heterogeneity: Q: 89.02, DF: 9 ($p < 0.0001$), I^2: 89.89% (95% CI: 83.54–93.79). CI, confidence interval; DF, degrees of freedom. **(C)** Incidence of renal-limited thrombotic microangiopathy. Test for heterogeneity: Q: 70.64, DF: 5 ($p < 0.0001$), I^2: 92.92% (95% CI: 87.31–96.05). CI, confidence interval; DF, degrees of freedom. **(D)** Incidence of systemic and renal-limited thrombotic microangiopathy. Test for heterogeneity: Q: 55.58, DF: 3 ($p < 0.0001$), I^2: 94.60% (95% CI: 89.25–97.29). CI, confidence interval; DF, degrees of freedom.

patients received PE or infusion, and 18 (29%) patients received eculizumab therapy. The follow-up periods, months (mean ± SD) were 19.10 ± 37.23, 14.81 ± 13.74, and 4.45 ± 4.68 among tacrolimus, cyclosporine, and sirolimus users, respectively. Finally, 8 of the 62 individuals showed graft loss, whereas 48 individuals showed improvement in serum creatinine levels.

Incidence of *De Novo* Thrombotic Microangiopathy

The detailed *de novo* TMA incidence in the individual studies is summarized in **Table 1**. Among the studies included in our analysis, 20 reported on the incidence of *de novo* TMA. Of them, 18 studies focused on *de novo* TMA in kidney allograft recipients, whereas the remaining 2 studies [16, 36] reported on *de novo* TMA detected in kidney allograft biopsies. Two studies reported only on the incidence of TMA, without specifying the number of kidney allograft recipients or biopsies involved [34, 36]. Therefore, these studies were excluded from the meta-analysis.

Among kidney allograft recipients, the overall incidence of *de novo* TMA was 3.2% (95% CI: 1.93–4.77) (**Figure 1A**). The incidence of systemic and renal-limited *de novo* TMA was 1.38% (95% CI: 0.65–2.39) and 2.79% (95% CI: 1.27–4.91), respectively (**Figures 1B, C**). The unknown type of TMA incidence was 3.64% (95% CI: 1.50–6.67) (**Figure 1D**). All the outcomes showed significant heterogeneity ($I^2 > 89\%$). Stratifying the analysis based on distinct follow-up periods provided information on the overall incidence of thrombotic microangiopathy (TMA). Within 5 years follow up time, the TMA incidence was 1.04% (95% CI: 0.16–2.68) with significant heterogeneity (I^2: 92.17%) (**Supplementary Figure**

TABLE 2 | Graft outcome in included cohort and single-arm studies.

Study	Study design	Graft loss							Graft survival						
		Total graft loss	Plasma exchange	Either tacrolimus or cyclosporine, shift to sirolimus (%)	Acute antibody-mediated rejection (%)	Acute cell-mediated rejection (%)	C4d+	C4d– (%)	1-year survival (%)	2-year survival (%)	3-year survival (%)	4-year survival (%)	5-year survival (%)	8-year survival (%)	10-year survival (%)
Baid, 1999 [33]	Single-arm								40						
Braet, 2016 [34]	Single-arm								32						
Caires, 2012 [35]	Single-arm											43			
Costa, 2013 [49]	Single-arm								73.30						
Dessaix, 2019 [36]	Single-arm								8						
Doradla, 2020 [37]	Single-arm	53% (9/17)			100	100			47				35		35
Fortin, 2004 [38]	Single-arm	30.77% (4/13)													
Gumber, 2014 [41]	Single-arm	17.65% (6/34)													
Le Quintrec, 2008 [50]	Single-arm	33.33% (8/24)							67						
Meehan, 2011 [51]	Single-arm						57%								
Oyen, 2006 [45]	Single-arm	28.57% (2/7)		28.6											
Ozedemir, 2018 [46]	Single-arm								83	51			51		
Reynolds, 2003 [3]	Cohort								47	35					
Santos, 2003 [47]	Single-arm	33.33% (2/6)													
Satoskar, 2010 [16]	Single-arm	40.68% (24/59)	35% (8/23)				40%	42							
Tasaki, 2019 [17]	Cohort	26.67% (4/15)													
Wu, 2016 [52]	Cohort								70.0	48.3				28.0	
Zarifian, 1999 [48]	Single-arm								81		69				

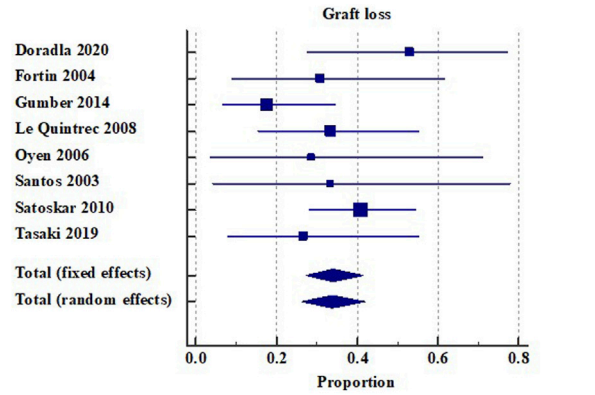

FIGURE 2 | Graft loss in patients with *de novo* thrombotic microangiopathy. Test for heterogeneity: Q: 8.54, DF: 7 ($p = 0.46$), I^2: 18.04% (95% CI: 0.00–60.87). CI, confidence interval; DF, degrees of freedom.

S4). As the follow-up duration extended to the 5–10 years, the TMA incidence was 3.02% (95% CI: 2.23–3.92) with low heterogeneity (I^2: 0.00%) (**Supplementary Figure S5**). If follow-up was more than 10 years, the TMA incidence was 4.15% (95% CI: 1.64–7.75) with significant heterogeneity (I^2: 95.54%) (**Supplementary Figure S6**). In a cohort study conducted in 2003, the incidence of *de novo* TMA in kidney allograft recipients was 4.9 episodes per 1,000 person-years [3].

The incidence of *de novo* TMA among kidney allograft biopsies ranged from 0.26% to 4.8% across the studies [34, 36, 39]. The incidence of systemic *de novo* TMA was 0.26% [39].

Graft Survival Rate in Patients With *De Novo* Thrombotic Microangiopathy

The detailed individual *de novo* TMA graft survival rate is summarized in **Table 2**. Our analysis included a total of 18 studies that reported on kidney allograft survival, of which, 8 were eligible for inclusion in the meta-analysis [16, 17, 37, 38, 41, 45, 47, 50]. The overall graft loss rate of *de novo* TMA was 33.79% (95% CI: 26.14–41.88). No significant heterogeneity (I^2 = 18.04%) was observed (**Figure 2**). The meta-analysis of seven studies reporting 1-year graft survival outcomes revealed a rate of 55.39% (95% CI: 36.46–73.54). However, a substantial degree of heterogeneity (I^2 = 88.12%) was observed (**Supplementary Figure S7**).

33% of patients with CNI-related TMA (4 out of 12) developed ESRD, while all patients with rejection-associated TMA developed ESRD [37]. The overall 1-year graft survival rate was 47%, whereas the 5- and 10-year graft survival rates were 35%. Additionally, there was no significant difference in the graft survival rate between the renal-limited and systemic TMAs ($p = 0.4$) [37].

In one study, among 33 C4d-positive TMA patients, 23 (70%) underwent plasmapheresis, with a graft loss rate of 35% (8 out of 23). Conversely, the remaining 30% (10 patients) did not receive plasmapheresis, and among these, the graft loss rate was higher at 50% [16].

Study Quality of Included Cohort Studies

All observational studies scored from 6 to 9 on the Newcastle–Ottawa Scale criteria and were included in the quantitative analysis (**Supplementary Table S3**). Five cohort studies were considered to be of high quality (Newcastle–Ottawa score ≥ 7).

DISCUSSION

Our systematic review and meta-analysis encompassed 75 studies, including 29 cohort or single-arm studies and 46 case series or case reports, to provide a comprehensive examination of the incidence and graft survival rate in kidney allograft recipients with *de novo* TMA. Among 14,410 kidney allograft recipients, 306 individuals developed *de novo* TMA, corresponding to an incidence of 3.20%, while the incidences of systemic TMA and renal-limited TMA were 1.38% and 2.80%, respectively. Among the 200 kidney allograft recipients who developed *de novo* TMA, 138 individuals remained dialysis-free 1 year after transplantation. However, among the 175 individuals with *de novo* TMA who were followed up for graft outcomes, 59 individuals eventually experienced graft loss, resulting in an overall graft loss rate of 33.79%.

Data on the incidence difference between kidney recipients with renal-limited TMA and systemic TMA are inconsistent. A study involving 21 individuals with pathology-proven kidney allograft TMA showed that 60% of the individuals had systemic TMA, and 40% had renal-limited TMA [4]. However, in contrast, only 5% of 43 individuals with pathology-proven lupus nephritis and concomitant TMA were found to have systemic TMA [53]. Moreover, comparative studies investigating differences in graft survival rates between patients with renal-limited and systemic TMA are scarce. In a case series involving 21 individuals with kidney allograft TMA, including 8 with renal-limited TMA and 13 with systemic TMA, Kaplan–Meier analysis revealed that those with renal-limited TMA had better graft survival than those with systemic TMA, with an average follow-up of 62 months [4]. Therefore, a well-designed future study is needed to examine the difference in incidence and outcomes between renal-limited and systemic TMA.

More than 90% of kidney recipients are treated with CNIs, and only a few develop *de novo* TMA. Therefore, caution should be exercised before attributing *de novo* TMA to CNIs until other predisposing factors have been ruled out [54]. The incidence of CNIs-related *de novo* TMA ranges from 1.29% to 3.7% [43, 55]. Several mechanisms explain the relationship between CNIs and *de novo* TMA. In CNI users, an imbalance of vasodilators (prostaglandin E2, prostacyclin I2, and nitric oxide) and vasoconstrictors (thromboxane A2 and endothelin) leads to glomerular arteriolar vasoconstriction and endothelial damage [56, 57]. The release of microparticles from CNIs-exposed

endothelium is reported to activate the complement alternative pathway, causing endothelial cell damage [11]. Our analysis of case reports and series revealed that tapering down the CNIs-dose is the most common strategy, followed by shifting to other CNIs or sirolimus. However, the graft survival rate remains unfavorable and is reported to be 28.6% [45].

C4d is an indicator of an activated classical complement pathway, and linear C4d staining in the peritubular capillary is a key diagnostic feature of AMR [58]. A retrospective study involving 59 individuals with kidney allograft TMA revealed that those with peritubular capillaries linear C4d staining had a nearly 4-fold higher incidence of TMA than did those without C4d staining (C4d+ vs. C4d−: 13.6% vs. 3.6%) [16]. However, the 2-year graft loss rate was similar between the two groups, with nearly 40% in each group. In contrast, another study of 74 individuals with kidney allograft TMA found that those with C4d+ had a higher graft loss rate than those without C4d staining (55.6% vs. 30%) [46]. Nevertheless, C4d deposits are not uncommon in kidney allograft TMA, particularly in the glomeruli. In a study of 32 individuals with renal TMA, which included 12 kidney allograft sections and 30 native kidney sections, C4d deposits were detected in 88% of TMA cases, while C5b-9 deposits were detected in 76% of TMA cases [58]. Notably, of the 12 kidney allograft TMA sections, C4d deposits were present in 75% of glomeruli, and C5b-9 deposits were present in 50%. The study showed that C4d and C5b-9 are common denominators in kidney allografts in patients with TMA and suggested that anti-terminal complement therapy may be beneficial in these patients. The management strategy for *de novo* TMA includes identifying and removing triggers, PE, and eculizumab therapy. However, the efficacy of PE in *de novo* TMA has not been fully established, owing to the heterogeneity of its etiologies. Although PE plays an important role in managing thrombotic thrombocytopenic purpura, it is also used as a bridging therapy to eculizumab in patients with aHUS. In a single-arm retrospective cohort study conducted in the United States in 2003 (pre-eculizumab era) to examine the efficacy of PE in 29 kidney allograft recipients with TMA, 6 (20%) of them suffered from graft loss. Among the 10 individuals who had histological acute rejection, 6 (60%) suffered from graft loss within 1 year [59]. In a comparative study conducted in 2010 (pre-eculizumab era), which aimed to explore the efficacy of PE with concurrent intravenous immunoglobulin in 33 kidney allograft recipients with *de novo* TMA and concomitant AMR, the graft loss rate was not different between those with and without PE + intravenous immunoglobulin (35% vs. 50%) [16]. In a single-arm retrospective cohort study conducted in Spain in 2020, which comprised 16 kidney allograft recipients with *de novo* aHUS, only 2 of 13 individuals who underwent PE achieved complete hematological and renal recovery. Eight individuals received rescue eculizumab owing to no or partial renal response to PE, and six (75%) of them achieved complete hematological and renal recovery after receiving rescue eculizumab [60]. Finally, according to our analysis of case reports and series, 52% of 48 individuals with *de novo* TMA who underwent PE achieved a renal response, whereas 83% of 18 individuals with *de novo* TMA receiving eculizumab achieved a renal response.

This study has few limitations. First, owing to the low prevalence of *de novo* TMA, there was a lack of randomized controlled trials, and the meta-analysis results were based on single-arm or observational cohort studies. Additionally, there was a wide variance in the number of cases among the enrolled studies. Second, there was heterogeneity in our meta-analysis of TMA incidence, which may be mainly attributed to the influence of the following three studies: a study by Tasaki et al., which included ABO-incompatible kidney allograft recipients and had a high TMA incidence; a study by Nava et al., which included older kidney allograft recipients and had a high TMA incidence; and a study by Zarifian et al., which had a higher proportion of individuals with chronic transplant nephropathy and was conducted in 1999 when all recipients were receiving cyclosporine for immunosuppression [17, 44, 53]. Finally, 9 of the 15 studies included in our TMA incidence meta-analysis and 5 of 8 studies included in our graft survival meta-analysis were conducted before 2013 (pre-eculizumab era). This may have led to a bias in both TMA incidence and graft survival rates in the current eculizumab era.

To conclude, the incidence of *de novo* TMA in patients with kidney allografts was 3.20%, whereas the incidences of systemic TMA and renal-limited TMA were 1.38% and 2.80%, respectively. The overall graft loss rate was 33.79%. These findings highlight the rare and complex nature of *de novo* TMA in kidney allograft recipients, which is associated with poor graft outcomes. Our study provides valuable insights into the incidence and graft outcomes.

AUTHOR CONTRIBUTIONS

H-YC conceived of the presented idea. S-HW and C-YHu verified the analytical methods and C-YHu performed data analysis. C-YHu and C-YHs wrote the manuscript with support from H-YC. C-YHs and H-YC investigated and supervised the findings of this work. 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.2024.12168/full#supplementary-material

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ORIGINAL RESEARCH
published: 24 January 2024
doi: 10.3389/ti.2024.12192

CMV Infection and Lymphopenia: Warning Markers of *Pneumocystis* Pneumonia in Kidney Transplant Recipients

Isabelle Eberl[1], Christine Binquet[2,3], Adrien Guilloteau[4], Mathieu Legendre[5], Frederic Dalle[6,7], Lionel Piroth[1,2,3], Claire Tinel[5,8†] and Mathieu Blot[1,2,3,9†]*

[1]Department of Infectious Diseases, Dijon-Bourgogne University Hospital, Dijon, France, [2]CHU Dijon-Bourgogne, INSERM, Université de Bourgogne, CIC 1432, Module Épidémiologie Clinique, Dijon, France, [3]LabEx LipSTIC, University of Burgundy, Dijon, France, [4]Côte d'Or Haematological Malignancy Registry (RHEMCO), Dijon-Bourgogne University Hospital, Dijon, France, [5]Department Nephrology and Kidney Transplantation, Dijon-Bourgogne University Hospital, Dijon, France, [6]Department of Parasitology-Mycology, Dijon Bourgogne University Hospital, Dijon, France, [7]UMR PAM Université de Bourgogne Franche-Comté (UBFC), AgroSup Dijon, Équipe Vin, Aliment, Microbiologie, Stress, Groupe Interactions Candida-muqueuses, Dijon, France, [8]Université Bourgogne Franche-Comté (UBFC), EFS BFC, Inserm UMR1098, RIGHT, Besançon, France, [9]Lipness Team, INSERM Research Centre LNC-UMR1231 and LabEx LipSTIC, University of Burgundy, Dijon, France

Pneumocystis pneumonia (PcP) remains life-threatening in kidney transplant recipients (KTR). Our study investigated risk factors one-year before PcP. We conducted a monocentric, case-control study including all KTR at the Dijon University Hospital (France) with a diagnosis of PcP between 2005 and 2022 (cases), and matched control KTR with no history of PcP (3 controls/case). Among all 1,135 KTR, 57 cases (5%) and 169 matched-controls were included. PcP was associated with 18% mortality. Compared to controls, cases were older, with a higher immunological risk, and CMV infection was more frequent in the year preceding the occurrence of PcP (23% vs. 4%; $p < 0.001$). As early as 1 year before PcP, lymphocyte counts were lower and serum creatinine levels were higher in cases, but immunosuppressive regimens were not significantly different. Multivariable analysis identified lymphocyte count, serum creatinine level, being treated by immunosuppressive therapy other than anti-rejection drugs, and CMV infection in the year preceding the time PcP as independently associated with the occurrence of PcP. PcP was associated with an increased risk of subsequent chronic rejection (27% vs. 3%; $p = 0.001$) and return to dialysis (20% vs. 3%; $p = 0.002$). The occurrence of CMV infection and a low lymphocyte count could redefine the indications for continuation or reinitiation of anti-*Pneumocystis* prophylaxis.

Keywords: kidney transplantation, pneumonia, lymphopenia, pneumocystis, CMV

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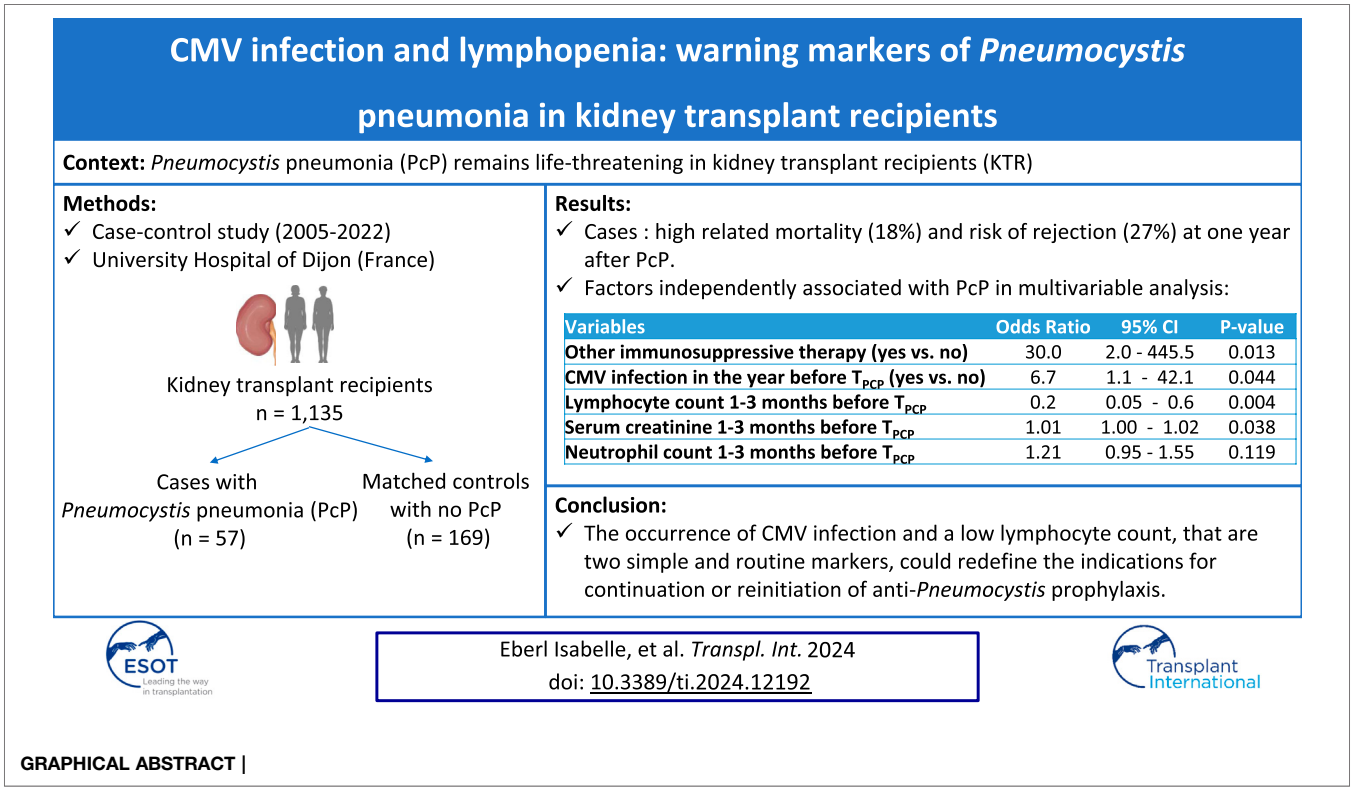
***Correspondence**
Mathieu Blot,
mathieu.blot@chu-dijon.fr

†These authors have contributed equally to this work

***Received:** 05 October 2023*
***Accepted:** 11 January 2024*
***Published:** 24 January 2024*

Citation:
Eberl I, Binquet C, Guilloteau A, Legendre M, Dalle F, Piroth L, Tinel C and Blot M (2024) CMV Infection and Lymphopenia: Warning Markers of Pneumocystis Pneumonia in Kidney Transplant Recipients. Transpl Int 37:12192. doi: 10.3389/ti.2024.12192

Abbreviations: CMV, Cytomegalovirus; HLA, Human Leukocyte Antigen; KTR, Kidney Transplant Recipients; PcP, *Pneumocystis* pneumonia; rATG, recombinant anti-lymphocyte depleting antibodies; TIS, Total Immunosuppression.



GRAPHICAL ABSTRACT |

INTRODUCTION

Infections are the third leading cause of death following kidney transplantation [1], and *Pneumocystis* pneumonia (PcP) is one of the most severe opportunistic causes. *Pneumocystis* infects 0.3%–2.6% of kidney transplant recipients (KTR), with a mortality rate reaching 14% in patients admitted to the ICU [2] and an increased risk of transplant loss in surviving patients [3]. The Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend universal initial PcP prophylaxis with cotrimoxazole for the first 3–6 months after kidney transplantation [4], while the American Society of Transplantation recommends prophylaxis for 6–12 months [5]. However, whether this prophylaxis should be prolonged or resumed in certain high risk situations remains unclear [5]. These recommendations have changed the epidemiology, and now most reports involve late post-transplant recipient PcP [6–9].

Pneumocystis infection elicits T-cell mediated responses including T helper (Th) 1, Th2 and Th17 responses [10], and lymphopenia has been frequently reported as an independent risk factor for PcP [6, 11–13]. Kaminski et al proposed targeted prophylaxis based on simple criteria such as chronic lymphopenia (i.e., < 1,000/μL) [6]. However, the factors that contribute to lymphopenia are not fully understood. Some studies showed that cumulative immunosuppression, corticosteroids pulses, or treated transplant rejection episodes are independent risk factors for PcP, with some conflicting results depending on the cohorts [3, 6, 11–19]. In addition, most study data are collected more than 1 year before the PCP. Identifying clinical and biological biomarkers in the year preceding the PcP could help guide clinicians regarding PcP prophylaxis.

Thus, the main objective of our study was to identify risk factors associated with PcP after kidney transplantation, with a particular focus on events occurring in the year prior PcP. The secondary objective was to study how PcP affects kidney transplant and patient outcomes.

MATERIAL AND METHODS

Study Design

We conducted a retrospective, case-control study at the University Hospital of Dijon (France) (1,200 beds). We included all KTR aged 18 years or older, with post-transplant PcP diagnosed between 2005 and 2022 (cases) and 3 matched-control KTR with no history of PcP during their follow-up (controls). Control patients were matched on the date of the active transplantation (±6 months) and selected if they had a functioning transplant at the time of PcP and with a minimal 1-year follow-up after the date of the matched PCP case. We used a simple matching strategy, using the date of transplantation to ensure a homogeneous clinical care and an equal distribution of exposure among cases and controls. Further matching variable candidates (such as induction therapy or lymphopenia) were not retained as the association of matched variables with outcomes

TABLE 1 | Baseline characteristics of cases and controls.

	Missing Data	Controls *n* = 169	Cases *n* = 57	*p*-value
Demographic data				
Age (years) at T_{PCP}, median (IQR)	0	57 (43–65)	61 (57–66)	0.043
Male sex, n (%)	0	70 (41)	28 (49)	0.282
Comorbidities at T_{PCP}				
Chronic heart disease, n (%)	0	36 (21)	17 (30)	0.197
Diabetes, n (%)	0	36 (21)	16 (28)	0.272
Chronic pulmonary disease, n (%)	0	6 (4)	3 (5)	0.566
Chronic liver disease, n (%)	0	12 (7)	2 (4)	0.43
Solid tumor, n (%)	0	26 (15)	10 (18)	0.751
Hematological cancer, n (%)	0	2 (1)	2 (4)	0.272
Cancer chemotherapy within a year before T_{PCP}, n (%)	0	2 (1)	2 (4)	0.272
Primary underlying nephropathy	0			0.909
- Vascular, n (%)		11 (7)	3 (5)	
- Tubulo-interstitial, n (%)		27 (16)	4 (7)	
- Glomerular, n (%)		60 (36)	26 (46)	
- Polycystic kidney, n (%)		43 (25)	13 (23)	
- Others, n (%)		10 (6)	3 (5)	
- Unknown, n (%)		18 (11)	8 (14)	
Transplant data				
First transplant, n (%)	0	144 (85)	49 (86)	0.913
Age (years) at active transplant, median (IQR)	0	51 (38–60)	56 (47–60)	0.04
Living donor transplant, n (%)	2	21 (12)	5 (9)	0.469
High immunological risk, n (%)	6	70 (42)	31 (60)	0.029
Anti-HLA antibodies	9			0.107
- Transitional, n (%)		13 (8)	9 (18)	
- Constant, n (%)		52 (32)	20 (40)	
Anti-HLA antibodies at the time of transplantation, n (%)	9	56 (34)	23 (46)	0.125
Antibodies to the donor at the time of transplantation, n (%)	9	1 (1)	0	NA
CMV Status D+/R-	0	47 (28)	12 (21)	0.403
Induction therapy				
Polyclonal antibodies, n (%)	2	114 (67)	37 (69)	0.851
Anti-IL2-R, n (%)	3	50 (30)	16 (30)	1
Other induction therapy, n (%)	4	3 (2)	5 (9)	0.018
Initial immunosuppressive regimen				
Corticosteroids, n (%)	1	169 (100)	55 (98)	0.561
Calcineurin inhibitors, n (%)	1	156 (92)	49 (88)	0.234
- Ciclosporin, n (%)	1	119 (70)	34 (61)	0.208
- Tacrolimus, n (%)	1	38 (22)	14 (25)	0.703
Antimetabolites, n (%)	1	168 (99)	54 (96)	0.24
- Azathioprine, n (%)	1	12 (7)	3 (5)	0.885
- Mycophenolic acid, n (%)	1	156 (92)	51 (91)	1
m-TOR inhibitors, n (%)	1	4 (2)	4 (7)	0.097
Prophylaxis against PCP				
Cotrimoxazole, n (%)	0	81 (48)	26 (48)	0.696
Atovaquone, n (%)		2 (1)	0 (0)	1
Prophylaxis duration (month), median (IQR)		8.2 (5.3–15.8)	6.0 (4.6–8.4)	0.027
Infectious and immunological complications before T_{PCP}				
Acute rejection, n (%)	1	23 (14)	8 (14)	0.482
Acute rejection in the year before T_{PCP}, n (%)	1	3 (2)	2 (4)	0.448
CMV infection, n (%)	1	26 (15)	21 (37)	<0.001
CMV infection in the year before T_{PCP}, n (%),	1	7 (4)	13 (23)	<0.001
Other infection, n (%)	7	41 (24)	21 (37)	
- Bacteriemia, n (%)		7 (4)	2 (4)	0.848
- Urinary tract infection (including pyelonephritis), n (%)		37 (22)	19 (33)	0.298
- Respiratory infection, n (%)		3 (2)	2 (4)	0.448
- Other infection in the year before T_{PCP}, n (%)		8 (5)	4 (7)	0.42
Immunosuppressive regimen at T_{PCP}, n (%)				
Corticosteroids, n (%)	0	161 (95)	57 (100)	0.208
Ciclosporin, n (%)	0	76 (45)	25 (44)	0.936
Tacrolimus, n (%)	0	52 (31)	16 (28)	0.717

(Continued on following page)

TABLE 1 | (*Continued*) Baseline characteristics of cases and controls.

	Missing	Controls	Cases	*p*-value
	Data	*n* = 169	*n* = 57	
m-TOR inhibitors, n (%)	0	22 (13)	14 (25)	0.041
Azathioprine, n (%)	0	14 (8)	8 (14)	0.262
Mycophenolic acid, n (%)	0	143 (85)	43 (77)	0.144
Corticosteroid pulses in the year before T_{PCP}, n (%)	0	5 (3)	4 (7)	0.177
Other immunosuppressive therapy[a], n (%)	0	3 (2)	5 (9)	0.025
Immunosuppression score				
Modified Vasudev total score, median (IQR)	0	5 (4–7)	5 (4–6.5)	0.822
TIS score, median (IQR)	0	22.5 (17.5–27.5)	25 (20–27.5)	0.17
Biological findings 1–3 months before TPCP				
Leukocytes (/mm³), median (IQR)	17	6.1 (4.7–7.4)	5.7 (4.4–8.1)	0.407
Neutrophils (/mm³), median (IQR)	20	4.2 (3.2–5.1)	3.9 (3.1–5.7)	0.032
Lymphocytes (/mm³), median (IQR)	19	1.1 (0.7–1.6)	0.7 (0.4–1)	0.001
Monocytes (/mm³), median (IQR)	20	0.6 (0.4–0.7)	0.5 (0.4–0.6)	0.137
Serum creatinine (µmol/L), median (IQR)	13	128 (103–155)	175 (133–225)	0.001
Calcemia (mmol/L), median (IQR)	18	2.4 (2.3–2.5)	2.4 (2.2–2.5)	0.536

[a]*Plasma exchanges (*n *= 2), anti-CD20 (*n *= 2), Sirolimus as anti-cancer therapy (*n *= 1), cyclophosphamide (*n *= 1), OKT3 (*n *= 1), azathioprine for an ulcerative colitis (*n *= 1).*
Abbreviations: CMV, Cytomegalovirus; IQR, Interquartile range; HLA, Human Leukocyte Antigen; mTOR, Mammalian Target of Rapamycin; PCP, Pneumocystis pneumonia; T_{PCP}, Time of PCP; TIS, total immunosuppression score.

cannot be examined. We defined T_{PcP} as the day of the microbiological confirmation of the PcP for each case and as the reference matched day from active transplantation for the matched control.

The criteria for PcP were (i) clinical signs of pneumonia (at least 2 signs among cough, sputum, chest pain, dyspnea, temperature >37.8°C or <36°C, crackles), and (ii) lung infiltration on chest x-ray or CT-scan, and (iii) a positive result on *Pneumocystis jirovecii* real-time polymerase chain reaction (PCR) testing [MycoGENIE® P. jirovecii Kit ADEMTECH, Bordeaux, France] or direct immunofluorescence testing, or direct examination (Gomori-Grocott and May-Grünwald-Giemsa staining) of respiratory microbiological samples (sputum, tracheal aspirate, broncho-alveolar lavage fluid (BALF)). A diagnosis of PcP was not retained in case of a more likely diagnosis and if the curative treatment for PcP was not pursued.

First, we identified cases with the International Classification of Diseases (ICD)-10 codes in the French hospital discharge database using codes associated with kidney transplantation (Z940) and *Pneumocystis* infection (B59). These data were cross-referenced with those of the Nephrology Department of the Dijon University Hospital to identify potential missing cases. The accuracy of the diagnosis was checked in individual medical files by a trained clinician and patients were not included if they did not meet the inclusion criteria. If a patient presented several episodes of PcP, only the first was considered.

Data Collection

Data from cases and controls were collected from medical records. A high immunological risk was defined as >1 allograft transplantation and/or positive anti-human leukocyte antigen (HLA) antibodies (before or on the day of transplantation). Cytomegalovirus (CMV) infection was defined as a positive whole blood CMV quantitative nucleic acid testing for patients from 2005 or as a positive CMV antigenemia (CMV-pp65 antigen) before that date, in accordance with the evolving diagnostic strategy in our center.

In the year before T_{PCP}, at several time points (6 months-1 year, 3–6 months, 1–3 months before T_{PcP}, and at T_{PcP}), we collected biological data, immunosuppressive regimen including mycophenolate mofetil (MMF) and azathioprine doses, and trough levels [T_0] for cyclosporine, tacrolimus, and mammalian target of rapamycin inhibitors (mTORi), and occurrence of infections. Clinical and radiological signs, and treatments received for PcP were collected for each case. One year after T_{PcP}, we collected immune status and renal function (serum creatinine levels and estimated glomerular filtration rate [eGFR] according to the chronic kidney disease-epidemiology collaboration [CKD-EPI]). We defined allograft failure as return to permanent dialysis. Each transplant rejection was histologically proven by allograft biopsy and immunohistological examination according to the Banff classification.

Immunosuppressive Regimen and Scoring Therapy-Related Immunosuppression

Immunosuppressive therapy strategy in our center is detailed in the **Supplemental Methods**.

We used a modified version of the score by Vasudev et al [20] to quantify the impact of immunosuppressive therapies, using the concept of an immunosuppression unit and based on the drug trough level (T0) instead of drug doses (**Supplemental Methods**). We also established the TIS (Total ImmunoSuppression) score, to

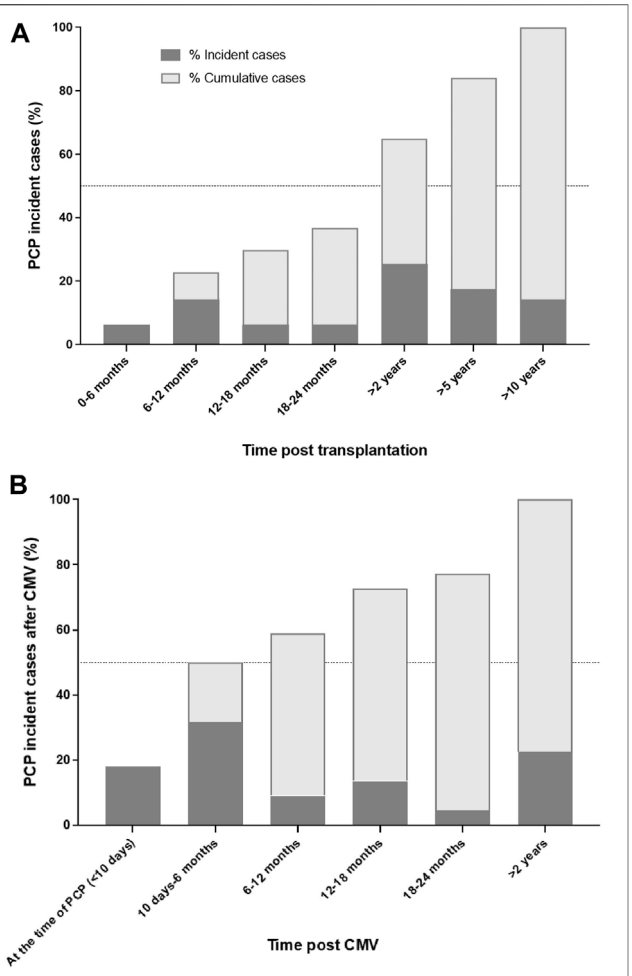


FIGURE 1 | Proportion of incident and cumulative *Pneumocystis* pneumonia cases according time post transplantation **(A)** and among cases with CMV infection in the year before *Pneumocystis* pneumonia **(B)**. Abbreviations: CMV, cytomegalovirus; PCP, *Pneumocystis* pneumonia.

take into account immunosuppressive therapies other than maintenance treatment, including corticosteroid pulses, chemotherapy treatment for solid cancer or hematological disease received in the year before T_{PCP} (**Supplemental Methods**). In our center, patients were treated with post-transplantation prophylaxis (oral cotrimoxazole or atovaquone if intolerance) but the duration of treatment was left to the physician's discretion.

Ethics

The study protocol and data collection are in accordance with French (Information Technology and Freedom Law n°78-17 of 6 January 1978) and European (GRPD EU 2016/679) good practice recommendations on data protection and patient information (Commitment of compliance MR004 n°2210228 of 3 December 2018), with written patient consent not being required for this non-interventional study. All personnel involved in organ donation and transplantation at the University Hospital of Dijon commit to respect the objectives, principles and recommendations of the Istanbul Declaration against organ trafficking and tourism in organ transplantation.

Statistical Analysis

Quantitative values were expressed by their medians and interquartile ranges (IQR), and qualitative variables by their level's size and percentages. Initial univariable analyses were performed using a conditional logistic regression on all available patient characteristics. In order to identify the variables independently associated with PcP, a conditional logistic regression was estimated with all the variables associated with the occurrence of PcP with a p-value <0.2 in univariable analysis and then a backward selection was performed using AIC. Patients with missing data were excluded. The log-linearity hypothesis for continuous variable was assessed by comparing two models, with and without the adjunction of a quadratic term, using the Likelihood Ratio Test (LRT). Results were expressed as odds ratios (OR) with 95% confidence intervals (95%CI). Stacked bar charts were plotted to represent the distribution of cases and controls according to the lymphocyte count and the occurrence of a CMV infection within the year before the time of *Pneumocystis* pneumonia. A p-value <0.05 was considered statistically significant. Analyses were performed using R (v4.1.3) and GraphPad Prism (v.9.1.1) software.

RESULTS

Demographic and Clinical Characteristics of the Study Population

Among 1,135 kidney transplant patients, 57 patients (5%) developed PcP after transplantation between 2005 and 2022, and were considered as cases. They were matched with 169 control renal transplant patients with no history of PcP. Not all cases could be matched to 3 controls (**Supplementary Figure S1**). Following active transplantation, PcP occurred after a median time of 40 months (IQR 13–92) and, after prophylaxis discontinuation if applicable, a median time of 18 months (4–34).

Cases were significantly older than controls at T_{PcP} but the sex ratio and comorbidity profile did not differ between groups (**Table 1**). Cases had a higher immunological risk, but induction and maintenance therapies were comparable. However, cases had received significantly more other adjuvant immunosuppressive therapies prior to their active transplantation (i.e., anti-CD20 or anti-CD3 antibodies or plasma exchange). Anti-*Pneumocystis* prophylaxis was administered in half of the patients (including 100% of patients transplanted after 2007), with no difference between the two groups, but with a shorter prescription in cases compared to controls (6.0 (4.6–8.4) vs. 8.2 (5.3–15.8) months; $p = 0.027$). Acute rejection was reported in 14% of patients, with no significant difference between the two groups.

Cases were significantly more likely to present CMV infection than controls (37% vs. 15%; $p < 0.001$), mainly in the year before T_{PcP} (23% vs. 4%; $p < 0.001$). Among cases with a CMV infection, 17/22 (77%) developed PcP in the 2 years following the infection (**Figure 1B**).

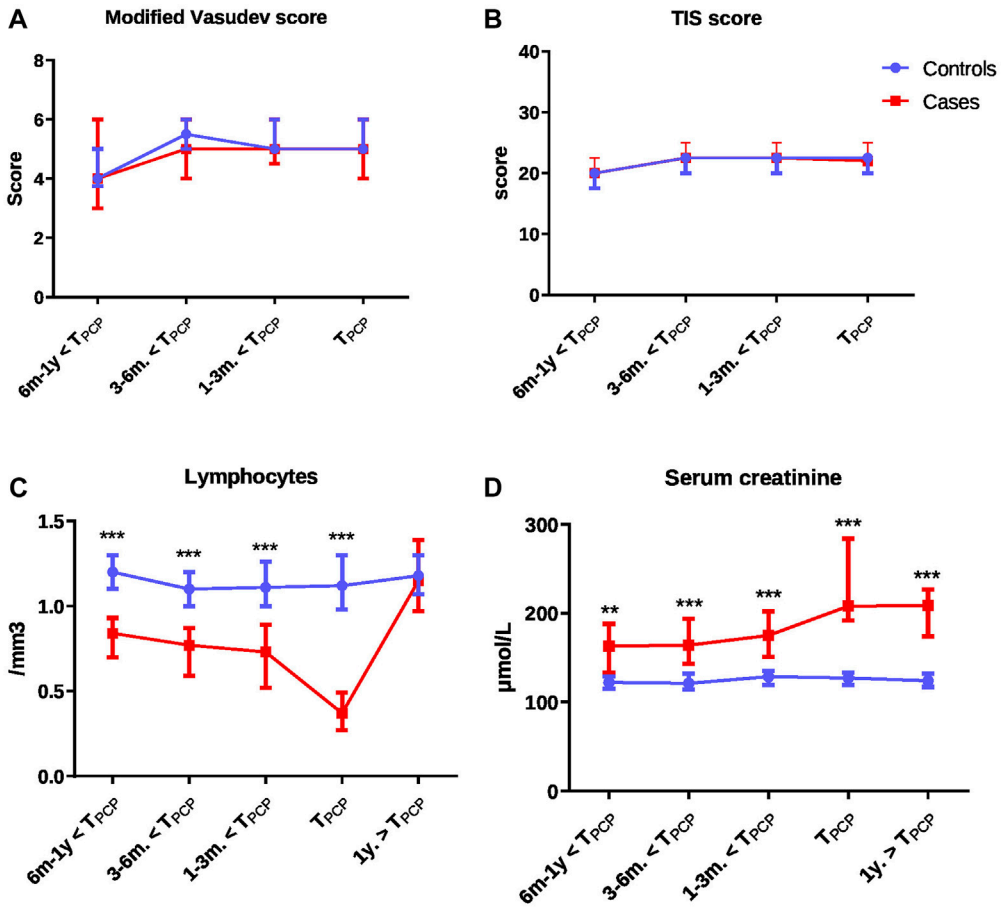


FIGURE 2 | Comparison of immunosuppression scores and biological values over time from 1 year before to 1 year after the time of PCP between cases and controls: modified Vasudev score **(A)**, Therapeutic Immunosuppression (TIS) score **(B)**, lymphocytes **(C)** and serum creatinine levels **(D)**. Comparisons were performed with Wilcoxon test for matched data for each time-point with false discovery rate to correct for multiple comparisons. All panels: $^{*}p < 0.05$ to <0.01; $^{**}p < 0.01$ to <0.001; $^{***}p < 0.001$. Abbreviations: TIS score, therapeutic immunosuppression score; PCP, *Pneumocystis* pneumonia; T_{PCP}, time of PCP.

TABLE 2 | Multivariable logistic regression analysis for factors associated with Pneumocystis pneumonia.

Variables	Odds ratio	95% CI	*p*-value
Other immunosuppressive therapy (yes vs. no)	30.006	2.021–445.451	0.013
CMV infection in the year before T_{PCP} (yes vs. no)	6.663	1.054–42.121	0.044
Lymphocyte count 1–3 months before T_{PCP}	0.174	0.054–0.563	0.004
Serum creatinine 1–3 months before T_{PCP}	1.009	1.000–1.017	0.038
Neutrophil count 1–3 months before T_{PCP}	1.214	0.951–1.549	0.119

Abbreviations: PCP, Pneumocystis pneumonia; T_{PCP}, Time of PCP.

The immunosuppressive regimen that was being administered at T_{PcP} did not differ between cases and controls, with the exception of m-Tor inhibitors, which were significantly more prescribed for cases. However, both immunosuppression scores TIS and modified Vasudev total scores did not significantly differ between cases and control at T_{PcP} and in the year before (**Table 1**; **Figures 2A, B**; **Supplementary Table S1**).

Lymphocyte counts were significantly lower and neutrophil counts and creatinine levels higher in cases compared to controls (**Table 1**). The differences in lymphocyte count and creatinine levels were present as early as 1 year before T_{PCP} (**Figure 2**; **Supplementary Table S2**).

Factors Independently Associated With the Occurrence of PcP

Due to missing data mainly on biological findings, the multivariable model was estimated on 44 cases and 157 controls. It showed that

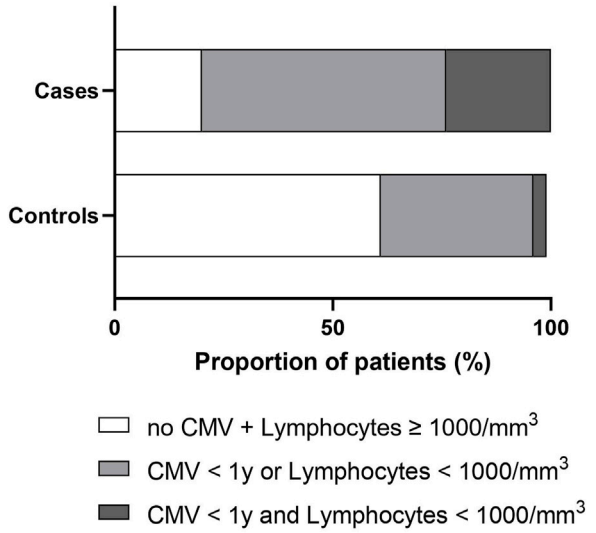


FIGURE 3 | Stacked bar charts showing the distribution of cases and controls according to the lymphocyte count and the occurrence of a CMV infection within the year before the time of *Pneumocystis* pneumonia (whole population $n = 226$). Cases and controls were represented as having no CMV infection within the year and lymphocytes count ≥1,000/mm^3 (white), CMV infection within the year or lymphocyte count <1,000/mm^3 (light gray), and CMV infection within the year and lymphocytes count <1,000/mm^3 (dark gray). Lymphocyte count was measured 3 months before the time of *Pneumocystis* pneumonia. Abbreviations: CMV, cytomegalovirus.

factors independently associated with PcP were: being treated by immunosuppressive therapy other than anti-rejection drugs, CMV infection in the year before T_{PcP}, lymphocyte count and creatinine levels 1–3 months before T_{PcP} (**Table 2**). Thus we observed that 24% of cases had a lymphocyte count <1,000/mm^3 and CMV infection in the year before T_{PcP}, compare with only 3% of control patients (**Figure 3**; **Supplementary Table S4**). In a sensitivity analysis in patients who systematically received anti-*Pneumocystis* prophylaxis after renal transplantation ($n = 104$), we observed that 32% of cases had a lymphocyte count <1,000/mm^3 and a CMV infection in the year before T_{PcP}, compared with only 4% of control patients (**Supplementary Table S5**). No deviation from the hypothesis of log-linearity was identified for continuous variable (age at T_{PcP}, neutrophils, lymphocytes, serum creatinine).

Outcomes Following PCP

At 1 year after T_{PcP}, we observed 12 (21%) deaths, including 10 (18%) related to PcP in cases and no deaths in control patients. In surviving patients, cases were more likely to have high creatinine levels, transplant rejection and return to dialysis 1 year after T_{PcP} (**Table 3**).

DISCUSSION

Our case-control study involving KTR yielded 2 main results. First, PcP occurred in 5% of KTR followed in our center and was associated with high related mortality (18%), an increased risk of subsequent chronic rejection, and a return to dialysis. Secondly, several factors were independently associated with PcP, including being treated by immunosuppressive therapy other than anti-rejection drugs, CMV infection in the year before T_{PcP}, low lymphocyte count, and high creatinine levels. Having a lymphocyte count <1,000/mm^3 and/or a CMV infection are two main factors associated with the occurrence of PcP within the year.

PcP is an opportunistic infection that occurs in patients suffering from CD4$^+$ T cell response deficiency, which is the case in KTR, who are thus eligible for PcP prophylaxis [4]. In our cohort, 5% developed PcP, which is within the range reported in other cohorts [2, 6, 16, 19]. However, the epidemiology has changed over the last 30 years as a result of updated recommendations and the systematic use of cotrimoxazole, leading to an increase in the proportion of late-onset PcP. It should be noted that PcP occurred in the median time of 40 months, i.e., well after the end of the theoretical prophylaxis recommendation. In this cohort, only half of patients, particularly the most recently included patients, received early prophylaxis with cotrimoxazole. By matching cases and controls on the date of the active transplantation, it is therefore not possible to study the effect of the prophylaxis variable (presence/absence) on the occurrence of PcP. However, the duration of prophylaxis was shorter for cases, suggesting that extending or reinitiating PcP prophylaxis could benefit some patients.

To identify such patients, several associated/risk factors for susceptibility to PcP have been previously identified, but with some discrepancies between studies [3, 6, 11–19]. In addition, events occurring during the year preceding PcP could be informative. As expected, cases were older than controls at the time of PcP, with frailty conferring a higher age-related risk of infection [21]. Cases were also more likely to have a higher creatinine level preceding PcP, supporting the concept of kidney impairment-associated immunosenescence [22]. They were more often considered as having a high immunological risk, raising the possibility of more likely transplant rejection. However, the proportion of acute rejection was similar in cases and controls (14% in each group).

We observed that CMV infection was independently associated with PcP, mainly in the year preceding T_{PcP}. This association has been reported in several studies [14–16], but not all [6]. In the meta-analysis by Hosseini-Moghaddam et al., CMV infection significantly increased the risk of post-transplant PcP (OR: 3.30, 95% CI: 2.07–5.26). In addition, Lee et al. showed that PcP and CMV co-infection is associated with an increased clinical severity and worse clinical outcomes [23]. The causal link between CMV infection and the occurrence of PcP cannot be asserted, but pathophysiological assumptions can be proposed. First, stronger immunosuppression could be responsible for both opportunistic infections. We observed that cases were more likely to have a low lymphocyte count, as described in other work [6, 12, 13, 18]. The intensity of cumulative immunosuppression remains a difficult variable to quantify. However, we observed no significant difference in the choice of anti-rejection molecules or in the intensity of therapeutic

TABLE 3 | Univariable comparisons of patient outcomes at 1 year after *Pneumocystis* pneumonia.

	Missing	Controls	Cases	
	Data	*n* = 169	*n* = 57	*p*-value
Mortality, n (%)	0	0	12 (21)	<0.001
PCP-related mortality, n (%)	0	0	10 (18)	<0.001
Leukocyte count (/mm^3), median (IQR)	32	6 (5.1–7.5)	5.8 (4.9–8.1)	0.352
Neutrophil count (/mm^3), median (IQR)	33	4 (3.2–5)	3.7 (3–5.4)	0.703
Lymphocyte count (/mm^3), median (IQR)	32	1.2 (0.9–1.6)	1.2 (0.8–1.5)	0.483
Monocyte count (/mm^3), median (IQR)	34	0.6 (0.5–0.7)	0.6 (0.5–0.8)	0.807
Serum creatinine levels (µmol/L), median (IQR)	28	124 (105–159)	209 (146–252)	0.001
GFR (mL/min)[a], median (IQR)	32	54 (38–70)	27 (23–42)	0.001
Proteinuria[b] (g/g), median (IQR)	46	0.28 (0.17–0.51)	0.48 (0.2–1.2)	0.486
Transplant rejection, n (%)	22	5 (3)	12 (27)	0.001
Transplant rejection with need for dialysis, n (%)	21	5 (3)	9 (20)	0.002

[a]*According o the CKD-EPI, formula.*

[b]*Proteinur/creatinuria ratio = Uprot [mg/L] x 8,84/Ucreat [µmol/L]) Abbreviations: GFR, Glomerular filtration rate; IQR, Interquartile range; T_{PCP}, Time of* Pneumocystis *pneumonia.*

immunosuppression, as assessed by modified Vasudev and TIS scores. Only mTOR inhibitors were more prescribed in cases compared to matched controls, as previously reported [6, 24]. As we discuss above, this association can be explained by the immunosuppressive effect of mTOR inhibitors but without ruling out the possibility of having included mTOR inhibitor-induced interstitial lung disease in some cases [24]. Furthermore, the administration of steroid pulses were not significantly associated with PcP, unlike in the study by Kaminski et al. [6, 13, 25]. However, this result should be interpreted in the light of a low frequency of acute rejection in the cohort. Other immunosuppressive therapies were more frequently prescribed in cases, mainly anti-cancer chemotherapy or anti-C5 therapies, highlighting the role of the cumulative immunosuppressive burden between transplantation and PcP. Secondly, cases were more likely to have impaired renal function, even when adjusted for age. This poorer renal function may reflect the altered terrain in which opportunistic infection occurs more frequently, as the incidence of infections increases linearly as renal function deteriorates [26]. Finally, we observed that for 3⁄4 of patients, PcP occurred within 2 years after CMV infection. CMV infection by itself can induce cellular immunodepression, through mobilization of cellular T immune defenses and secondary immunoparalysis. This hypothesis is reinforced by the results of an *in vivo* study in mice inoculated with CMV and *Pneumocystis*, showing that CMV infection induces a decrease in lung cells expressing MHC class II, and in activated T-CD4 lymphocytes in lymphoid organs and the alveolar compartment, associated with a defect in *Pneumocystis* clearance [27].

Our study confirms that PcP is associated with a poor prognosis in KTR [2, 28, 29], with an attributable mortality rate of 18% and transplant loss in 20% of surviving patients. It is therefore crucial to better understand the risk factors associated with this infection in order to define at risk-situations where anti-*Pneumocystis* prophylaxis is highly recommended. Global management of PcP involves several nephrotoxic interventions (high dose cotrimoxazole, contrast agent. . .) and the tapering of immunosuppressive regimen that may further elicit chronic rejection, contribute to the decline in transplant function and precipitate the return to dialysis.

In the end, we identified simple and routine biomarkers (serum creatinine, lymphocyte count) and a frequent opportunistic infectious event (CMV infection) that were associated with the occurrence of PcP. Among cases who received initial anti-*Pneumocystis* prophylaxis, 22 of 25 (88%) infections could have been prevented if prophylaxis had been restarted or continued in the presence of CMV infection and/or lymphopenia <1,000/mm^3. This strategy would have been associated with excess treatment in of 23 out of 79 controls (29%), but is supported by the excellent tolerability of such low doses in real practice, the low cost of the drug, and the good compliance of patients.

The limitations of this study are related to its retrospective and monocentric nature. Some data are missing, even if this number is very limited for most variables. It is possible that over this period of 17 years, unmeasured changes in clinical practice may have influenced the risk to contract PCP, but such difference have been minimized by the controls pairing strategy. We did not provide CD4 and CD8 lymphocytes count since lymphocyte immunophenotyping has only become part of routine follow-up in more recent years and CD4 counts are thus not available for all patients. However, in the study of Kaminsky et al. lymphopenia was identified as the most significantly associated lymphocytic marker of PCP [6]. Patient prognosis could only be partially evaluated and is potentially biased insofar as the matching imposed a follow-up time for controls that was at least equal to that of the index case plus 1 year. Some patients had not received anti-*Pneumocystis* prophylaxis, but our sensitivity analysis confirmed the same findings in the subgroup of patients who received prophylaxis.

CONCLUSION

PcP is associated with high mortality and transplant loss in patients who have undergone a kidney transplant. We identified factors that were independently associated with

PcP, including immunosuppressive therapy other than anti-rejection drugs, CMV infection in the year before T_{PcP}, low lymphocyte count and high serum creatinine levels. These risk factors remain unchanged with or without anti-*Pneumocystis* prophylaxis. Based on these results and previous literature, the occurrence of CMV infection and/or lymphopenia <1,000/mm^3 could redefine the indications for continuation or reinitiation of anti-*Pneumocystis* prophylaxis, which is an inexpensive and well-tolerated treatment.

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 study protocol and data collection are in accordance with French (Information Technology and Freedom Law n°78-17 of 6 January 1978) and European (GRPD EU 2016/679) good practice recommendations on data protection and patient information (Commitment of compliance MR004 n°2210228 of 3 December 2018). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board also waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin as this was a non-interventional study.

AUTHOR CONTRIBUTIONS

Concept and design: IE, CB, AG, CT, and MB. Recruitment of patients: IE, CT, and MB. Acquisition, analysis, or interpretation of data: IE, CB, AG, CT, and MB. Drafting of the manuscript: IE, CT, and MB. Critical revision: IE, CB, AG, ML, FD, LP, CT, and MB. Supervision: CT and MB. 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.

ACKNOWLEDGMENTS

The authors thank the patients, Maud Carpentier from the DRCI (Direction de la Recherche Clinique et de l'Innovation), Michèle Vourc'h for extracting data from the French Diagnosis Information System database and Suzanne Rankin for proofreading and editing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12192/full#supplementary-material

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ORIGINAL RESEARCH
published: 12 January 2024
doi: 10.3389/ti.2024.12417

Assessing Health-Related Quality of Life in Non-Directed Versus Directed Kidney Donors: Implications for the Promotion of Non-Directed Donation

*Assaf Vital[1,2], Maya Siman-Tov[3], Gadi Shlomai[4,5], Yana Davidov[4,6], Keren Cohen-Hagai[4,7], Moshe Shashar[8], Enosh Askenasy[4,9], Ronen Ghinea[4,9], Eytan Mor[4,9] and Tammy Hod[4,9]**

[1]Arrow Program for Medical Research Education, Sheba Medical Center, Tel-Hashomer, Israel, [2]Adelson School of Medicine, Ariel University, Ariel, Israel, [3]Department of Emergency and Disaster Management, School of Public Health, Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel, [4]Faculty of Medicine, Tel-Aviv University, Tel Aviv, Israel, [5]Department of Internal Medicine D and Hypertension Unit, Division of Endocrinology, Diabetes and Metabolism, Sheba Medical Center, Tel-Hashomer, Israel, [6]Liver Disease Center, Sheba Medical Center, Tel Hashomer, Israel, [7]Department of Nephrology and Hypertension, Meir Medical Center, Kfar Saba, Israel, [8]Department of Nephrology and Hypertension, Laniado Hospital, Netanya, Israel, [9]Renal Transplant Center, Sheba Medical Center, Tel-Hashomer, Israel

Living kidney donation has increased significantly, but little is known about the post-donation health-related quality of life (HRQoL) of non-directed donors (NDs) vs. directed donors (DDs). We thus examined the outcomes of 112 living kidney donors (82 NDs, 30 DDs). For the primary outcomes—namely, the mean physical component summary (PCS) and mental component summary (MCS) scores of the 12-item Short Form Survey (SF-12) questionnaire—scores were significantly higher for the NDs vs. the DDs (PCS: +2.69, MCS: +4.43). For secondary outcomes, NDs had shorter hospital stays (3.4 vs. 4.4 days), returned to physical activity earlier (45 vs. 60 days), exercised more before and after donation, and continued physical activity post-donation. Regression analyses revealed that donor type and white blood cell count were predictive of the PCS-12 score, and donor type was predictive of the MCS-12 score. Non-directed donation was predictive of a shorter hospital stay (by 0.78 days, $p < 0.001$) and the odds of having PCS-12 and MCS-12 scores above 50 were almost 10 and 16 times higher for NDs, respectively ($p < 0.05$). These findings indicate the safety and potential benefits of promoting non-directed donation. However, careful selection processes must be maintained to prevent harm and exploitation.

Keywords: non-directed kidney donors, living kidney donors, directed kidney donors, quality of life, length of stay

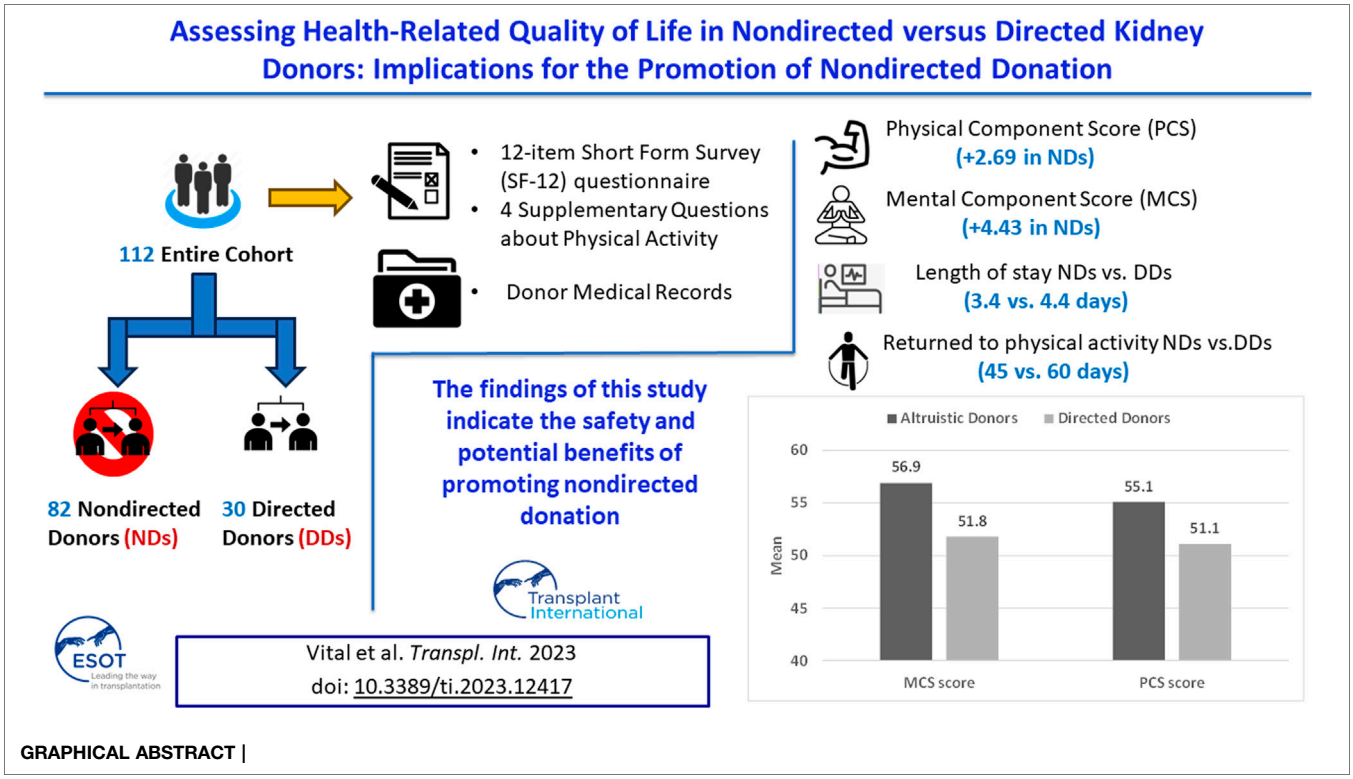
OPEN ACCESS

***Correspondence**
Tammy Hod,
tamar.hod@sheba.health.gov.il

Received: *14 November 2023*
Accepted: *02 January 2024*
Published: *12 January 2024*

Citation:
Vital A, Siman-Tov M, Shlomai G, Davidov Y, Cohen-Hagai K, Shashar M, Askenasy E, Ghinea R, Mor E and Hod T (2024) Assessing Health-Related Quality of Life in Non-Directed Versus Directed Kidney Donors: Implications for the Promotion of Non-Directed Donation. Transpl Int 37:12417. doi: 10.3389/ti.2024.12417

Abbreviations: BMI, body mass index; BPACR, biopsy proven acute cellular rejections; eGFR, estimated glomerular filtration rate; ESRD, end stage renal disease; HLA MM, human leukocyte antigen mismatch; IQR, interquartile range; LOS, length of stay; MCS, mental component summary; OR, odds ratio; PCKD, polycystic kidney disease; PCS, physical component summary; RTRs, renal transplant recipients; Scr, serum creatinine; SF-12, 12-item Short Form Survey; WBC, white blood cell.



GRAPHICAL ABSTRACT |

INTRODUCTION

Prelude

I [Assaf Vital] am a 28 years-old medical student, currently in my third year of studies at Ariel University in Israel. At the age of 16, I was diagnosed with stage 4 chronic kidney disease, which has remained stable to this day. My nephrologist advised me that at some point in the future, I would likely need a kidney transplant, and I should start looking for a donor. The thought of asking someone for such a major gift was daunting, and I felt I needed to understand more about what it would entail before doing so.

Under the guidance of Dr. Hod, I undertook research to explore the implications of kidney donation on the lives of donors, both in terms of their physical health and their mental wellbeing. Through my investigations, I hope to provide physicians and patients with a clearer understanding of what donation involves and what the potential consequences might be. As part of my research, I spoke with several individuals who had donated a kidney to a loved one or to a stranger. Their insights and experiences gave me a deeper appreciation of the sacrifices involved in kidney donation, as well as the extraordinary generosity and resilience of the donors themselves. For instance, I remember speaking with one donor who apologized for being breathless on the phone since she had just finished a half-marathon with a group of other kidney donors. Another donor shared with me that recovering from laser eye surgery had been more difficult than recovering from kidney donation.

Ultimately, my research helped me to feel more informed and empowered in facing my own kidney transplant journey. While I have not yet found a donor, "I am heartened by the knowledge that there are many compassionate and courageous people out there who are willing to give the gift of life to others."

Background

The rate of living kidney donation has increased significantly over the years, accounting for a global increase to 38% of all kidney transplants in 2021 [1]. This welcome trend is helping to bridge the gap between the shortage of deceased donor organs and the growing number of transplant candidates on waiting lists. In addition, there are clear advantages of living over deceased kidney donation, including minimization of the recipient's waiting time and shorter cold and warm ischemic times, with consequent improved graft quality and transplant outcomes. An additional advantage is that the surgery is elective, enabling optimization of the recipient's health before the transplant [2–6].

Living kidney donation may be directed or non-directed. Directed kidney donation is donation to a recipient with whom the donor has a genetic and/or emotional relationship pre-transplant, while non-directed kidney donation is donation to a recipient with whom the donor has no previous acquaintance. It is notably more straightforward for medical professionals and policymakers to endorse directed kidney donation, where a family member, close friend, or anyone with an emotional connection to the recipient donates a

kidney out of a sense of obligation or personal will. However, non-directed kidney donation presents a distinct challenge.

The number of non-directed donors has increased sharply in recent years [7], contributing significantly to the feasibility of kidney paired or pooled exchange programs and facilitating transplants for high immunological risk recipients [8, 9]. Yet, clinicians express skepticism about motivations for non-directed donation and concerns about long-term physical and psychological outcomes for non-directed donors and hence hesitate to actively promote it [10–12]. Thus, non-directed kidney donation remains uncommon, being limited to a minority of European countries due to legal constraints and moral objections and accounting for only 10% and 3% of all living donations in the United Kingdom and the United States, respectively [13].

In Israel, a non-profit organization, known as Matnat Chaim (meaning the Gift of Life), has emerged as a major force encouraging living—mainly non-directed—kidney donation. The organization has facilitated 1,398 live kidney donations since its founding in February 2009 (up to the end of February 2023), thereby contributing to a steady increase in the number of living kidney donations per year in Israel from 71 in 2010 to 319 in 2022. These 319 living donations comprised 68.75% of the total of 464 kidney transplants in Israel in 2022, with non-directed donors contributing 58.3% (186/319) of the kidneys.

To shed light on the dilemma of whether living kidney donation, specifically non-directed kidney donation, should be encouraged, this study aimed to evaluate the health-related quality of life (HRQol) of living donors after donation. Specifically, we compared the HRQol of directed vs. non-directed donors, alongside examining differences between the two groups in hospital length of stay (LOS), time to return to normal activity, and time to physical activity post donation.

MATERIALS AND METHODS

Study Population

The study is of a cross sectional design. All 179 individuals who underwent laparoscopic kidney donation at the Sheba Medical Center between the end of June 2019 and the beginning of October 2022 were eligible to participate in the study. Three donors were excluded, one due to first year recipient graft loss and two due to deaths of the recipients in the first year after transplant. A total of 176 donors—130 non-directed and 46 directed—were contacted via phone and asked to participate in the study. Donors who consented were required to confirm receipt of our questionnaire—based on SF-12 plus four **Supplementary Questions**—via WhatsApp or email through a Google form. Participants were provided with a designated phone number for assistance with questionnaire completion or for any queries.

Eighty-two (63.1%) of the 130 non-directed donors and 30 (65.2%) of the 46 directed donors returned the completed study questionnaires and comprised our final study cohort (**Figure 1**). A comparison of age, sex, and year of donation between study participants and non-participants showed no significant differences. Similarly, there were no significant differences in the participation rates between non-directed and directed donors. The protocol was approved by our institutional review board (7053-20-SMC).

Pre-Donation Evaluation

The evaluation process for donors involves a comprehensive medical, social, and psychological assessment. Directed donors are subject to approval by a local independent committee at the Sheba Medical Center, while non-directed donors are referred to a national independent committee. Before deciding on a particular donor-recipient pair, the relevant committee requests information from the transplant center about the potential recipient and the donor as well as an independent psychological evaluation. It is important to note that the transplant center medical team provides all donors with the assurance that they can choose to withdraw from the donation process at any point, without any guilt or negative consequences.

Primary and Secondary Outcomes

Our primary outcomes were the physical component summary (PCS) score and the mental component summary (MCS) score calculated from the 12-item Short Form Survey (SF-12) questions, with health outcomes grouped into eight domains, namely, physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health [14]. These scores were normalized to a mean score of 50 and a standard deviation of 10 [15], meaning that a score of 50 represents the average HRQol of the general population, and a score of 40 or 60 represents a HRQol one standard deviation lower or higher, respectively, than the average. We conducted a comparative analysis of the PCS and MCS scores between non-directed and directed donors, and further investigated the variations in the eight domains that constitute the PCS and MCS scores for the two groups.

Donors participating in the study were required to provide written consent by answering "yes" to the first question on our questionnaire (**Supplementary Figure S1**). Before filling out the SF-12 questionnaire, participants were requested to respond to four additional questions regarding the time to return to normal activity post-donation, pre-donation exercising status, exercising status at the time of questionnaire completion, and time to return to exercising post-donation. In addition, we modified the SF-12 questionnaire by requesting participants to take "the day they reported being back to normal activity after kidney donation" as their baseline for answering questions, rather than "during the past 4 weeks," as stated in the original questionnaire.

To fortify the HRQoL evaluation, we also compared several secondary outcomes between non-directed and directed donors. These outcomes are pertinent to HRQoL or influenced by it and include hospital LOS for kidney donation, times to return to normal activity and physical activity post-donation, post-donation cessation of exercising, starting physical activity post-donation, and continuation of physical activity post-donation.

Data Extraction and Study Assessments

The following information was extracted from donor medical records: donor type, smoking status and relevant family

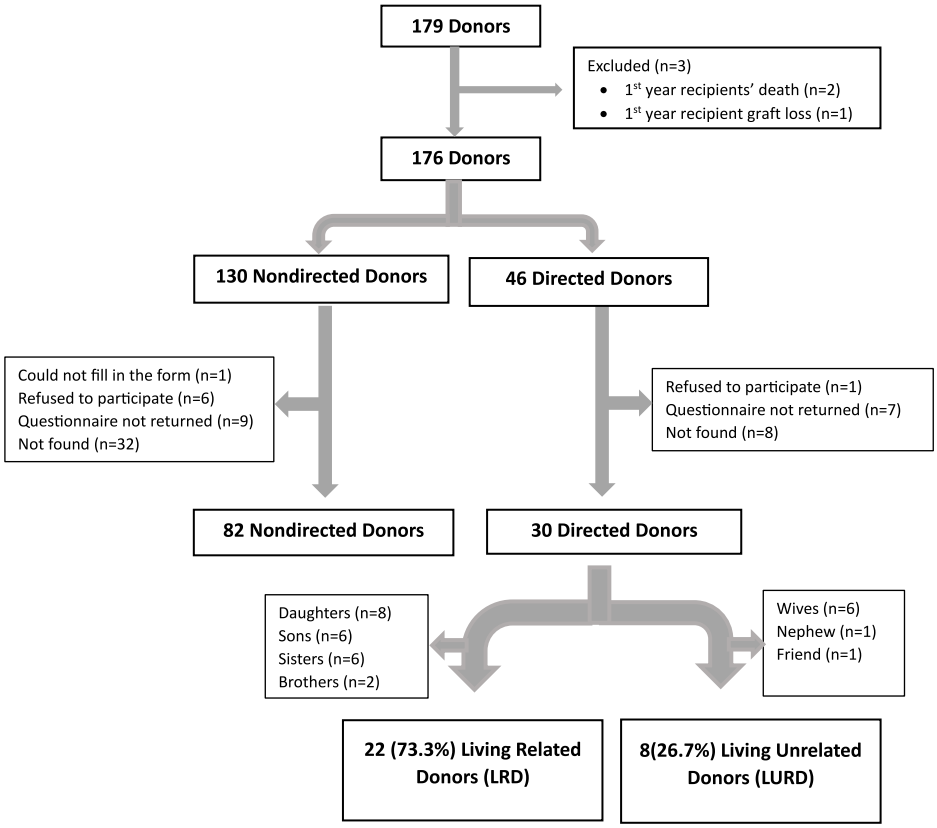


FIGURE 1 | Consort diagram.

history—specifically of diabetes, hypertension, ischemic heart disease, malignancy, and nephrolithiasis. Additionally, donor information was obtained from the electronic patient records in the MDClone data acquisition system of the Sheba Medical Center. This system facilitated retrieval of relevant clinical information for donors, including age, gender, weight, and body mass index (BMI) pre-donation, hospital LOS for kidney donation, average systolic blood pressure and diastolic blood pressure in the 6 months pre-donation and in the first month post-donation. The following biochemical parameters were also retrieved from MDClone: average serum creatinine in the 6 months pre-donation and in the first week and 6 months post-donation, average uric acid in the 6 months pre-donation and in the first week post-donation, and average total white blood cell (WBC) count, hemoglobin, platelet count, globulins, albumin, glucose, HbA1C, lipid profile, urine protein/creatinine and urine albumin/creatinine in the 6 months pre-donation.

In view of the fact that both directed and non-directed donors are acquainted with their recipients (non-directed donors meet their recipients for the first time post-donation, during admission for kidney donation), we also determined whether there were any significant differences between the recipients of non-directed donors and directed donors that could impact the HRQoL of donors following donation. The following information about the recipients was retrieved from electronic records: transplant number, underlying cause of end-stage renal disease (ESRD), renal replacement therapy pre-transplant (yes/no), duration of dialysis, past medial history of diabetes, hypertension, ischemic heart disease, congestive heart failure, peripheral vascular disease, and malignancy, smoking status, human leukocyte antigen (HLA) match between the donor and recipient, delayed graft function (yes/no), slow graft function (yes/no), perioperative complications, and peri-transplant biopsy proven acute cellular rejections (BPACR). Additional clinical and biochemical parameters for the recipients were retrieved from MDClone, including age, gender, average weight and BMI for the 1–12 months post-transplant, serum creatinine on postoperative day 5, and at 1, 3, and 6 months and 1 year post-transplant.

Statistical Analysis

Donors' and recipients' demographic, clinical and biochemical covariates of interest were tabulated and compared between non-directed and directed donors. Categorical variables were compared using the Chi-squared test, or Fisher's test if the expected count number was less than 5. For continuous variables, we first checked for normality using the

TABLE 1 | Demographic and clinical characteristics of donors, stratified by donor type.

Variable	Entire cohort (*n* = 112)	Nondirected donors (*n* = 82)	Directed donors (*n* = 30)	*p*-value
Donor characteristic				
Age (years)	43.0 ± 10.7	43.1 ± 10.2	42.8 ± 12.2	0.45
Male sex	66 (58.9%)	57 (69.5%)	9 (30.0%)	<**0.001****
Weight (kg)	71.4 ± 10.4	72.2 ± 9.8	69.2 ± 11.9	0.09
BMI (kg/m^2)	24.2 ± 2.5	24.1 ± 2.4	24.5 ± 2.7	0.25
Family history of diabetes	30 (26.8%)	18 (22.2%)	12 (40.0%)	0.056
Family history of hypertension	25 (22.5%)	16 (19.5%)	9 (31.0%)	0.202
Family history of ischemic heart disease	24 (21.4%)	14 (17.1%)	10 (33.3%)	0.063
Family history of malignancy	28 (25.0%)	20 (24.4%)	8 (26.7%)	0.805
Family history of nephrolithiasis	12 (10.7%)	7 (8.5%)	5 (16.7%)	0.218
Smoking status				
Current smoker	14 (12.5%)	8 (9.7%)	6 (20.0%)	0.338
Past smoker	7 (6.3%)	5 (6.1%)	2 (6.7%)	
Never smoked	91 (81.3%)	69 (84.1%)	22 (73.3%)	
Average vital signs in the 6 months pre-donation				
SBP	122.6 ± 10.3	123.4 ± 10.5	120.3 ± 9.6	0.087
DPB	75.7 ± 7.3	76.0 ± 6.9	74.6 ± 8.4	0.091
Average vital signs in the first month post-donation				
SBP (max)	145.8 ± 15.5	145.9 ± 16.1	145.7 ± 13.9	0.481
SBP (min)	79.3 ± 14.0	78.7 ± 13.4	80.9 ± 15.7	0.239
SBP (average)	108.7 ± 10.2	108.8 ± 9.9	108.5 ± 11.3	0.448
DBP (max)	87.0 ± 9.1	86.2 ± 7.9	89.1 ± 12.0	0.118
DBP (min)	40.6 ± 9.4	40.6 ± 8.6	40.5 ± 11.6	0.483
DBP (average)	62.1 ± 8.4	62.0 ± 8.2	62.6 ± 8.9	0.369

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Continuous variables are presented as means ± standard deviations, categorical variables are presented as numbers (%).

*p < 0.05; **p < 0.01.

The bold values are all the p *values which are significant, either below 0.05 or below 0.01.*

Shapiro-Wilks test and for equality of variances (using Levene's test). We then used a t-test for normally distributed variables, and non-parametric tests (Mann-Whitney) for non-normally distributed variables. Differences in PCS and MCS index and components were analyzed using an independent sample t-test.

PCS score, MCS score and LOS were selected as the major dependent variables for linear regression analyses. The variables entered into the model were chosen after checking for multicollinearity and association with donation type. Variables that were significant ($p \leq 0.05$) and/or those with clinical importance were entered into multivariate models. Logistic regression was also conducted for predicting PCS and MSC scores after dividing the index into two categories based on a threshold value of 50, followed by calculating the odds ratio (OR) with 95% CI. The data was analyzed using SPSS version 28.

RESULTS

Donor Cohort Characteristics

A total of 112 living kidney donors comprised our final cohort. Mean age was 43.0 ± 10.7 years; 66 (58.9%) were males; and mean BMI was 24.2 ± 2.5 kg/m^2. Of the donors, 30 (26.8%), 25 (22.5%), 24 (21.4%), 28 (25.0%), and 125 (10.7%) had a family history of diabetes, hypertension, ischemic heart disease, malignancy and nephrolithiasis, respectively; 14 (12.5%) were current smokers and 7 (6.3%) were past smokers. All cohort characteristics including average vital signs in the 6 months pre-donation and in the 1 month post-donation are shown in **Table 1**.

Of the 112 donors, 90 (80.4%) were healthy without any past medical history. Relevant past medical histories of 22 donors (14 non-directed and 8 directed) included hypertension (in 2), prediabetes (in 2), dyslipidemia (in 5), hypothyroidism (in 4), bariatric surgery (in 2), asthma (in 2), osteoporosis (in 1), celiac disease (in 1), motor cerebral palsy (in 1) and full recovery from breast carcinoma (in 1 directed donor). None of the donors had any mental disorder. Laboratory results including renal function tests of all donors in the 6 months pre-donation and at 1 week and 6 months post-donation are shown in **Table 2**.

Univariate Comparison of Non-Directed vs. Directed Donors

Our cohort consisted of 82 non-directed donors and 30 directed donors. Directed donors comprised 22 (73.3%) living related donors (8 daughters, 6 sons, 6 sisters, and 2 brothers) and 8 (26.7%) living unrelated donors (6 wives, 1 nephew, and 1 friend) (**Figure 1**). There were significantly more males among non-directed vs. directed donors (69.5% vs. 40%, $p < 0.001$). Rates of family history of diabetes and of ischemic heart disease were higher among directed compared to non-directed donors (40% vs. 22.2% and 33.3% vs. 17.1%, respectively, with p values approaching significance). There were no other differences between non-directed and directed donors, including no statistically significant differences in systolic and diastolic blood pressures in the 6 months pre-donation and 1 month post-donation, as shown in **Table 1**.

TABLE 2 | Biochemical characteristics of donors, stratified by donor type.

Variable	Entire cohort (*n* = 112)	Non-directed donors (*n* = 82)	Directed donors (*n* = 30)	*p*-value
Average laboratory results in the 6 months pre-donation				
WBC (K/μL)	7.4 ± 4.9	6.5 ± 1.4	10.0 ± 8.9	**0.023***
Hemoglobin (g/dL)	14.0 ± 1.1	14.2 ± 1.0	13.6 ± 1.3	**0.017***
Platelets (K/μL)	216.5 ± 43.3	213.0 ± 40.0	226.3 ± 50.9	0.082
Creatinine (mg/dL)	0.83 ± 0.15	0.85 ± 0.15	0.75 ± 0.15	**0.002****
eGFR (CKD-EPI)[a]	101.7 ± 13.5	100.9 ± 13.9	103.8 ± 12.1	0.159
CCT urine collection (mL/min)	130.1 ± 25.5	132.1 ± 23.6	122.2 ± 31.7	0.085
Glucose (mg/dL)	90.9 ± 7.9	90.9 ± 6.4	90.8 ± 11.2	0.486
HbA1C (g/dL)	5.1 ± 0.4	5.1 ± 0.4	5.0 ± 0.5	0.105
Albumin (g/dL)	4.4 ± 0.3	4.5 ± 0.3	4.4 ± 0.3	**0.03***
Globulins (g/dL)	2.8 ± 0.3	2.7 ± 0.3	2.8 ± 0.3	0.063
Uric acid (mg/dL)	5.2 ± 1.3	5.4 ± 1.2	4.6 ± 1.1	**0.025***
Total cholesterol (mg/dL)	174.5 ± 27.7	171.1 ± 27.6	184.6 ± 26.4	**0.042***
LDL cholesterol (mg/dL)	109.4 ± 22.7	106.6 ± 22.9	118.2 ± 20.3	**0.027***
HDL cholesterol (mg/dL)	53.1 ± 11.3	51.5 ± 8.9	58.1 ± 16.0	0.055
Triglycerides (mg/dL)	88.6 ± 43.2	86.4 ± 40.9	95.5 ± 50.3	0.208
Urine protein/creatinine (g/g creatinine)	0.06 ± 0.03	0.06 ± 0.03	0.08 ± 0.05	0.083
Urine albumin/creatinine (mg/g creatinine)	3.8 ± 4.4	3.2 ± 3.7	5.8 ± 6.1	**0.03****
Laboratory results in the first week post-donation				
Uric acid (mg/dL) average	4.3 ± 1.1	4.5 ± 1.1	3.8 ± 1.0	**0.002****
Creatinine (mg/dL) max	1.4 ± 0.3	1.41 ± 0.3	1.2 ± 0.2	**0.001****
Creatinine (mg/dL) min	1.3 ± 0.3	1.3 ± 0.3	1.1 ± 0.2	<**0.001****
Creatinine (mg/dL) average	1.3 ± 0.3	1.4 ± 0.3	1.2 ± 0.2	<**0.001****
eGFR average (CKD-EPI)[a]	61.3 ± 11.1	59.5 ± 10.8	67.0 ± 10.4	**0.002****
Laboratory results in the 6 months post-donation				
Creatinine (mg/dL)	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.02	**0.034***
eGFR (CKD-EPI)[a]	69.3 ± 14.3	69.3 ± 14.8	69.0 ± 12.3	0.464

Abbreviations: WBC, white blood cell; CCT, creatinine clearance; eGFR, estimated glomerular filtration rate.

Continuous variables are presented as means ± standard deviations, categorical variables are presented as numbers (%).

*p < 0.05; **p < 0.01.

[a]*eGFR was calculated according to the following CKD-EPI formula: eGFR = 141* min (Scr/k, 1)α * max (Scr/k, 1)–1.209 * 0.993Age * 1.018 * 1.159 (if black) (where Scr—standardized serum creatinine; k = 0.7 if female, 0.9 if male; α = –0.329 if female, –0.411 if male; min = the minimum of Scr/k of 1; max = the maximum of Scr/k or 1).*

The bold values are all the p *values which are significant, either below 0.05 or below 0.01.*

Biochemical characteristics differed between non-directed and directed donors in pre-donation total WBC count (6.5 ± 1.4 vs. 10.0 ± 8.9, $p = 0.023$) and in lipid profile (total cholesterol and LDL cholesterol), which were both higher in directed donors. Urine albumin/creatinine was higher in directed compared to non-directed donors, but values were in the normal range for both groups. The variations observed in hemoglobin, uric acid, albumin, creatinine and eGFR between non-directed and directed donors were primarily due to gender differences, with the higher proportion of female donors among the directed group contributing to lower levels of hemoglobin, uric acid, albumin and creatinine. All other biochemical characteristics were not statistically different between the two groups, as shown in **Table 2**.

Univariate Comparison for Renal Transplant Recipients Who Received a Kidney From Non-Directed vs. Directed Donors

Renal transplant recipients (RTRs) who received a kidney from a non-directed vs. a directed donor were younger (49.7 ± 13.4 years vs. 56.1 ± 13.2 years, $p = 0.013$), had spent a longer time on dialysis pre-transplant [1.8 years (0.8–3.5) vs. 0.7 (0.3–2.0) years, $p = 0.002$], and exhibited a lower rate of hypertension (75.6% vs. 93.3%, $p = 0.037$) and a higher degree of human leukocyte antigen mismatch (HLA MM) (5–6 MM in 55.6% vs. 25.9% and 0% 0–2 MM vs. 25.9%, $p < 0.001$). No statistically significant differences were observed in hospital LOS, rates of delayed or slow graft function, peri-operative complications and peri-transplant BPACR between the two groups. All other demographic and clinical characteristics are shown in **Table 3**. Renal allograft function on postoperative day 5, and at 1, 3, 6 and 12 months post-transplant did not differ significantly between the groups (**Table 4**).

Primary and Secondary Outcomes in the Entire Donor Cohort

Mean time from donation to questionnaire completion was 1.07 ± 0.65 years. Mean PCS-12 and MCS-12 scores were both higher than those in the general population (54.1 ± 4.1 and 55.5 ± 5.8, respectively). Median time to normal activity post-donation was 30 days [interquartile range (IQR) 14–42]. Of the donors, 77 (68.8%) reported exercising before donation and 78 (69.6%) post-donation, with a median time to physical activity post-donation of 48 days (IQR 30–90); 66 (58.9%) continued exercising, 11 (9.8%) stopped exercising, and 12 (10.7%) started physical activity post-donation. Mean hospital LOS for kidney donation was 3.7 ± 0.9 days (**Table 5**).

TABLE 3 | Demographic and clinical characteristics of renal transplant recipients, stratified by donor type.

Variable	Entire cohort (*n* = 112)	Non-directed donors (*n* = 82)	Directed donors (*n* = 30)	*p*-value
RTR characteristics				
Age (years)	51.4 ± 13.6	49.7 ± 13.4	56.1 ± 13.2	**0.013***
Sex –Male	71 (63.4%)	52 (63.4%)	19 (63.3%)	0.994
Weight, average of 1–12 months post-transplant (kg)	75.8 ± 15.7	75.6 ± 16.2	76.4 ± 14.7	0.407
BMI, average of 1–12 months post-transplant (kg/m^2)	26.8 ± 5.1	26.5 ± 5.0	27.4 ± 5.2	0.206
ESRD etiology				
Diabetic nephropathy	16 (14.3%)	11 (13.4%)	5 (16.7%)	0.13
Glomerulonephritis	25 (22.3%)	17 (20.7%)	8 (26.7%)	
Nephrosclerosis	10 (8.9%)	4 (4.9%)	6 (20.0%)	
PCKD	17 (15.2%)	14 (17.1%)	3 (10.0%)	
Other	27 (24.1%)	22 (26.8%)	5 (16.7%)	
Unknown	17 (15.2%)	14 (17.1%)	3 (10.0%)	
Pre-transplant dialysis				
Dialysis before transplant	84 (75.0%)	64 (78.0%)	20 (66.7%)	0.218
Time on dialysis (years)	1.4 (0.6–2.9)	1.8 (0.8–3.5)	0.7 (0.3–2.0)	**0.002****
Medical history				
Diabetes	31 (27.7%)	20 (24.4%)	11 (36.7%)	0.198
Hypertension	90 (80.4%)	62 (75.6%)	28 (93.3%)	**0.037***
Ischemic heart disease	21 (18.8%)	13 (15.9%)	8 (26.7%)	0.194
Congestive heart failure	12 (10.7%)	8 (9.8%)	4 (13.3%)	0.731
Peripheral vascular disease	4 (3.6%)	2 (2.4%)	2 (6.7%)	0.291
Malignancy	6 (5.4%)	4 (4.9%)	2 (6.7%)	0.658
Smoking status				
Current smoker	9 (8.1%)	4 (4.9%)	5 (16.7%)	0.124
Past smoker	23 (20.6%)	17 (20.7%)	6 (20.0%)	
Never smoked	80 (71.4%)	61 (74.4%)	19 (63.3%)	
Transplant number				
1	101 (90.2%)	74 (90.2%)	27 (90.0%)	0.496
2	7 (6.3%)	4 (4.9%)	3 (10.0%)	
3	3 (2.7%)	3 (3.7%)	0	
4	1 (1.2%)	0	0	
HLA MM				
0–2	7 (6.5%)	0	7 (25.9%)	**<0.001****
3–4	49 (45.4%)	36 (44.4%)	13 (48.1%)	
5–6	52 (48.1%)	45 (55.6%)	7 (25.9%)	
Peri-transplant data				
Hospital LOS for transplant (days)	8 (8–10)	8 (8–10)	8.5 (8–10)	0.276
Delayed graft function	1 (0.9%)	0	1 (3.3%)	0.268
Slow graft function	10 (8.9%)	7 (8.5%)	3 (10.0%)	0.726
Peri-operation complications				
CVS	4 (3.6%)	4 (4.9%)	0	0.221
ID	18 (16.1%)	15 (18.3%)	3 (10.0%)	
Vascular	5 (4.5%)	2 (2.4%)	3 (10.0%)	
Other	1 (0.9%)	1 (1.2%)	0	
None	84 (75.0%)	60 (73.2%)	24 (80.0%)	
Peri-transplant BPACR	11 (9.8%)	10 (12.2%)	1 (3.3%)	0.283

Abbreviations: BMI, body mass index; BPACR, biopsy proven acute cellular rejections; CVS, cardiovascular; ESRD, end stage renal disease; HLA MM, human leukocyte antigen mismatch; ID, infectious diseases; PCKD, polycystic kidney disease; RTRs, renal transplant recipients.

Continuous variables are presented as means ± standard deviations, categorical variables are presented as numbers (%).

*p < 0.05; **p < 0.01.

The bold values are all the p *values which are significant, either below 0.05 or below 0.01.*

Univariate Comparison of Primary and Secondary Outcomes in Non-Directed vs. Directed Donors

Comparisons for all primary and outcomes secondary are presented in **Table 5**. Mean PCS-12 and MCS-12 scores were significantly higher in non-directed compared to directed donors (55.1 ± 3.1 vs. 51.1 ± 5.2, $p < 0.001$ and 56.9 ± 4.1 vs. 51.8 ± 7.9, $p < 0.001$, respectively) (**Figure 2A**). There were also significant differences between the two groups in six of the eight domains of the SF-12 questionnaire (general health, bodily pain, and role physical for the PCS score, and mental health, vitality, and social functioning for the MCS score) (**Figure 2B**). Time to resumption of normal activity was not significantly different between the two groups. However, time to resumption of physical activity was shorter for the non-directed donors than for the directed donors [45 days (IQR 30–90) vs. 60 days (IQR 34–90)], but significant difference could not be shown due to the small size of the two groups (**Figure 3A**). More non-directed than directed donors engaged in physical activity before and after kidney donation and

TABLE 4 | Renal allograft function of renal transplant recipients, stratified by donor type.

Variable	Entire cohort (*n* = 112)	Non-directed donors (*n* = 82)	Directed donors (*n* = 30)	*p*-value
Postoperative day 5				
Creatinine (mg/dL) on	1.7 ± 1.3	1.7 ± 1.2	1.6 ± 1.6	0.379
1 month post-transplant				
Creatinine (mg/dL)	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.4	0.452
eGFR (CKD-EPI)[a]	62.8 ± 21.3	63.2 ± 20.8	61.6 ± 22.4	0.366
3 months post-transplant				
Creatinine (mg/dL)	1.3 ± 0.7	1.4 ± 0.8	1.3 ± 0.4	0.283
eGFR (CKD-EPI)	63.7 ± 21.7	64.0 ± 21.2	63.0 ± 23.4	0.418
6 months post-transplant				
Creatinine (mg/dL)	1.3 ± 0.6	1.3 ± 0.7	1.2 ± 0.4	0.267
eGFR (CKD-EPI)	65.7 ± 20.1	65.8 ± 19.5	65.6 ± 21.9	0.474
1 year post-transplant				
Creatinine (mg/dL)	1.3 ± 0.8	1.3 ± 0.9	1.2 ± 0.4	0.231
eGFR (CKD-EPI)	68.3 ± 20.0	67.8 ± 19.8	69.6 ± 21.0	0.254

Abbreviations: eGFR, estimated glomerular filtration rate.

Continuous variables are presented as means ± standard deviations.

[a]*eGFR was calculated according to the following CKD-EPI formula: eGFR = 141* min (Scr/k, 1)α * max (Scr/k, 1)–1.209 * 0.993Age * 1.018 * 1.159 (if black) (where Scr—standardized serum creatinine; k = 0.7 if female, 0.9 if male; α = –0.329 if female, –0.411 if male; min = the minimum of Scr/k of 1; max = the maximum of Scr/k or 1).*

TABLE 5 | Primary and secondary outcomes for donors, stratified by donor type.

Variable	Entire cohort (*n* = 112)	Non-directed donors (*n* = 82)	Directed donors (*n* = 30)	*p*-value
Primary outcome—questionnaire results				
Time from donation to questionnaire completion (years)	1.07 ± 0.65	1.02 ± 0.56	1.21 ± 0.85	0.141
PCS-12 score	54.1 ± 4.1	55.1 ± 3.1	51.1 ± 5.2	<**0.001****
MCS-12 score	55.5 ± 5.8	56.9 ± 4.1	51.8 ± 7.9	<**0.001****
Secondary outcomes				
Time to normal activity post-donation (days)	30 (14–42)	30 (14–40)	30 (16–45)	0.117
Physical activity before donation	77 (68.8%)	58 (70.7%)	19 (63.3%)	0.454
Physical activity after donation	78 (69.6%)	61 (74.4%)	17 (56.7%)	0.071
Time to physical activity post-donation (days)	48 (30–90)	45 (30–90)	60 (34–90)	0.306
Change in physical activity post-donation				
Continued	66 (58.9%)	52 (63.4%)	14 (46.7%)	0.287
Stopped	11 (9.8%)	6 (7.3%)	5 (16.7%)	
Started	12 (10.7%)	9 (11.0%)	3 (10.0%)	
Never did	23 (20.5%)	15 (18.3%)	8 (26.7%)	
Hospital LOS for kidney donation (days)	3.7 ± 0.9	3.4 ± 0.7	4.4 ± 1.1	<**0.001****

Continuous variables are presented as means ± standard deviations or as median (interquartile range), categorical variables are presented as numbers (%).

**p < *0.01.*

The bold values are all the p *values which are significant, either below 0.05 or below 0.01.*

continued exercising post-donation. A higher rate of directed vs. non-directed donors did not exercise before or after kidney donation or stopped exercising post-donation (**Figure 3B**). Hospital LOS for kidney donation was significantly longer for directed than for non-directed donors (4.4 ± 1.1 vs. 3.4 ± 0.7 days, $p < 0.001$) (**Figure 4**).

Multivariable Linear Regression Analysis of PCS-12 Score in Kidney Donors

In a multivariable linear regression analysis of the PCS-12 score in kidney donors (adjusted for donor type, age, gender, donor family history of diabetes and of ischemic heart disease, average eGFR in the first week post-donation, WBC count in the 6 months pre-donation, and hospital LOS for kidney donation), only donor type and WBC count were found to be significant predictors for PCS-12 score. Being a non-directed donor vs. a directed donor is associated with a 2.69 (1.02) points higher mean PCS-12 score, $p = 0.01$. For every increase of 1 K/μL in WBC count in the 6 months pre-donation, PCS-12 score decreased by 0.18 (0.08), ($p = 0.02$; **Table 6**).

Multivariable Linear Regression Analysis of MCS-12 Score in Kidney Donors

In a multivariable linear regression analysis of the MCS-12 score in kidney donors adjusted for the same variables as those listed above, donor type alone was found to be a significant predictor for MCS-12 score. Mean MCS-12 score increased by 4.43 (1.53) in non-directed compared to directed donors ($p = 0.005$). Increases in WBC counts pre-donation and in hospital LOS for kidney donation reduced the MCS-12 score, with p values approaching significance (**Table 6**).

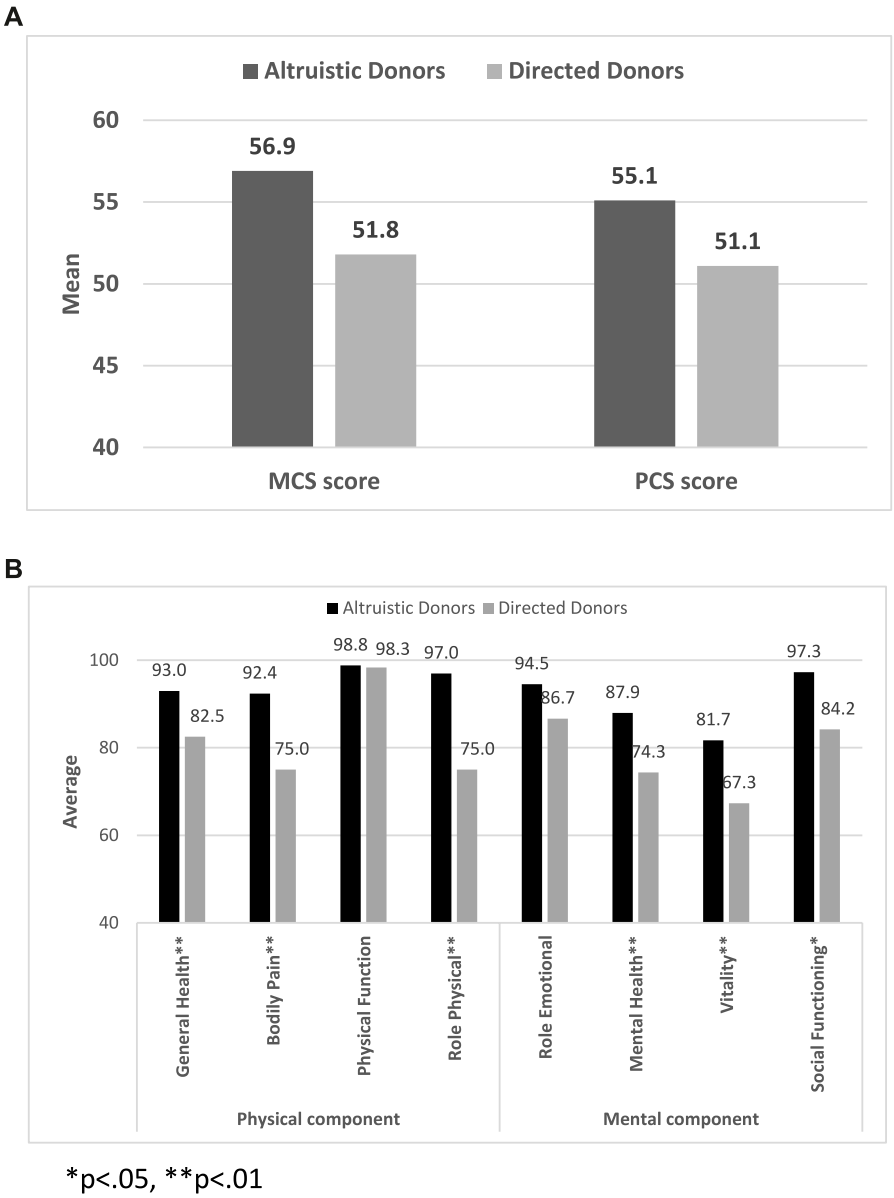


FIGURE 2 | SF-12 questionnaire results: **(A)** Mean MCS-12 and PCS-12 scores for non-directed vs. directed donors. **(B)** PCS-12 and MCS-12 scores for non-directed vs. directed donors for the eight domains of the SF-12 questionnaire.

Multivariable Linear Regression Analysis of Hospital LOS for Kidney Donation

In a multivariable linear regression analysis of hospital LOS for kidney donation adjusted for the same variables as those listed above, donor type and family history of diabetes were found to be significant predictors for LOS. LOS was shorter by 0.78 (0.22) days in non-directed compared to directed donors ($p < 0.001$). Family history of diabetes prolonged the LOS by 0.54 (0.19) days ($p = 0.007$; **Table 6**). There were no intraoperative surgical problems or any postoperative complications during hospitalization in our study cohort of living kidney donors.

Multivariable Logistic Regression Analysis of PCS-12 and MCS-12 Scores Above 50 in Kidney Donors

In a multivariable logistic regression analysis of PCS-12 and MCS-12 scores above 50 adjusted for the same variables as those listed above, donor type alone was found to be significantly associated with PCS-12 and MCS-12 score above 50. The odds for PCS-12 score to be above 50 were almost 10 times higher in non-directed compared to directed donors (OR 9.9, 95% CI 1.48–66, $p = 0.018$). The odds for an MCS-12 score above 50 were more than 16 times higher in non-directed vs. directed donors (OR 16.23, 95% CI 2.37–111.02, $p = 0.005$).

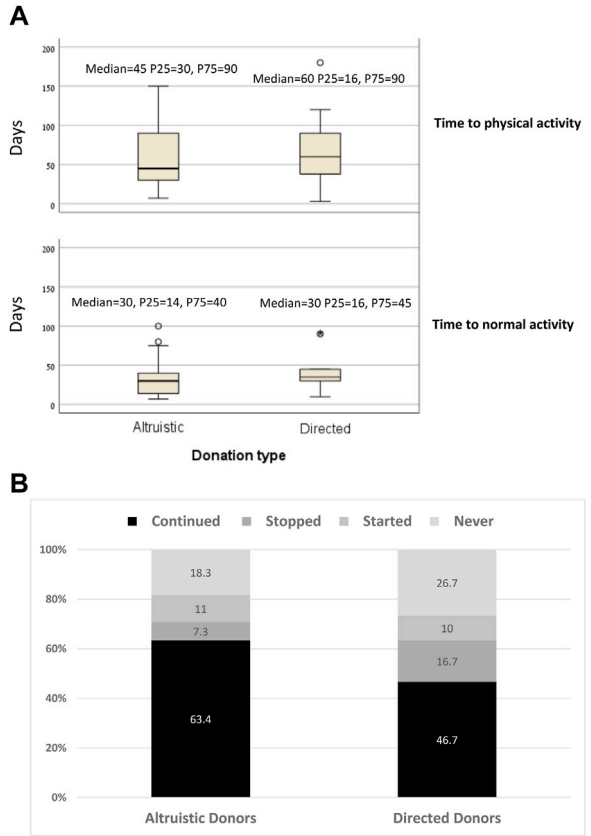


FIGURE 3 | (A) Time to normal activity and to physical activity for non-directed vs. directed donors. **(B)** Rates of kidney donors who continued, stopped, started exercising after donation and of donors who did not exercise before or after donation for non-directed vs. directed donors.

DISCUSSION

As the number of live kidney donations, particularly non-directed donations, continues to rise, it is becoming imperative to conduct a comprehensive analysis of donor outcomes, including a thorough comparison of outcomes between non-directed and directed donors in terms of both physical and mental health, as reflected in HRQol.

Our assessment of HRQol was based on a variety of factors, primarily PCS-12 and MCS-12 scores, but also time to resumption of normal activity, changes in the rate of physical activity, and the time taken to return to physical activity after donation. Our findings indicate that live kidney donors experience better HRQol than the general population with mean PCS-12 and MCS-12 scores surpassing the average score of 50. The median time for donors to return to normal activity and to physical activity was 30 and 48 days, respectively, and 58.9% of donors continued to exercise post-donation, while another 10.7% started exercising post-donation. Our analysis revealed that non-directed donors had a significantly higher HRQol than directed donors, as demonstrated by both PCS-12 and MCS-12 scores. Moreover, a higher proportion of non-directed donors continued with physical activity and they resumed exercising sooner after donation compared to directed donors. Mean hospital LOS for kidney donation was 3.7 days, with LOS being significantly shorter for non-directed than for directed donors. Our multivariable analyses demonstrated that non-directed donation was an independent predictor of higher PCS-12 and MCS-12 scores as well as a shorter hospital LOS.

The literature shows that, in general, most living donors exhibit excellent medical heath and enjoy high levels of HRQol [16–21]. However, studies investigating the

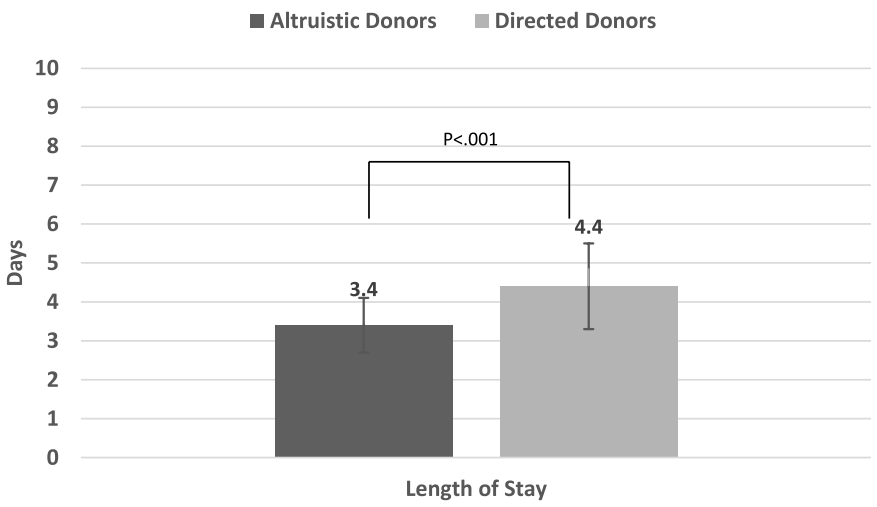


FIGURE 4 | Mean in-hospital length of stay (LOS) for kidney donation for non-directed vs. directed donors.

TABLE 6 | Multivariate linear regression analysis for PCS-12, MCS-12 and hospital LOS for kidney donors.

Effect	Mean (SD)	*p*-value
Multivariate linear regression analysis for PCS-12		
Donor type (non-directed vs. directed)	2.69 (1.02)	**0.01***
Age (for every increase of 1 year)	0.02 (0.04)	0.58
Gender (male vs. female)	(–)0.33 (0.90)	0.72
Donor family history of diabetes (Yes vs. No)	(–)0.07 (0.90)	0.94
Donor family history of ischemic heart disease (Yes vs. No)	(–)0.82 (0.95)	0.39
eGFR average in the first week post-donation (for every increase of 1 mL/min)	0.02 (0.04)	0.6
WBC count in the 6 months pre-donation (for every increase of 1 K/μL)	(–)0.18 (0.08)	**0.02***
Hospital LOS for kidney donation (for every increase of 1 day)	(–)0.14 (0.46)	0.77
Multivariate linear regression analysis for MCS-12		
Donor type (non-directed vs. directed)	4.43 (1.53)	**0.005****
Age (for every increase of 1 year)	0.01 (0.07)	0.89
Gender (male vs. female)	(–)0.19 (1.35)	0.89
Donor family history of diabetes (Yes vs. No)	(–)0.20 (1.35)	0.88
Donor family history of ischemic heart disease (Yes vs. No)	0.58 (1.43)	0.69
eGFR average in the first week post-donation (for every increase of 1 mL/min)	0.07 (0.06)	0.24
WBC count in the 6 months pre-donation (for every increase of 1 K/μL)	(–)0.23 (0.12)	0.05
Hospital LOS for kidney donation (for every increase of 1 day)	(–)1.22 (0.69)	0.08
Multivariate linear regression analysis for hospital LOS for kidney donation		
Donor type (non-directed vs. directed)	(–)0.78 (0.22)	**<0.001****
Age (for every increase of 1year)	(–)0.01 (0.01)	0.13
Gender (male vs. female)	(–)0.20 (0.20)	0.33
Donor family history of diabetes (Yes vs. No)	0.54 (0.19)	**0.007****
Donor family history of ischemic heart disease (Yes vs. No)	0.00 (0.22)	1.00
eGFR average in the first week post-donation (for every increase of 1 mL/min)	(–)0.01 (0.01)	0.44
WBC count in the 6 months pre-donation (for every increase of 1 K/μL)	0.01 (0.02)	0.67

Abbreviations: eGFR, estimated glomerular filtration rate; WBC, white blood cell.

*p < 0.05; **p < 0.01.

The bold values are all the p *values which are significant, either below 0.05 or below 0.01.*

psychological outcomes after non-directed kidney donation are limited. Sadler et al. conducted an early investigation (1971) of 18 living unrelated kidney donors that revealed that the donors did not exhibit any unusual characteristics or significant mental illness during the donation process. However, a retrospective follow-up conducted 4–6 years later showed that three of the donors had developed psychiatric disorders, including two cases of alcoholism and one of anti-social personality disorder [22]. A later study of 24 non-directed donors reported a considerable positive impact of donation on psychological wellbeing and very high satisfaction with the donation [23]. However, in another study of 49 unspecified living donors, psychologic symptoms increased after donation [24]. In the only study to date comparing non-directed donors to directed donors (39 vs. 52), similar positive outcomes were observed after donation. The majority of non-directed donors reported feeling content with the donation process and expressed a strong willingness to make the same decision again, with the caveat that three non-directed donors did regret their decision to donate [25]. Our study is the first to demonstrate superior HRQol experienced by a substantial group of non-directed donors compared to directed donors.

In our study, the significant disparity in the MCS-12 score between non-directed and directed donors probably derives from the distinctive characteristics of the non-directed donor population in Israel. In Israel, most non-directed donors are Orthodox Jews whose "point of contact" is the Matnat Chaim organization. Their religious conviction to assist others and fulfill a righteous duty probably plays a crucial role in promoting non-directed donation, as saving person's life is considered a significant religious obligation. This world view is exemplified by a passage in the Babylonian Talmud, Tractate Sanhedrin on page 37a, which states, "He who saves one life is as if he has saved the entire world." Indeed, non-directed donors scored significantly higher in the mental health and vitality domains of the MCS-12 score (**Figure 2B**), suggesting that belief and faith contribute to feelings of calmness, completeness, and energy. Furthermore, non-directed donors exhibited better social functioning than directed donors. While it is possible that the strong religious faith of non-directed donors makes them mentally more resilient than directed donors, further research is required to confirm this premise.

Non-directed donors showed higher energy levels and better PCS-12 scores, potentially explaining the shorter time to the resumption of physical activity post-donation, the greater likelihood of continuing physical activity and initiating exercise after donation compared to directed donors. In terms of the duration of hospital stay post-donation, patients' complaints of pain and willingness to extend their stay were the main factors determining LOS in the absence of any surgical or post-operative complications. Notably, non-directed donors had a shorter hospital stay, probably due to their faster physical recovery associated with less pain (**Figure 2B**) and their superior mental wellbeing.

Interestingly, an increase in WBC count was found to be associated with the PCS-12 score. This finding is in line with prior research demonstrating a link between excessive inflammatory activity and physical health problems, including cardiovascular disease, stroke, certain cancers and autoimmune disorders [26], with substantial morbidity and mortality being attributable to

inflammation-related conditions [27, 28]. Donor family history of diabetes was found to be associated with an increase in hospital LOS. This observation has no obvious explanation currently.

When interpreting our findings, it is important to consider the study's strengths and its limitations. The strengths include the use of the widely validated SF-12 questionnaire, which provides a strong foundation for evaluating HRQol. The study sample is comprised of a large group of donors, which enhances the reliability of the findings. Additionally, by examining both donor and recipient characteristics, this study was able to consider multiple confounders, including clinical and biochemical factors collected both before and after donation or transplantation. However, additional confounders cannot be excluded. An additional limitation is that the use of patient questionnaires can introduce subjective elements, which can be a drawback compared to direct assessments of inpatients. Living donors are a select group chosen for their good health and we did not evaluate the HRQol of the donors prior to donation; it is thus possible that these donors already had good HRQol before donation and any improvement was not necessarily linked to the kidney donation itself. It is also possible that those who declined to participate or those who we could not reach would have affected our psychosocial and functional outcomes had they been included in the study.

Importantly, the findings of this study endorse the continued use of non-directed donors, given the enhanced physical and mental HRQoL observed after donation, indicating that the donation process has no negative impact on their physical or mental wellbeing. In fact, carefully screened donors do not suffer any adverse physical or psychological consequences from donating to a stranger. Nevertheless, it is crucial to emphasize the benefits of living related donors, such as the improved HLA matching within families that leads to lower rejection rates and improved long-term outcomes. As healthcare providers, we strongly believe that safeguarding the wellbeing of all donors, particularly those motivated by altruism, is our fundamental duty. To minimize the risk of adverse health consequences post-donation and prevent any potential future harm, selecting non-directed donors should involve meticulous screening and a more stringent process. Moreover, it is imperative to ensure that the eagerness of non-directed donors to help others is not exploited or manipulated in any way. Therefore, the use of non-directed kidney donation should be considered only as a last resort after exhausting all possible options to secure a donation within the family.

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 humans were approved by the Institutional Review Board 7053-20-SMC. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AV: data acquisition, data interpretation; MS-T: data analysis; GS: conception and design; YD: data interpretation; KC-H: data interpretation; MS: conception and design; EA: data acquisition; RG: data acquisition; EM: data interpretation, revising; TH: conception and design, data acquisition, data interpretation, writing, revising. 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.

ACKNOWLEDGMENTS

We thank Ms. Inez Mureinik for critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12417/full#supplementary-material

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ORIGINAL RESEARCH
published: 18 January 2024
doi: 10.3389/ti.2024.11900

Glucose Concentration in Regulating Induced Pluripotent Stem Cells Differentiation Toward Insulin-Producing Cells

Chencheng Wang[1,2], Shadab Abadpour[1,2], Petter Angell Olsen[2,3], Daxin Wang[4], Justyna Stokowiec[2], Simona Chera[5], Luiza Ghila[5], Helge Ræder[5,6], Stefan Krauss[2,3], Aleksandra Aizenshtadt[2] and Hanne Scholz[1,2]**

[1]Department of Transplant Medicine, Institute for Surgical Research, Oslo University Hospital, Oslo, Norway, [2]Hybrid Technology Hub, Center of Excellence, University of Oslo, Oslo, Norway, [3]Department of Immunology and Transfusion Medicine, Oslo University Hospital, Oslo, Norway, [4]Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, [5]Department of Clinical Science, University of Bergen, Bergen, Norway, [6]Department of Pediatrics, Haukeland University Hospital, Bergen, Norway

The generation of insulin-producing cells from human-induced pluripotent stem cells holds great potential for diabetes modeling and treatment. However, existing protocols typically involve incubating cells with un-physiologically high concentrations of glucose, which often fail to generate fully functional IPCs. Here, we investigated the influence of high (20 mM) versus low (5.5 mM) glucose concentrations on IPCs differentiation in three hiPSC lines. In two hiPSC lines that were unable to differentiate to IPCs sufficiently, we found that high glucose during differentiation leads to a shortage of NKX6.1+ cells that have co-expression with PDX1 due to insufficient *NKX6.1* gene activation, thus further reducing differentiation efficiency. Furthermore, high glucose during differentiation weakened mitochondrial respiration ability. In the third iPSC line, which is IPC differentiation amenable, glucose concentrations did not affect the *PDX1/NKX6.1* expression and differentiation efficiency. In addition, glucose-stimulated insulin secretion was only seen in the differentiation under a high glucose condition. These IPCs have higher KATP channel activity and were linked to sufficient *ABCC8* gene expression under a high glucose condition. These data suggest high glucose concentration during IPC differentiation is necessary to generate functional IPCs. However, in cell lines that were IPC differentiation unamenable, high glucose could worsen the situation.

Keywords: stem cell-derived beta cells, mitochondria, glucose, stem cell differentiation, induced pluripotent stem cells

OPEN ACCESS

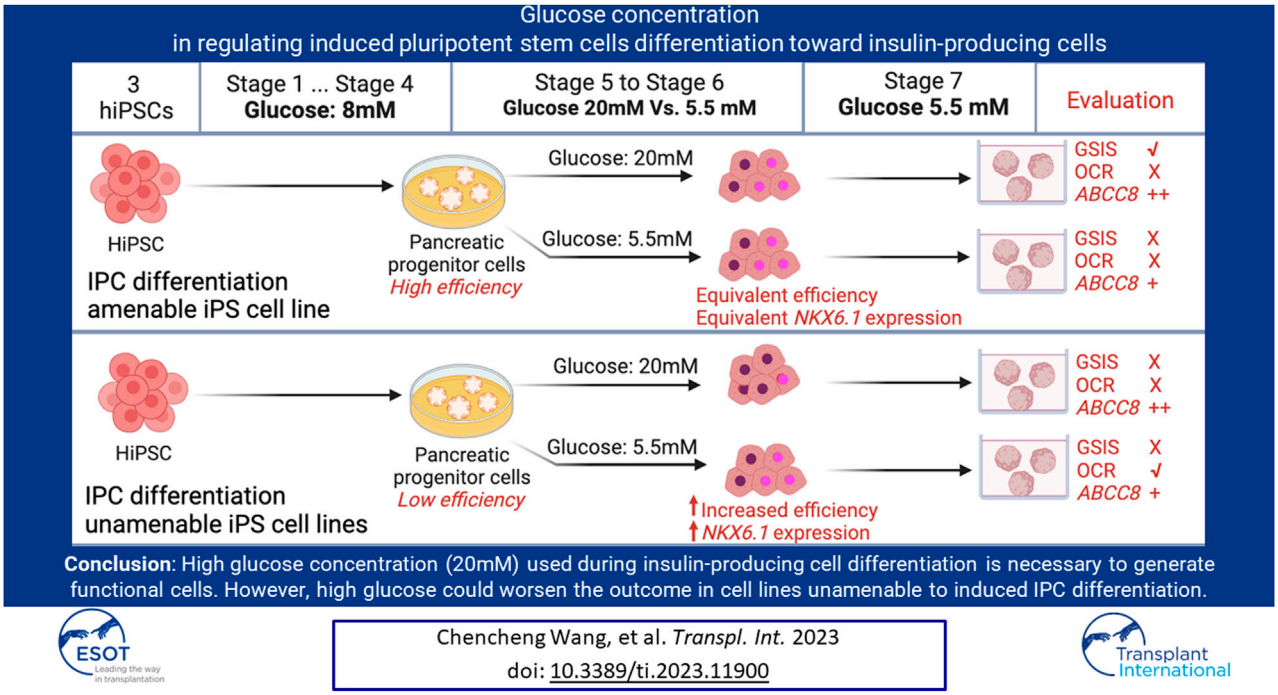
***Correspondence**
Chencheng Wang,
chencheng.wang@medisin.uio.no
Hanne Scholz,
hanne.scholz@medisin.uio.no

Received: *07 August 2023*
Accepted: *02 January 2024*
Published: *18 January 2024*

Citation:
Wang C, Abadpour S, Olsen PA, Wang D, Stokowiec J, Chera S, Ghila L, Ræder H, Krauss S, Aizenshtadt A and Scholz H (2024) Glucose Concentration in Regulating Induced Pluripotent Stem Cells Differentiation Toward Insulin-Producing Cells. Transpl Int 37:11900. doi: 10.3389/ti.2024.11900

INTRODUCTION

Cellular therapy as a treatment option for type 1 diabetes (T1D) may benefit from improving current protocols for generating insulin-producing cells (IPCs) from human-induced pluripotent stem cells (hiPSC). Existing studies have shown the possibility of using hiPSC for differentiating functional IPCs *in vitro* [1]. Glucose is an important energy source and a primary physiological regulator of insulin biosynthesis and secretion for IPCs [2]. IPCs differentiation from early reports [3, 4] to state-of-the-art protocols has relied on non-



GRAPHICAL ABSTRACT

physiological high glucose concentrations during the differentiation. These differentiation protocols applied a glucose concentration of 8–15 mM until the pancreatic progenitor (PP) stage (stage 4), followed by incubation in a differentiation medium containing 20–25.5 mM glucose in stage 5/6 but often reduced in the final maturation stage. The above protocols endowed IPCs with functional properties, but showed metabolic abnormalities, lower oxidative phosphorylation levels, and an immature mitochondria morphology [5–9]. It is known that high glucose causes adverse effects on human primary islets [10, 11], but why high glucose is needed during IPC differentiation has not been well studied.

To gain insights into the impact of different glucose concentrations in regulating IPCs differentiation from hiPSCs, we studied three hiPSC lines from different sources in our model by following a seven-stage protocol with minor modifications [3, 12]. In which, after reaching the pancreas progenitor stage (stage 4), a low (5.5 mM), non-physiological high (20 mM) and an insufficient energy condition mimicked by 5–6, 2-deoxy-D-glucose (2-DG) were applied to continue the differentiation until maturation stage. We analyzed the IPC differentiation efficiency, gene expression profiles, and co-localization of transcription factors such as NKX6.1 and PDX1. Furthermore, glucose's impacts on IPCs functionalities, including glucose-stimulated insulin secretion (GSIS), calcium flux, and oxygen consumption.

MATERIALS AND METHODS

Human iPSC Differentiation and Human Islets

The information on hiPSC and human islets was displayed in **Supplementary Tables S1, S2**. Human primary islets were maintained in CMRL 1066 (Corning, 15-110-CV) supplemented with 5% human AB serum (PAN-Niotech GmbH), L-Glutamine, 1% penicillin/streptomycin, 10 mM HEPES (all from Gibco) on ultra-low attachment plates (Corning, CLS3261). The hiPSCs were cultured in E8 Medium (Gibco, A1517001), and confirmed to be mycoplasma-free. The differentiation was done using the seven stages protocol [3] with modification [12]. On day 1 of the suspension culture, Rho Kinase inhibitor Y27632 (StemCell Technologies, 72304) was added to prevent cell death (**Supplementary Table S3**).

Flow Cytometry and Immunofluorescence Analysis

Primary and secondary antibodies were incubated for 45–60 min at RT or overnight at 4°C (**Supplementary Table S4**). LSR-II or LSRFortessa (BD Biosciences) and FlowJo (v.10.8.1, Treestar) were used for flow cytometry analysis. Images were taken with Leica TCS SP8 microscope and analyzed with Fiji (v.2.3.0). Trainable WEKA segmentation plugin [13] was used to identify particles in the images, and Fiji ROI manager was used to map the co-localization.

Glucose-Stimulated Insulin Secretion (GSIS)

Cell clusters were hand-picked into cell culture inserts (Merck, CLS3414) placed in 24-well cell culture plates. Cells were equilibrated in Krebs-Ringer buffer (KRB) with 1.67 mM glucose for 1 h at 37°C before being subjected to sequential 1-h incubation of 1.67 mM (Low), 20 mM (High) and 1.67 mM (Low) glucose, and then 20 mM glucose with 30 mM KCl in KRB for 30 min. Dynamic GSIS was performed using a perfusion system (Suprafusion 1000, BRANDEL). Sixty hand-picked cell clusters and 20 hand-picked human islets were used for each channel. Samples were collected every 6 min, and insulin was measured using human insulin ELISA kits (Mercodia, 10-1113-10).

Oxygen Consumption and Calcium Flux Analysis

The seahorse XFe24 analyzer (Agilent) was used to measure oxygen consumption, as described [14]. 40–60 cell clusters were picked for analysis. The oxygen consumption values were normalized to the baseline. Calcium imaging was performed as previously described [4]. Stage 7+ cell clusters were attached to 1: 100 diluted Geltrex-coated chambers (Ibidi, 80827), incubated at 37°C overnight, and labeled with 20 μM Fluo4-AM (Molecular Probes, F14201). Time series images were acquired every 15 s with Leica TCS SP8 and analyzed with Fiji. The Fiji plugin Register Virtual Stack Slices [15] was used for image alignment.

Insulin Contents, Lactate, and Glucose Measurement

Cells were lysis by CellTiter-Glo 3D Cell Viability Assay Kit (Promega, G9681) and measured with human insulin ELISA kits. The insulin content was normalized to the total protein. The lactate and glucose levels in the cell culture supernatant were measured by a blood gas analyzer (Radiometer, ABL800 FLEX). Glucose uptakes were calculated by glucose supplemented in medium minus glucose left in the daily cell culture supernatant. Uptake ratios were calculated from glucose uptakes divided by glucose supplemented.

Western Blot and qRT-PCR

Total proteins and RNA were isolated with TRIzol (Invitrogen, 15596026). Protein samples were separated with 8% Midi Protein Gels (Invitrogen, WG1001A). Primary and HRP-conjugated secondary antibodies were incubated at 4°C overnight or RT for 1 h. Images were developed in the ChemiDoc MP System (Bio-Rad). Semi-quantification analysis was conducted by using Fiji. A cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) was used for cDNA synthesis. PowerUp SYBR Green (Applied Biosystems, A25780) based RT-PCR was performed with Viia 7 RT-PCR system (Applied Biosystems). Gene expression was normalized to Tbp (TATA box binding protein) and human islets' gene expression profile. Heatmap was analyzed and plotted with Heatmapper [16] with the average linkage clustering method, and Manhattan clustering algorithms were selected to compute distances.

Mitochondrial Contents and Membrane Potential Analysis

Stage 6 cells were dissociated as single cells and incubated with 100 mM MitoTracker DeepRed (Invitrogen, M22426). To analyze insulin + subpopulation, samples were incubated with the anti-insulin antibody and analyzed with LSRFortessa. Undifferentiated hiPSC without dye or only with secondary antibodies was performed as the negative control. For mitochondrial membrane potential analysis, 2 μM JC-1 dye was incubated with cells for 30 min. Cells incubated with 4 μM CCCP were set up as negative control. For mtDNA/gDNA ratio analysis, the total DNA was extracted using the Mammalian Genomic DNA Miniprep Kits (Sigma, G1N70) and determined with SYBR Green-based qPCR. Primers are listed in **Supplementary Table S5**.

Statistical Analysis

Data were plotted as mean ± SD unless otherwise indicated. A two-tailored Student's t-test was used for the analysis of statistical significance by using GraphPad Prism 8.4.0 software. Sample size (n) is specified in each figure caption and indicates biological replicates unless otherwise noted.

RESULTS

Differentiation Under a High Glucose Concentration Medium Decreased NKX6.1/PDX1 Co-Localization in Non-Pancreatic Preferable Cell Lines

To study the impact of glucose concentration during IPC differentiation, three hiPSC lines derived PP cells (stage 4) continued to differentiate in high glucose (20 mM) and low glucose (5.5 mM) medium till stage 6 (**Figure 1A**). The cell line differentiation efficiency till stage 4 was exanimated (**Supplementary Figure S1A**). The InsCherry iPSC line differentiated stage 6 cells (InsCherry-stage 6 cells) under high glucose showed a stronger insulin signal, but the differentiation efficiency was unaffected (**Figures 1B, C**; **Supplementary Figure S2A**). PDX1 and NKX6.1 are critical transcription factors to maintain beta cell identity [17]. It has been shown PDX1-/NKX6.1+ cells can also continue to differentiate into IPCs [18]. However, low levels of *Pdx1* accompany IPCs' dysfunction in experimental models of glucotoxicity and diabetes [19]. Therefore, we quantified the NKX6.1/PDX1 subcellular co-localization in InsCherry-stage 6 cells. Over 95% of NKX6.1+ cells were co-localized with PDX1 among all NKX6.1+ cells (**Figure 1D**).

In contrast to InsCherry-stage 6 cells, the Babk2 and WTC cell lines demonstrated a lower IPC differentiation efficiency (**Supplementary Figure S1A**) and had less than 10% IPCs at stage 6 (**Supplementary Figures S2B, C**). The lower efficiency

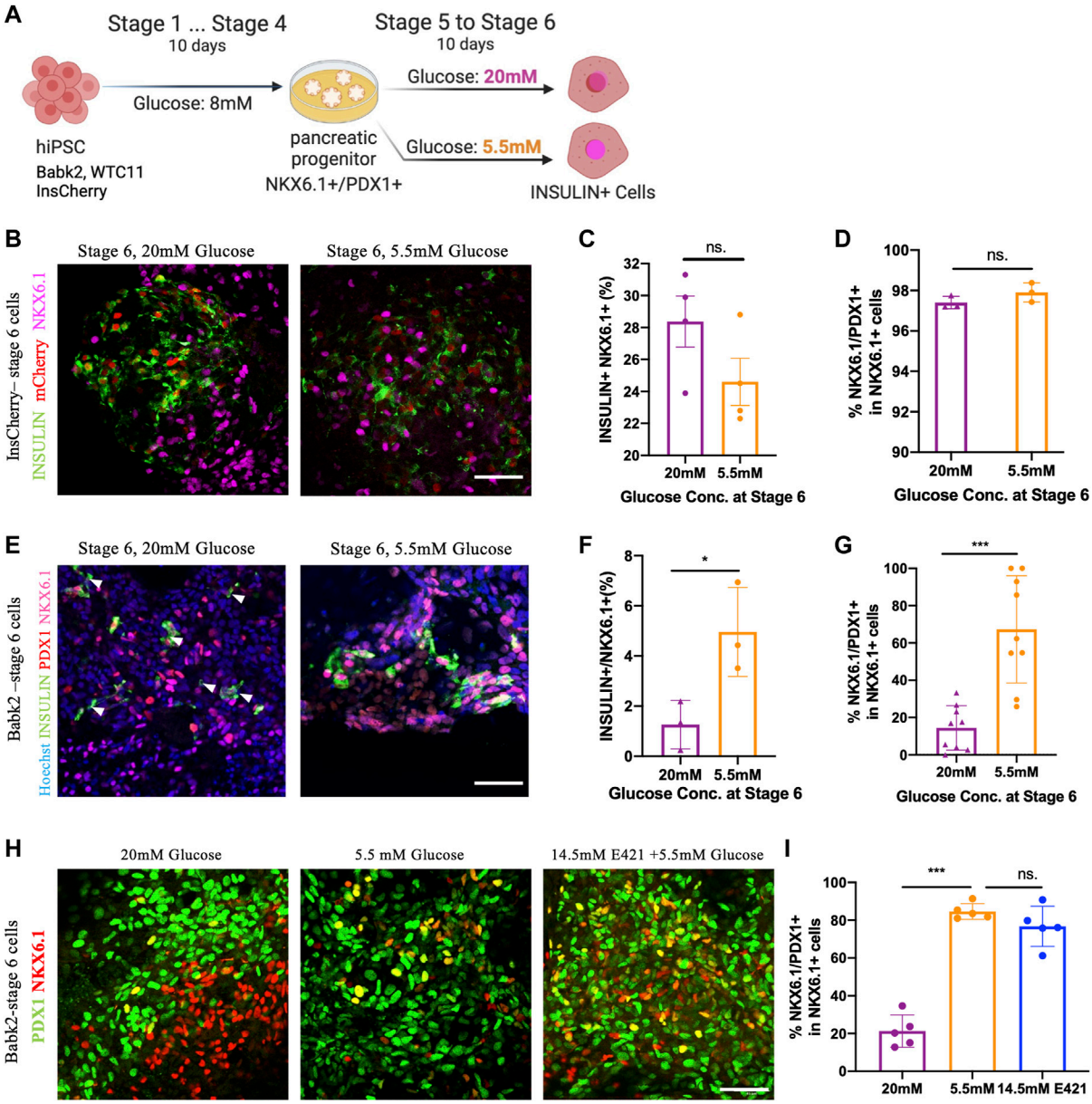


FIGURE 1 | High glucose concentration differentiation decreased NKX6.1/PDX1 co-localization in non-pancreatic preferable cell lines. **(A)** Outline of the experiment design: cells after the PP stage (stage 4) were cultured in stage 5 and 6 medium containing 5.5 mM or 20 mM of glucose for 10 days. Created with BioRender.com. **(B)** Immunostaining of INSULIN and NKX6.1 in InsCherry-stage 6 cells, mCherry also representing insulin + cells. **(C)** Flow cytometry quantification of InsCherry-stage 6 cells staining for INSULIN and NKX6.1, $n = 4$. **(D)** PDX1/NKX6.1 co-localization percentage among NKX6.1+ cells in InsCherry-stage 6 cells, $n = 3$. **(E)** Immunostaining for Babk2-stage 6 cells, the white arrowheads indicated the insulin + cells which have no NKX6.1 detected. **(F)** Flow cytometry analysis of Babk2-steg6 cells staining for INSULIN and NKX6.1, $n = 3$. **(G)** Immunostaining quantification analysis of PDX1 and NKX6.1 co-localization percentage among NKX6.1+ cells in Babk2-stage 6 cells, $n = 9$. **(H)** Immunostaining for Babk2-stage 6 cells for PDX1 and NKX6.1. **(I)** Immunostaining quantification analysis of NKX6.1/PDX1 co-localization percentage among NKX6.1+ cells in Babk2-stage 6 cells. E421 (mannitol), 14.5 mM mannitol supplemented in 5.5 mM glucose medium. $n = 5$. Scale bars represent 50 μm; ns. Non-significant; $^{*}p < 0.05$, $^{***}p < 0.001$ by unpaired two-way t-tests.

obtained from Babk2 and WTC11 cell lines is consistent with previous reports showing variations among cell lines [3, 4]. Interestingly, a low glucose differentiation of the Babk2 and WTC11 cell lines at stages 5–6 resulted in a higher percentage of IPCs at stage 6 (**Figure 1F**; **Supplementary Figures S2B, C**). In Babk2-stage 6 cells, the quantitative analysis of NKX6.1 subcellular co-localization showed less than 30% of NKX6.1+ cells co-localized with PDX1 when cells were differentiated in a high glucose medium (**Figure 1G**). A similar effect of high glucose impact in WTC11-stage 6 cells was observed (**Supplementary Figures S2D, E**). Furthermore, we frequently observed babk2-stage 6 INSULIN + cells with

undetectable NKX6.1 through immunostaining under high glucose differentiation (**Figure 1E**; **Supplementary Figure S2F**). Significantly more glucagon+/insulin + cells can be observed in Babk2-stage 6 cells differentiated under high glucose (**Supplementary Figures S3A, B**). Reduced proliferation is an important hallmark of mature beta cells [9]. The cell cycle distribution in the cells differentiated under different glucose conditions has no significant difference, but the InsCherry-stage 6 cells had a significantly higher proportion of phase G1 cells compared to Babk2-stage 6 cells differentiated under high glucose (**Supplementary Figures S3C, D**).

To determine whether the effect of abnormal co-expression of PDX1/NKX6.1 depends on glucose concentration but not osmotic pressure, we supplemented 14.5 mM mannitol in 5.5 mM glucose medium at stages 5–6 to mimic an equivalence osmotic pressure. In Babk2-stage 6 cells, there was no significant difference in NKX6.1/PDX1 co-localization between the low glucose and osmotic control group (**Figures 1H, I**). However, Babk2-stage 6 cells differentiated under high glucose constantly had significantly less NKX6.1/PDX1 co-localization among NKX6.1+ cells (**Figure 1I**). The results indicated the loss of PDX1 expression among NKX6.1+ cells was because of high glucose applied during differentiation in non-pancreatic preferable cell lines.

The recently published protocols decreased the glucose concentration from above 20 mM at the maturation stages [6, 8], in which the maturation stage was comparable with stage 7 and stage 7+ in this study. Therefore, we investigated whether decreasing the glucose concentration at the maturation stages could rescue the reduced NKX6.1/PDX1 co-localization (**Supplementary Figure S3E**). The stage 6 cells differentiated under high glucose did not show significant differences in NKX6.1/PDX1 co-localization after 7 days of incubation, regardless of the glucose concentrations applied (**Supplementary Figures S3F, G**). Furthermore, the cells showed less than 20% of NKX6.1/PDX1 co-localization on average, suggesting that the loss of co-localization may be irreversible *in vitro* by lowering the glucose concentration for non-pancreatic preferable cell line differentiation.

The analysis of Babk2 and WTC11 cell lines suggested a negative impact of differentiating IPCs in a non-physiological high glucose medium, leading to a lower IPC differentiation efficiency and less co-localization of NKX6.1/PDX1, in which high glucose shows a long-term negative impact on differentiation efficiency *in vitro*. Of note, the above effect may have been overlooked by focusing on improving IPC differentiation efficiency by amenable PSC lines, such as the InsCherry cell line.

The High Glucose Slows Down *NKX6.1* Gene Activation in Non-Pancreatic Preferable Cell Lines

To determine how different energy statuses could impact the IPCs differentiation during stages 5 and 6, 2-DG, a competitive inhibitor of glucose phosphorylation, was adopted to mimic a fasting condition during differentiation (**Figure 2A**). Given that prolonged incubation with 2-DG induced severe cell death (data not shown), 20 mM of 2-DG was added in the first 24 h in the stage 5 medium containing 5.5 mM glucose. The three iPSC lines derived stages 4, 6, and 7+ cells were collected for a pancreatic lineage specification gene expression analysis.

The gene expression analysis showed a significantly lower *NKX6.1* expression in Babk2-and WTC11-stage 6 cells under a high glucose condition (**Figure 2B**, left), and no significant difference was detected in *PDX1* expression at stage 6 (**Supplementary Figures S4A, B**). In addition, no significant difference in *NKX6.1* expression was found in InsCherry-stage 6 cells (**Figure 2B**, right), consistent with the immunostaining image quantification that shows no significant difference in NKX6.1/PDX1 co-localization (**Figure 1D**). The results indicate that NKX6.1/PDX1 co-localization reduction is due to insufficient *NKX6.1* activation under the high glucose differentiation at stage 6. However, the *NKX6.1* expression shows no significant difference for Babk2-stage 7+ cells, which revealed that the high glucose might slow down *NKX6.1* activation.

Pancreatic Lineage Gene Expression Analysis Revealed a Delayed *NGN3* Activation in Non-Pancreatic Preferable Cell Lines

NGN3 is critical in the specification of endocrine cell development [20]. Babk2-stage 4 cells had a lower *NGN3* expression than InsCherry-stage 4 cells (**Figure 2C**). At stage 6, upregulation of *NGN3* expression was only detected in the Babk2 cell line. The *NGN3* upregulation was more significant in a low glucose condition and was highly elevated in a nutrient-deficient condition mimicked by supplementing 2-DG in a 5.5 mM glucose medium. The applied protocol in this study conducted stage 5 cells as pancreatic endocrine precursors and stage 6 cells as immature beta cells, whereas *NGN3* should be activated in the early days of stage 5 [3]. Therefore, we concluded that *NGN3* has a delayed activation pattern in non-pancreatic preferable cell lines, such as the Babk2 in this study.

Although a possibly delayed *NGN3* activation was found in non-pancreatic preferable cell lines, a significantly higher *NGN3* expression was found in Babk2 (**Figure 2C**) and WTC11 (**Supplementary Figure S4B**) cell lines at stage 6 under low glucose. The Babk2 and WTC11 cell lines in low glucose differentiation had a higher IPC differentiation efficiency (**Figure 1F**; **Supplementary Figure S4B**), indicating that a low glucose condition could increase *NGN3* activation and thus improve IPCs differentiation efficiency in non-pancreatic preferable cell lines. This result provided insight into further optimizing the IPCs differentiation protocol, especially for cell lines such as Babk2 or WTC11.

Varied Glucose Concentrations Are the Primary Cause of the Different Gene Expression Profiles

Islet-1 (ISL-1) is critical for ensuring the differentiation of pancreatic endocrine progenitors [21]. Its expression in Babk2

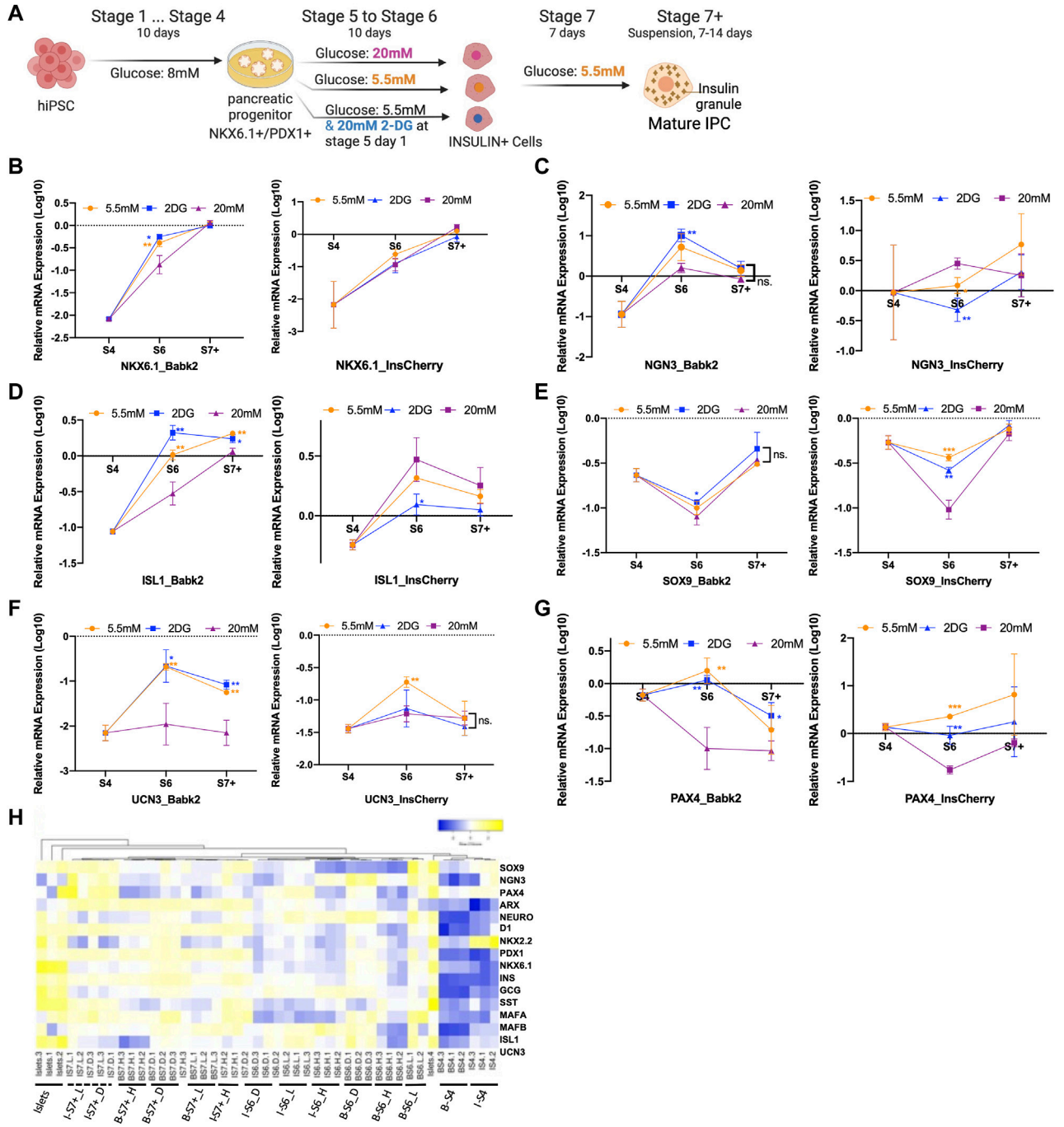


FIGURE 2 | Gene expression profile analysis. **(A)** Outline of the experiment design: cells after the PP stage (stage 4) were cultured in stage 5 and 6 medium containing 5.5 mM glucose with/without 20 mM 2DG, and 20 mM of glucose for 10 days, the cells were then entered to stage 7 and stage 7+ medium containing 5.5 mM glucose respectively. **(B–G)** Real-time PCR gene expression analysis of Babk2 and InsCherry cell line differentiated cells at different stages ($n = 3$). Data were normalized to *TBP* and then human islets ($n = 4$), "*Y*-axis = 0" representing the mean value of each gene expression in human islets. **(H)** Heatmap of 15 endocrine-related genes expression at different stages. "I", InsCherry cell line; "B", Babk2 cell line; "L", differentiation medium containing 5.5 mM low glucose; "H", differentiation medium containing 20 mM high glucose; "D", 20 mM 2-DG was added on the first day in the differentiation medium containing 5.5 mM glucose. 2-DG, 2-deoxy-D-glucose; S4, stage 4; S6, stage 6; S7+, cell clusters at stage 7+; ns, non-significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired two-way t-tests.

(**Figure 2D**, left) and WTC11 (**Supplementary Figure S4B**) cell lines benefited from low glucose and maintained a higher expression level until the end. In contrast, the glucose concentration had the opposite impact on *ISL1* expression in the InsCherry cell line (**Figure 2D**, right). Several other gene expressions displayed similar levels between cell lines. For

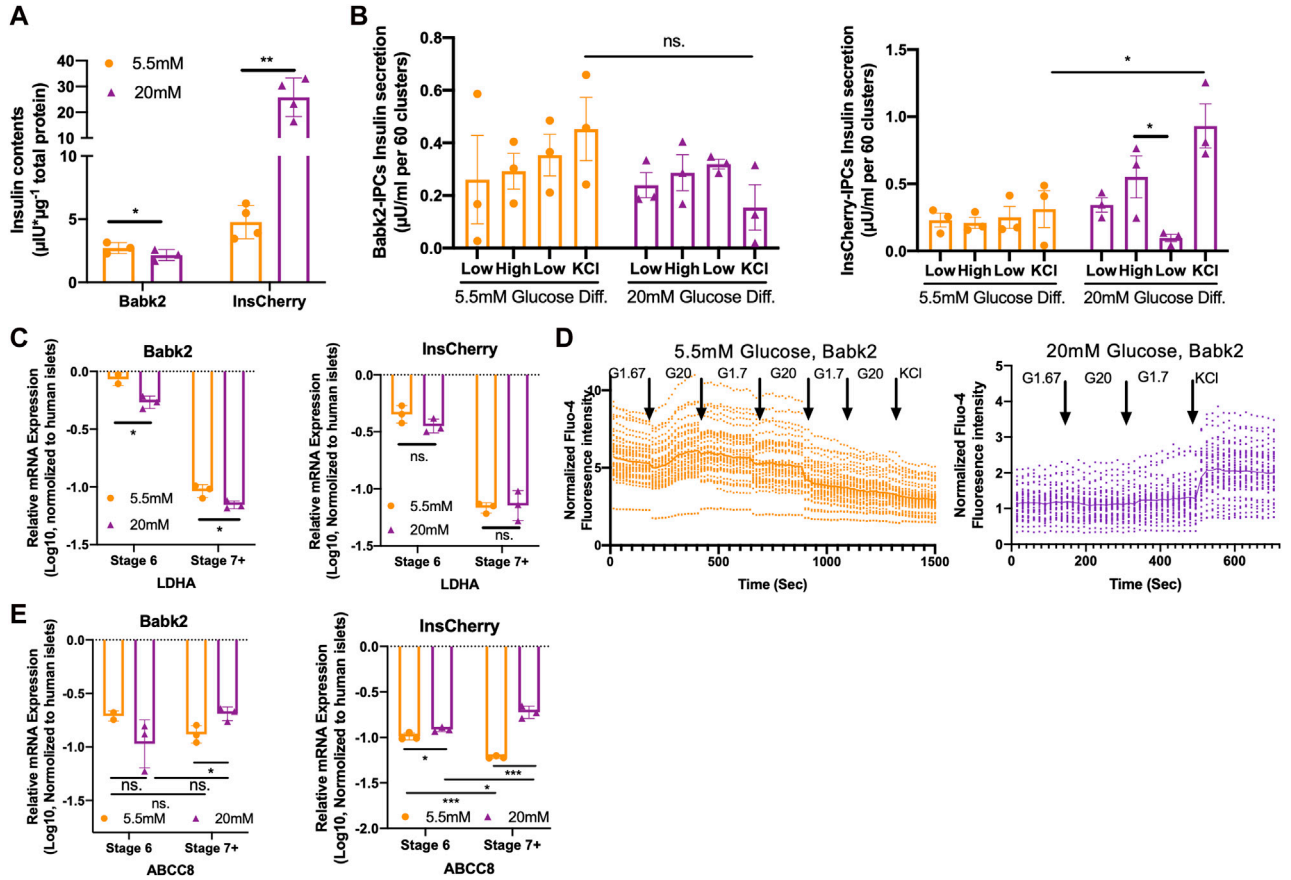


FIGURE 3 | High glucose concentration helps functional IPCs development. **(A)** Total insulin contents in Babk2-stage 7+ cells ($n = 3$) and InsCherry-stage 7+ cells ($n = 4$) differentiated under low (5.5 mM) and high (20 mM) glucose conditions. Insulin contents values were normalized to total protein. **(B)** GSIS of Babk2-stage 7+ cells (left) and InsCherry-stage 7+ cells (right) differentiated under low (5.5 mM) and high (20 mM) glucose conditions, $n = 3$. **(C)** *LDHA* expression analysis for Babk2 (left) and InsCherry (right) cell line differentiated cells at different stages ($n = 3$), data were normalized to *TBP* and human primary islets ($n = 4$). "*Y*-axis = 0" represents the mean value of *LDHA* expression in human islets. **(D)** The calcium flux analysis for Babk2-stage 7+ cells differentiated under low (5.5 mM, left) and high (20 mM, right) glucose conditions. Over 30 independent cells were traced and analyzed in each group, and the data were normalized to background light intensity. The solid line shows average Fluo-4 intensity. **(E)** *ABCC8* expression analysis for Babk2 (left) and InsCherry (right) cell line differentiated cells at different stages ($n = 3$), data were normalized to *TBP* and then human islets ($n = 4$), "*Y*-axis = 0" representing the mean value of *ABCC8* expression in human islets. Ns. Non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired two-way t-tests.

instance, *SRY-Box Transcription Factor 9 (SOX9)*, specific toward the non-endocrine cell lineage differentiation at the later stage of pancreatic development [22], had a lower expression at stage 6 under high glucose differentiation, suggesting that the high glucose concentration during differentiation inhibits non-endocrine cell development (**Figure 2E**). However, this short-term impact was not maintained till the end. Other beta cell maturation markers, including *Urocortin 3 (UCN3)* and *Paired box 4 (PAX4)*, had higher expression levels in a low glucose condition in both Babk2 and InsCherry differentiated cells (**Figures 2F, G**).

The gene expression correlation analysis revealed that Babk2 and InsCherry differentiated cells at the different stages were clustered separately (**Figure 2H**). The stage 7 cells formed as a separate group and were closer to human primary islets, meaning that the cells were successfully differentiated towards islet-like populations. Notably, the cells differentiated under low and high glucose conditions were clustered separately at each stage. Furthermore, Babk2-stage 6 cells in the osmotic control group clustered closer with the cells in the low glucose group but separated from cells differentiated under high glucose (**Supplementary Figure S4C**). Thus, variations in glucose concentrations during differentiation appeared to be a primary cause for the different gene expression profiles.

High Glucose Differentiation Improves Functional IPC Development

To investigate how the different glucose concentrations at stages 5 and 6 affect the functionality of the differentiated cells, we decreased the glucose concentration from 20 to 5.5 mM at stage 7 in the following studies (**Figure 2A**). The Babk2-stage 7+ cells had lower total insulin contents under high glucose differentiation (**Figure 3A**), which could be a consequence of

its lower IPC differentiation efficiency in high glucose. In contrast, InsCherry-stage 7+ cells had significantly higher total insulin contents in the cells differentiated under high glucose (**Figure 3A**).

The Babk2-stage 7+ cells did not show an activated insulin secretion in response to glucose stimulation, which is consistent with the InsCherry cell line differentiated under a low glucose condition (**Figure 3B**). Of note, the Babk2-Stage 7+ cells derived from high glucose differentiation showed a decreased but not significant KCl-mediated insulin secretion (3B, left), which might be because of the significantly lower IPCs yielding (**Figure 1F**) and insulin contents (**Figure 3A**). In contrast, the InsCherry differentiated under 20 mM glucose achieved an increased insulin secretion upon high glucose stimulation, and then the insulin secretion significantly decreased in response to the following incubation in a low glucose environment (**Figure 3B**, right). InsCherry-stage 7+ cells differentiated under high and low glucose conditions can respond to 20 mM glucose stimulation in dynamic GSIS evaluation but were not comparable with human primary islets (**Supplementary Figures S5A, B**).

High Glucose Concentration During Differentiation Efficiently Suppressed *LDHA* Gene Expression

We observed that the cell culture medium changed to bright-yellow under a high glucose condition at stages 5–6. Therefore, we hypothesized that the cells under a high-glucose differentiation had higher glycolytic activity and thus produced more lactate acid, which decreased the pH of the cell culture medium. The lactate measurements in daily medium supernatant supported that the cells differentiated under high glucose had a dramatically higher lactate production than in low-glucose conditions. In addition, the cells differentiated under low glucose have higher glucose utilization percentages (**Supplementary Figures S5C, D**).

Lactate dehydrogenase A (LDHA) is a so-called "disallowed" gene in beta cells due to its deficient expression in healthy beta cells [23]. We found that the *LDHA* was efficiently suppressed alongside the differentiation (**Figure 3C**). However, significantly less *LDHA* mRNA was detected in Babk2-derived cells under a high glucose condition (**Figure 3C**), suggesting a high glucose concentration could induce more efficient *LDHA* expression suppression in non-pancreatic preferable cell lines.

High Glucose Differentiation Improves KATP Channel Formation Through the Upregulation of *ABCC8* Gene Expression

Increased glucose levels lead to beta cell membrane depolarization, causing calcium ions influx and eventually triggering insulin secretion [4]. Therefore, we monitored the intracellular calcium flux in Babk2-stage 7+ cell clusters at the single-cell level. The Babk2-stage 7+ cells differentiated at low glucose responded to sequential glucose challenge by increasing intracellular calcium but failed to have increased intracellular calcium in response to cell membrane depolarization induced by 30 mM KCl (**Figure 3D**, left). In contrast, Babk2-stage 7+ cells differentiated under high glucose revealed few changes in Fluo-4 fluorescence intensity towards glucose stimulation but had an increased calcium influx after cell membrane depolarizing (**Figure 3D**, right).

To investigate why stage 7+ cells have a different calcium ions influx profile, we analyzed metabolism-related gene expressions under different glucose conditions (**Supplementary Figure S6**). Notably, the *ABCC8*, which encodes Sulfonylurea receptor-1 (SUR1) protein as a part of the KATP channel in regulating insulin secretion [24], was found to be significantly upregulated under high glucose differentiation (**Figure 3E**). In contrast, *ABCC8* expression decreased during stage 6 to stage 7+ under low glucose. Taken together with the lower expression of *ABCC8* and its failed activation during the maturation stages under low glucose, our results revealed the important role of glucose in regulating *ABCC8* expression, and a low glucose differentiation failed to trigger the KATP channel's efficient forming due to the inadequate activation of *ABCC8* expression.

High Glucose Differentiation Mediates the Inhibition of the Hippo Signaling Pathway

The YAP (Yes-associated protein) activation—Hippo signaling pathway inhibition after the PP stage could facilitate functional beta cell generation [25]. It has been reported that insufficient nutrient inhibits the Hippo signaling via YAP S127 phosphorylation that involves AMPK-mediated regulation of Angiomotin-like 1 (AMOTL1) protein and excludes YAP from the nucleus [26]. The phosphor-AMPKalpha (pAMPKa, phosphor-T172) to AMPKalpha (AMPKa) ratio was significantly higher in cells differentiated under high glucose, whereas total AMPKalpa remains no difference (**Figure 4A**; **Supplementary Figures S7A–C**). Due to the basal media without glucose supplementary was not commercially available and prolonged 2-DG supplementary inducing severe cell death during differentiation, we could not further investigate how the pAMPKa/AMPK ratio under a nutrient deprivation condition. However, it has been shown that lactate treatment upregulates the pAMPKa/AMPK ratio [27]. Thus, the upregulation of the pAMPKa/AMPKa might be due to the dramatically higher lactate produced by cells under high glucose conditions rather than the high glucose concentration applied during the differentiation (**Supplementary Figure S5C**).

AMOT was significantly higher in Babk2-and InsCherry-stage 6 cells differentiated under low glucose (**Figure 4B**). AMOT family proteins are YAP-binding partners that directly interact with YAP regulation, and AMOTL1 knockdown causes less YAP phosphorylation [28]. Thus, we investigated how glucose variations impact the Hippo by looking at the YAP protein. Babk2-and InsCherry-stage6 cells have a higher YAP expression under low glucose (**Figures 4C, D**). The total YAP protein did not show a substantial difference regardless of the glucose concentrations applied (**Figure 4E**; **Supplementary Figures S8A, B**). However, under low glucose conditions, the phosphorylated-YAP (p-YAP) to total-YAP ratio was higher in InsCherry- and Babk2-stage6 cells (**Figure 4F**). Thus, we identified a suppressed Hippo signaling pathway activity evidenced by a lower p-YAP/YAP ratio, which involves less stabilized AMOT protein (**Figure 4B**) under a high glucose

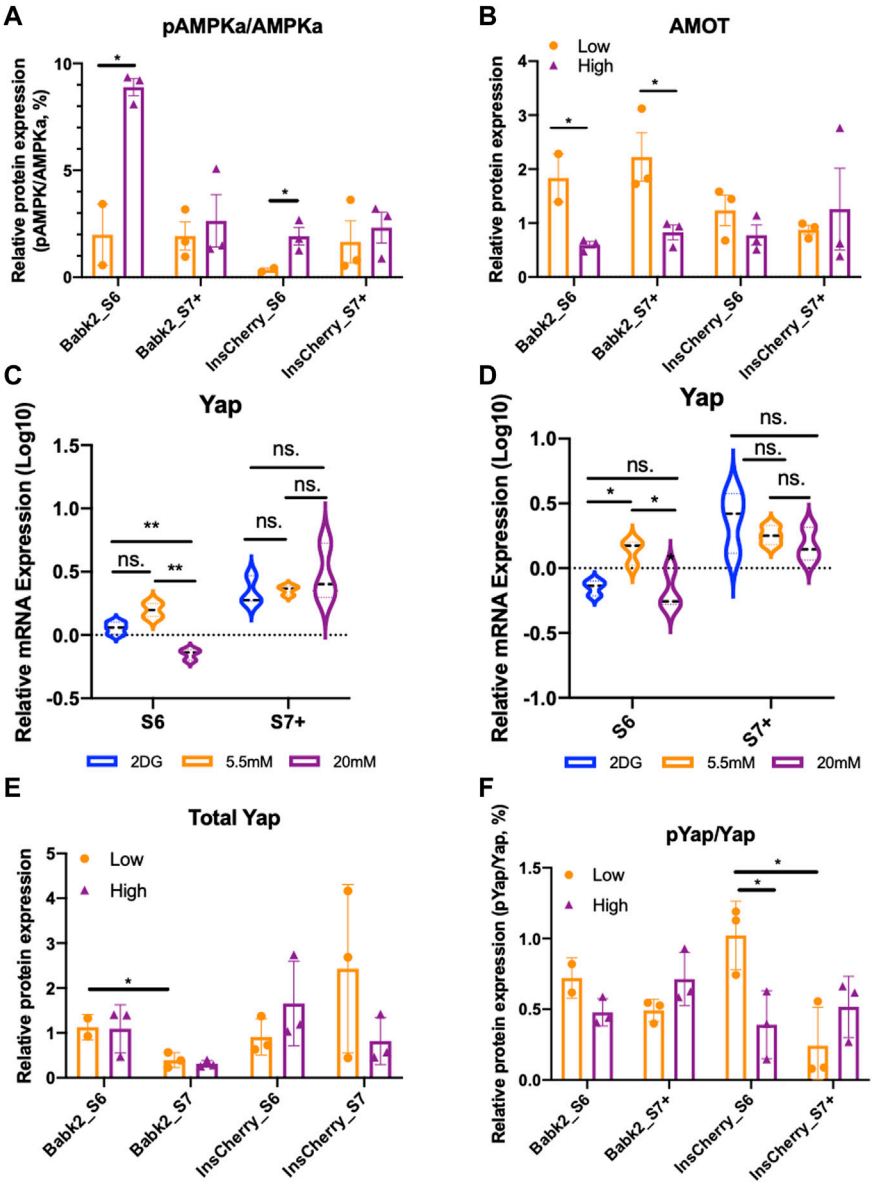


FIGURE 4 | The hippo signaling pathway was transiently regulated by different glucose levels. **(A)** Semi-quantification analysis of pAMPKa to AMPKa protein ratio from Babk2 and InsCherry cell line differentiated under low (5.5 mM) or high (20 mM) glucose conditions (unpaired one-way t-tests). **(B)** Semi-quantification analysis of AMOT protein from Babk2 and InsCherry cell line differentiated in low (5.5 mM) or high (20 mM) glucose conditions (unpaired one-way t-tests). **(C)** *Yap* mRNA expression analysis for Babk2 cell line differentiated cells at different stages ($n = 3$), data were normalized to *TBP* and human islets ($n = 4$), "*Y*-axis = 0" representing the mean value of *Yap* expression in human islets. **(D)** *Yap* mRNA expression analysis for and InsCherry cell line differentiated cells at different stages ($n = 3$). **(E)** Semi-quantification analysis of Yap protein in cells differentiated from Babk2 and InsCherry cell line in low (5.5 mM) or high (20 mM) glucose conditions. **(F)** Semi-quantification analysis of pYap to total Yap protein ratio from Babk2 and InsCherry cell line differentiated in low (5.5 mM) or high (20 mM) glucose conditions. ns, Non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$ by unpaired two-way t-tests.

condition at stage 6. Meanwhile, the total YAP protein stayed unchanged, suggesting that high glucose differentiation inhibited the Hippo signaling pathway, thus decreasing the IPCs differentiation efficiency in the Babk2 cell line. Of note, in the InsCherry cell line, even though a significant Hippo signaling pathway inhibition in a high glucose condition was found, the IPCs differentiation efficiency was not impacted.

High Glucose Weakened Mitochondrial Respiration Capacity

To investigate the effect of glucose levels on the mitochondrial contents during stepwise IPC differentiation, flow cytometry analysis with MitoTacker staining was used. There was no difference in the mitochondrial contents in stage 6 cells under different glucose concentrations (**Figure 5A**). Mitotracker

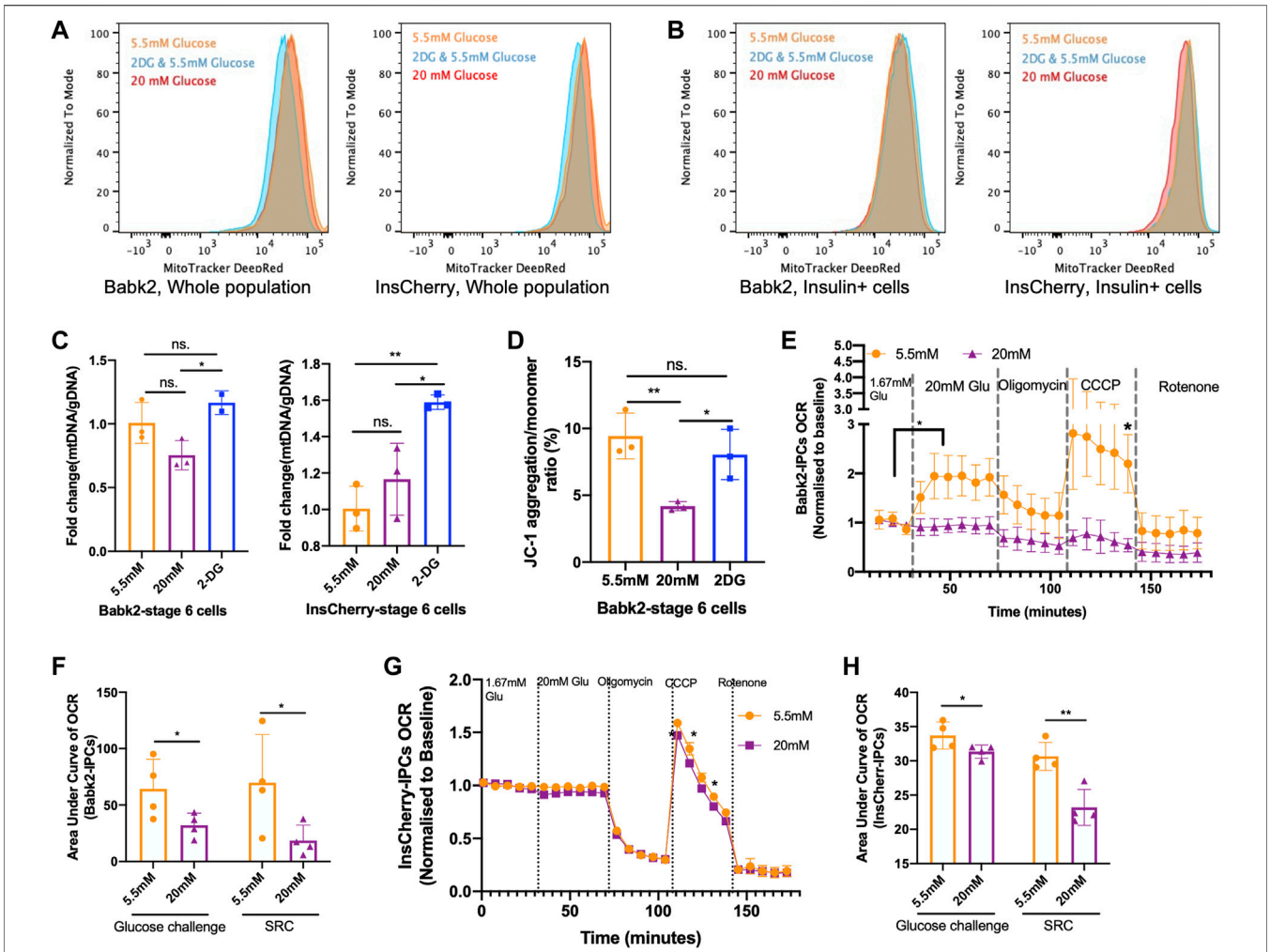


FIGURE 5 | High glucose weakened mitochondrial respiration capacity. **(A)** Flow cytometry assessment of the total mitochondrial contents in Babk2-stage 6 cells (left) and InsCherry-stage 6 cells differentiated at different glucose conditions. **(B)** The mitochondrial contents analysis among insulin + cells in Babk2-stage 6 cells (left) and InsCherry-stage 6 cells (right). **(C)** Mitochondrial DNA (mtDNA) to genomic DNA (gDNA) ratio analysis for Babk2-stage 6 cells (left) and InsCherry-stage 6 cells (right) differentiated at different glucose conditions ($n = 2$–4). **(D)** Flow cytometry quantification of JC-1 aggregation/monomer ratio in Babk2-stage 6 cells differentiated at different glucose conditions ($n = 3$). **(E)** Oxygen consumption rate (OCR) measurements of Babk2-stage 7+ cells under basal conditions and after sequential injections of glucose until final concentration reached 20 mM, Oligomycin 5 μM, CCCP 5 μM, and Rotenone 5 μM, values were normalized to average basal oxygen consumptions ($n = 4$). Data plotted as means ± SEM. **(F)** Area under the curve (AUC) analysis of OCR for Babk2-stage 7+ cells ($n = 4$, unpaired one-way t-tests). SRC, spare respiratory capacity (unpaired one-way *t*-test). **(G)** OCR measurements of InsCherry-stage 7+ cells. Values were normalized to average basal oxygen consumption ($n = 4$). Data plotted as means ± SEM. **(H)** AUC analysis of OCR for InsCherry-stage 7+ cells ($n = 4$, unpaired one-way t-tests). ns, Non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired two-way t-tests.

DeepRed was stained together with an anti-insulin antibody, but there was no significant difference in mitochondrial content among INSULIN + cells in Bbak2-stage 6 cells (**Figure 5B**, left). However, InsCherry-stage 6 cells under low glucose have more mitochondrial contents, as indicated by the peaks of Mitotracker DeepRed signal slightly right shifted among insulin + cells (**Figure 5B**, right; **Supplementary Figure S9A**), suggesting that the INSULIN + cells may have more mitochondrial in number under low glucose differentiation. A higher mtDNA/gDNA ratio was found in Babk2-stage 6 cells differentiated under low glucose but did not show statistical significance (**Figure 5C**). Furthermore, staining with JC-1, a mitochondrial membrane potential probe, revealed that Babk2-stage 6 cells had significantly higher JC-1 aggregation upon active mitochondria when differentiated under low glucose, which suggests that the mitochondria bioactivity in cells differentiated at high glucose might have been inhibited (**Figure 5D**; **Supplementary Figure S9B**).

The oxygen consumption rate (OCR) can better predict islets' clinical transplantation outcomes in a dose-dependent manner than GSIS [29]. In Babk2-and InsCherry-stage 7+ cells, we found no significant increase in oxygen consumption upon high glucose stimulation (**Figures 5E, G**). Similar OCR patterns have been reported by others [7, 30]. Opposite, the low glucose-induced

Babk2-stage 7+ cells showed a robust oxygen consumption (**Figure 5E**). InsCherry-stage 7+ cells showed no significant difference in OCR regardless of differentiation under low or high glucose (**Figure 5G**). The area under the curve (AUC) of the OCR analysis showed that the cells differentiated under low glucose had a significantly higher total oxygen uptake upon glucose challenge, and the spare respiratory capacity was significantly higher in both cells differentiated at a low glucose condition (**Figure 5F, H**). Our data suggested that the high glucose differentiation weakened the mitochondrial metabolic function.

DISCUSSION

Hyperglycemia, or high glucose exposure, can adversely affect beta cells [11, 31]. However, the effects of high glucose concentration in *in vitro* beta cell regeneration from hiPSCs have not been studied. By studying the stepwise IPCs differentiation model with three hiPSC lines from different sources, we demonstrated that the effect of glucose concentrations on the IPCs differentiation was cell line dependent, and we unveiled the unintended consequences of high glucose on IPCs differentiation. We showed that the iPS cell line (InsCherry cell lines) benefited from high glucose for IPC differentiation, but the non-pancreatic preferable cell lines (Babk2 and WTC11 cell lines) benefited from low glucose.

In healthy human pregnancies, the fetus's glucose supply depends on maternal circulation [32]. It is well known that fetal blood glucose levels are usually lower and fluctuate with maternal levels; lower glucose levels correlate with growth-retarded fetal, whereas high blood glucose may cause fetal over-growth [33–36]. In addition, a recent study showed that fetal insulin secretion depends on amino acids rather than glucose [37]. Thus, no evidence has been found *in vivo* that an unphysiologically high glucose level is required for beta cell development. Interestingly, the beta cell maturation process was accelerated when human embryonic stem cell (hESC) derived pancreatic progenitors were exposed to chronic hyperglycemia in mice models [38, 39]. However, in a recently published clinical trial with PP cell transplantation (clinicaltrials.gov: NCT02239354), the enrolled patients were directed to continue comparable insulin therapy to maintain blood glucose well-controlled peri- and post-transplant [40]. In human primary islet transplantation, keeping blood glucose concentrations between 4 and 7 mM peri- and post-transplant is recommended to minimize the loss of islets graft [41]. Thus, relying on high glucose levels to improve beta cell differentiation and maturation may need more concrete evidence, and it seems less practical *in vivo*. Nonetheless, our study revealed that high glucose is required to generate functional beta cells *in vitro* for the IPC differentiation amenable cell line—even though this may represent an artificial condition of the applied protocol and may not replicate the beta cell developmental biology.

Multiple studies have reported that the IPC differentiation efficiency varies from different cell lines [8, 42], thus reducing the flexibility of developing a universal protocol for customized autologous cell transplantation. InsCheery cell line and its parental cell lines have been well studied and characterized previously for IPC differentiation [42, 43]. To the best of our knowledge, WTC11 and Babk2 cell lines have not been used in IPC differentiation before. We recognized that the iPSC shows heterogeneity among cell lines, cells within a line, and temporal states of individual cells [44]. The above variations and complexity of IPC differentiation can influence the findings' reproducibility and scope of applicability to clinical settings. A dedicated project to investigate the heterogeneity of different clones from single-donor materials generated hiPSC, different donor derived hiPSC, and the comparison with human embryonic stem cells is ongoing and, thus, unable to be covered. Another limitation is the insufficient differentiation efficiency observed at the early stages for Babk2 and WTC11 cell lines, and the significant contributions from the off-target differentiation might dope to our observation even if the same differentiation matrix is applied. Thirdly, we found that an unphysiological high level of glucose (20 mM) was needed for the IPC differentiation amenable cell line differentiation, yet the most suitable glucose concentration remained to be defined. Finally, this study provides essential insight and may raise the attention to glucose concentration during IPC differentiation for future clinical applications, and more investigation is ongoing.

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 authors.

ETHICS STATEMENT

Human primary islets were provided by the Nordic Network for Islet Transplantation (Oslo University Hospital, Norway) after appropriate informed consent from relatives for multi-organ donation and for use in research, and approved by the Regional Ethics Committees (REK 67671 and REK 270665).

AUTHOR CONTRIBUTIONS

CW and HS conceived and designed the experiments. CW, SA, PO, DW, JS, and AA conducted the experiments, analyzed, and interpreted data. CW and HS wrote the manuscript, which was reviewed and edited by all the authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work has been supported by UiO: Life Science, the Research Council of Norway through its Centers of Excellence funding scheme, project number 262613, the Norwegian Diabetes Association, and Southern and Eastern Norway Regional Health Authority, project number 2023028.

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.

ACKNOWLEDGMENTS

We thank Siqing Liu, Anna Lång, Merete Høyem, and Yan Zhang for technical assistance in this study. Flow cytometry assays and confocal microscopy experiments were performed at The Norwegian Center for Stem Cell Research, Oslo University Hospital.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.11900/full#supplementary-material

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ORIGINAL RESEARCH
published: 31 January 2024
doi: 10.3389/ti.2024.12320

Peri-Transplant Inflammation and Long-Term Diabetes Outcomes Were Not Impacted by Either Etanercept or Alpha-1-Antitrypsin Treatment in Islet Autotransplant Recipients

*Tasneem R. Abdel-Karim[1], James S. Hodges[2], Kevan C. Herold[3], Timothy L. Pruett[4], Karthik V. Ramanathan[4], Bernhard J. Hering[4], Ty B. Dunn[4,5], Varvara A. Kirchner[4,6], Gregory J. Beilman[4] and Melena D. Bellin[1,4]**

[1]Department of Pediatrics, University of Minnesota, Minneapolis, MN, United States, [2]Division of Biostatistics, School of Public Health, University of Minnesota, Minneapolis, MN, United States, [3]Departments of Immunobiology and Internal Medicine, Yale University, New Haven, CT, United States, [4]Department of Surgery, University of Minnesota, Minneapolis, MN, United States, [5]Department of Surgery, Medical College of Wisconsin, Milwaukee, WI, United States, [6]Department of Surgery, Stanford University, Palo Alto, CA, United States

The instant blood-mediated inflammatory response (IBMIR) causes islet loss and compromises diabetes outcomes after total pancreatectomy with islet autotransplant (TPIAT). We previously reported a possible benefit of etanercept in maintaining insulin secretion 3 months post-TPIAT. Here, we report 2-year diabetes outcomes and peri-operative inflammatory profiles from a randomized trial of etanercept and alpha-1 antitrypsin (A1AT) in TPIAT. We randomized 43 TPIAT recipients to A1AT (90 mg/kg IV x6 doses, n = 13), etanercept (50 mg then 25 mg SQ x 5 doses, n = 14), or standard care (n = 16). Inflammatory cytokines, serum A1AT and unmethylated insulin DNA were drawn multiple times in the perioperative period. Islet function was assessed 2 years after TPIAT with mixed meal tolerance test, intravenous glucose tolerance test and glucose-potentiated arginine induced insulin secretion. Cytokines, especially IL-6, IL-8, IL-10, and MCP-1, were elevated during and after TPIAT. However, only TNFα differed significantly between groups, with highest levels in the etanercept group (p = 0.027). A1AT increased after IAT in all groups ($p < 0.001$), suggesting endogenous upregulation. Unmethylated insulin DNA ratios (a marker of islet loss) and 2 years islet function testing were similar in the three groups. To conclude, we found no sustained benefit from administering etanercept or A1AT in the perioperative period.

Keywords: islet transplantation, diabetes, beta cell death, anti-inflammatory, TPIAT

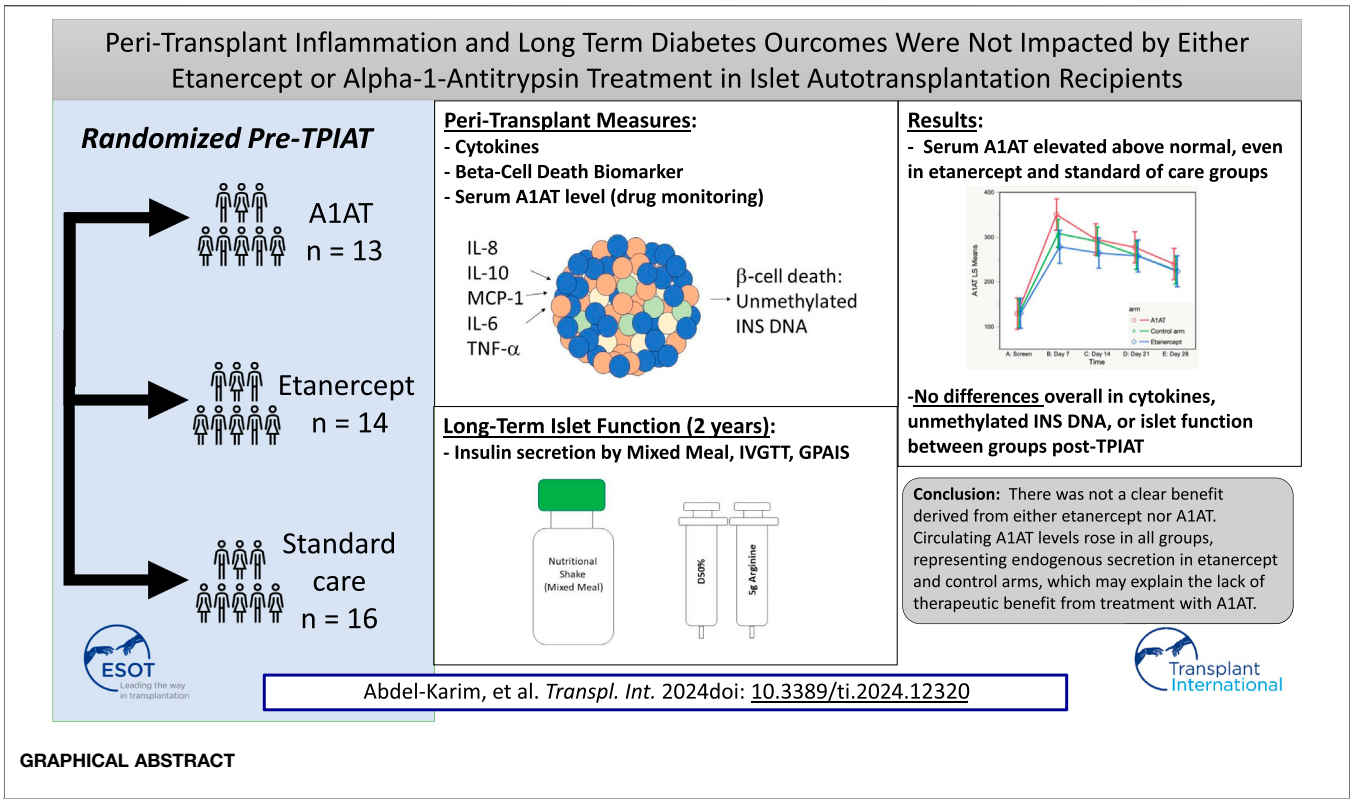
***Correspondence**
Melena D. Bellin,
bell0130@umn.edu

Received: 27 October 2023
Accepted: 11 January 2024
Published: 31 January 2024

Citation:
Abdel-Karim TR, Hodges JS, Herold KC, Pruett TL, Ramanathan KV, Hering BJ, Dunn TB, Kirchner VA, Beilman GJ and Bellin MD (2024) Peri-Transplant Inflammation and Long-Term Diabetes Outcomes Were Not Impacted by Either Etanercept or Alpha-1-Antitrypsin Treatment in Islet Autotransplant Recipients. Transpl Int 37:12320. doi: 10.3389/ti.2024.12320

INTRODUCTION

Total pancreatectomy (TP) is an effective treatment to relieve or reduce severe pain in patients with chronic pancreatitis or recurrent acute pancreatitis who do not respond to medical or endoscopic therapies. Islet auto transplantation (IAT) prevents or ameliorates brittle diabetes after total pancreatectomy [1–6].



GRAPHICAL ABSTRACT

Long-term insulin independence is an important goal in TPIAT as it reduces the burden of diabetes management and improves quality of life [4]. Many patients, however, never reach insulin independence. In one report, only 30% of patients undergoing TPIAT achieved insulin independence 3 years post-TPIAT [5]; this percentage showed a downward trend over time, dropping to about 11% 10 years post-TPIAT [7]. Successful engraftment of transplanted islet cells into the liver is crucial for long-term insulin independence. The survival and function of transplanted islet cells is negatively impacted by inflammation in the peri-transplant period, the so-called instant blood mediated inflammatory reaction (IBMIR) [8]. Therefore, blockading the innate inflammatory response could substantially improve engrafted islet mass and thereby reduce diabetes risk after TPIAT [9].

Aiming to reduce islet loss from IBMIR, we studied two promising anti-inflammatory therapies: the TNFα inhibitor etanercept, and alpha-1 antitrypsin (A1AT). Etanercept is a potent anti-inflammatory drug that targets TNFα, a key central inflammatory mediator in beta loss after transplant. Etanercept is widely used as an anti-inflammatory drug in intraportal *allo*transplantation for type 1 diabetes, with studies suggesting efficacy in this setting [10]. A1AT is a serine protease inhibitor indicated for treating alpha-1 antitrypsin deficiency. Multiple preclinical studies, including autologous islet transplant models in non-human primates, suggested that A1AT enhances islet engraftment and prevents beta cell apoptosis by suppressing the instant blood mediated inflammatory reaction [11–15]. We previously reported early outcomes with both drug therapies in a randomized pilot clinical trial, which suggested a greater first phase insulin response at 3 months only in the etanercept group [16]. It was not clear, however, whether any benefit was sustained 1 year post-TPIAT.

This current analysis reports 2-year outcomes for insulin use and islet function following TPIAT in our clinical trial participants treated with etanercept and A1AT. We also evaluated potential mechanistic pathways targeted by these agents by measuring cytokine levels, beta cell death measured by unmethylated insulin DNA, and circulating levels of A1AT during the peri-transplant period.

PATIENTS AND METHODS

Participants

Forty-four adult patients between 18 and 68 years old, who were scheduled for TPIAT at the University of Minnesota (UMN) from 12/2016 through 3/2020, were enrolled. Exclusions included pre-existing diabetes or other medical contraindications that could compromise participant safety; please refer to our first publication from this study for details about the exclusion criteria [16]. One individual did not meet inclusion criteria for randomization based on labs obtained at the screening visit, so 43 participants were randomized to receive etanercept (n = 14), A1AT (n = 13), or no treatment (controls, n = 16) as detailed below.

Informed consent was obtained from all participants before screening. The study protocol was reviewed and approved by the University of Minnesota's Institutional Review Board. This study

was performed under an Investigational New Drug Application (IND #119828) from the Food and Drug Administration and registered on clinicaltrials.gov (NCT#02713997).

Surgical and Islet Isolation Procedure

Participants underwent total pancreatectomy with partial duodenectomy, splenectomy, cholecystectomy, and roux-en-Y duodenojejunostomy [17]. Islet cells were extracted through enzymatic digestion using Vitacyte CIzyme Collagenase HA (Vitacyte LLC, Indianapolis, IN) with SERVA/Nordmark Neutral Protease NB (SERVA Electrophoresis GmbH, Heidelberg, Germany) followed by mechanical disruption using the semi-automated Ricordi method [18]. These isolated islet cells were then introduced into the liver's portal vein. Throughout the infusion of islet cells, portal pressures were closely monitored. In cases where elevated portal pressures were observed, some islets were directed to other locations, primarily within the peritoneal cavity. Heparin was administered at the time of islet infusion in the form of a 70 unit/kg bolus, with 35 u/kg incorporated into the islet preparation and 35 u/kg given to the patient. Subsequently, low dose heparin was administered either intravenously or subcutaneously (enoxaparin) and continued for 1 week post-transplantation. Islet mass was quantified as islet equivalents (IEQ) or IEQ per kilogram of recipient body weight (IEQ/kg), which standardizes islet mass to a size of 150 μm, consistent with established practices in islet research. We also evaluated islet number (IN) and IN/kg as measures of the total count of islets without adjusting for islet volume.

Treatment Provided

Patients were randomized in a 1:1:1 ratio to A1AT, or etanercept, or standard care. Alpha-1 antitrypsin (Aralast NP) was administered intravenously at a dose of 90 mg/kg, with the first dose administered 1 day before surgery and subsequent doses on days 3, 7, 14, 21, and 28 after infusion. Etanercept was given at a dose of 50 mg subcutaneously on day 0 (pre-operatively), and 25 mg subcutaneously on days 3, 7, 10, 14, 21.

Randomization was stratified on BMI (< or ≥27 kg/m^2). The investigational pharmacist dispensed study medication according to a randomization schedule provided by the biostatistician. Because the drugs are administered intravenously and subcutaneously, this pilot study was not blinded.

Study Visits and Assessments

Participants attended 2-day study visits to assess islet function before surgery ("Baseline"), at 3 months and at 1 and 2 years after TPIAT. Multiple blood draws were performed in the perioperative period for mechanistic assays, as detailed below. All study participants contributed to the mechanistic data, while 42 had remote or in-person follow up (32 in person, with metabolic testing). The "2 years" visit was complicated by COVID restrictions and an institutional-mandated pause in study visits so the 2-year window was expanded to 2–3 years post-TPIAT to capture participants who were able and willing to return. The average time of this visit was 2.2 years post TPIAT. For participants unwilling or unable to travel back, as much data as possible were collected virtually.

At each study visit, participants underwent comprehensive metabolic testing with mixed meal tolerance testing (MMTT), intravenous glucose tolerance testing (IVGTT), and glucose-potentiated arginine-induced insulin secretion (GPAIS) studies as described below. Hemoglobin A1c (HbA1c) level was also measured. Efficacy of islet graft function was assessed based on metabolic testing measures, HbA1c, insulin use and insulin dose. Blinded continuous glucose monitoring (iPro2, Medtronic) data were collected for 6 days following the 3-month, 1-year and 2-year visits to assess mean glucose, standard deviation, and percent of time in hypo- and hyperglycemia. Because iPro was discontinued by the manufacturer shortly before the conclusion of the study, CGM is available for fewer participants.

Mixed Meal Tolerance Testing

For the mixed-meal tolerance test (MMTT), measurements of glucose and C-peptide levels were taken at time 0, 30, 60, 90, and 120 min. Participants were given Boost HP, at a dose of 6 mL/kg (maximum 360 mL), consumed within 5 minutes after the initial time 0 blood draw. The area under the curve (AUC) for glucose (AUC glucose) and C-peptide (AUC C-peptide) were calculated using the trapezoidal rule, which included the baseline.

Intravenous Glucose Tolerance Testing

For the intravenous glucose tolerance test (IVGTT), a bolus of 0.3 g/kg of dextrose was administered at time 0. Samples of insulin, C-peptide, and glucose were drawn at times −10, −5, −1, and 1, 2, 3, 4, 5, 7, and 10 min. The AUC for the 10-min insulin or C-peptide values, minus the baseline, was used to determine the acute insulin response to glucose (AIRglu) and acute C-peptide response to glucose (ACRglu), respectively.

Glucose-Potentiated Arginine-Induced Insulin Secretion

The glucose-potentiated arginine stimulation (GPAIS) test was conducted after the IVGTT. A 20% dextrose solution was administered via infusion, commencing at +20 min after the IVGTT dextrose bolus. The infusion was maintained at a variable rate to attain a blood glucose target of approximately 230 mg/dL until the test was completed. Blood glucose levels were monitored every 5 minutes, using a bedside autoanalyzer to maintain glucose within the targeted range. At +60 min, after a minimum of 30 min of maintaining the target blood glucose level, baseline samples for glucose, insulin, and C-peptide were drawn at three intervals over 5 min. A 5-gram bolus of arginine was then administered, and samples for glucose, insulin, and C-peptide were taken at 2, 3, 4, 5, 7, and 10 min following the arginine bolus. The test results were used to calculate the glucose-potentiated acute insulin response to arginine (AIRpot) and glucose-potentiated acute C-peptide response to arginine (ACRpot) as surrogate markers for islet mass.

Sample Collection

Inflammatory cytokines were measured at various intervals throughout the study: twice during pre-operative screening, once immediately before islet infusion, and then at 1, 3, 6, 12, 24 h, and 3 and 7 days after IAT. These cytokines included interferon gamma-induced protein 10 (IP-10), interleukin

TABLE 1 | Characteristics of the treatment groups, displayed as mean (SD) or N (%).

Characteristic	All participants	Control	Etanercept	A1AT	*p*-value
n	43	16	14	13	
Age, years	38.5 (12.0)	37.4 (12.1)	40.8 (12.0)	37.3 (13.1)	0.70
Sex (male)	17 (40%)	3 (19%)	4 (29%)	9 (75%)	**0.009**
White	40 (93%)	16 (100%)	11 (79%)	12 (100%)	0.051
Hispanic	4 (9%)	1 (6%)	1 (7%)	2 (17%)	0.66
BMI Pre-TPIAT (kg/m^2)	24.9 (4.2)	24.1 (4.5)	24.7 (3.7)	25.8 (4.7)	0.58
Etiology					0.20
Genetic	20 (47%)	7 (44%)	4 (29%)	8 (67%)	
Obstructive	9 (21%)	2 (13%)	6 (43%)	1 (8%)	
Idiopathic	10 (23%)	6 (38%)	2 (14%)	2 (17%)	
Other	4 (19%)	1 (6%)	2 (14%)	1 (8%)	
Total IEQ (x 10^5)	2.4 (1.3)	2.2 (1.4)	2.7 (1.3)	2.4 (1.3)	0.58
IEQ/kg	3,313 (1987)	3,341 (2,396)	3,800 (1899)	2,864 (1,460)	0.50
Intraportal IEQ/kg	3,046 (1766)	3,096 (2,172)	3,260 (1,592)	2,752 (1,455)	0.78
Total islet number (x 10^5)	2.7 (1.4)	2.8 (1.6)	3.2 (1.5)	2.3 (1.1)	0.28
Islet number per kg body weight	3,851 (2,239)	4,329 (2,758)	4,485 (1964)	2,687 (1,140)	0.075
Tissue volume (mL)	14.1 (9.8)	13.5 (10.3)	18.5 (10.3)	10.4 (7.2)	0.10
All islets intraportal	31 (72%)	12 (75%)	6 (43%)	12 (100%)	**0.004**
Pre-Op labs					
Hba1c	5.3 (0.4)	5.3 (0.4)	5.2 (0.4)	5.3 (0.4)	0.51
ACRglu (ng/mL*min)	30.8 (18.9)	25.9 (12.5)	39.6 (26.8)	27.0 (10.7)	0.097
ACRpot (ng/mL*min)	8.3 (4.5)	7.2 (4.1)	9.2 (4.4)	8.7 (5.3)	0.47
AIRglu (mU/L*min)	472 (398)	382 (270)	632 (579)	398 (179)	0.18
AIRpot (mU/L*min)	176 (115)	158 (107)	186 (133)	187 (109)	0.74
AUC_glucose (10^4 mg/dL*min)	1.38 (0.23)	1.40 (0.26)	1.35 (0.19)	1.34 (0.20)	0.73
AUC_C-peptide (ng/mL*min)	600 (408)	545 (432)	730 (502)	525 (230)	0.37

P-*values are in bold for statistically significant differences.*

TABLE 2 | Two-year outcomes.

	N	Control	Etanercept	A1AT	*p*-value
2 Year outcomes, *N (%) or mean (SE)*					
On Insulin	42	14 (87.50%)	9 (64.29%)	10 (83.33%)	0.32
Insulin dose (u/day)	40	13.0 (3.5)	16.8 (3.9)	16.1 (4.3)	0.75
Hba1c (%)	37	6.3 (0.4)	7.1 (0.4)	7.0 (0.4)	0.45
FSIVGTT, ACRglu (ng/mL*min)	32	5.53 (2.10)	7.96 (2.39)	5.77 (2.52)	0.72
FSIVGTT, AIRglu (Mu/L*min)	32	112.0 (37.6)	149.2 (42.8)	105.7 (45.2)	0.74
GPAIS, ACRpot (ng/mL*min)	31	1.38 (0.45)	2.03 (0.51)	2.60 (0.57)	0.25
GPAIS, AIRpot (mU/L*min)	31	34.3 (11.8)	47.4 (13.5)	56.9 (15.1)	0.49
MMTT, AUC_glucose (10^4 mg/dL*min)	32	21,493 (1921)	18,040 (2,190)	21,846 (2,309)	0.40
MMMT, AUC_C-peptide (ng/mL*min)	32	165 (39)	276 (44)	235 (47)	0.18
Mean glucose on CGM, mg/dL	27	146.6 (11.7)	136.6 (14.0)	129.0 (15.9)	0.66
% Time >180 mg/dL	27	25.6 (7.5)	19.2 (9.0)	12.2 (10.2)	0.56
% Time <70 mg/dL	27	2.0 (0.8)	1.3 (0.9)	2.7 (1.1)	0.66

1 alpha (IL-1 α), interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor alpha (TNF-α).

Levels of serum Alpha 1 antitrypsin (sA1AT) were assessed at five times: before surgery and at 7, 14, 21, and 28 days following islet infusion.

Unmethylated INS DNA levels were measured 18 times, at baseline before MMTT and IVGTT and before islet infusion and then at 15 min, 30 min, 1, 3, 6, 12, and 24 h and at days 3, 7, 14, 21 and 28 post TPIAT. Unmethylated insulin DNA levels were assessed after that at 3 months, 1 year and 2–3-year visit.

Serum samples were collected and stored in separate aliquots at −80°C until assayed.

Mechanistic Assays

Cytokines

Cytokine samples were tested by the Cytokine Reference Laboratory (CRL, University of Minnesota). Samples were analyzed for human specific IL-6, IL-8, IL-1α, IL-1β, MCP-1, TNFα, IL-10, and IP10 using the Luminex platform and performed as a multi-plex. The magnetic bead set (cat. # FCSTM18-08; Lot # 1598610) was purchased from R&D Systems, Minneapolis MN.

Serum Alpha-1 Antitrypsin

The sA1AT levels were processed by the M Health Fairview laboratory and sent to the Mayo Diagnostic Laboratory for analysis using Siemens Nephelometer II.

Unmethylated INS DNA

The unmethylated INS DNA assay used droplet digital polymerase chain reaction (ddPCR). Quick-cfDNA Serum & Plasma kits and EZ DNA Methylation kits (Zymo Research,

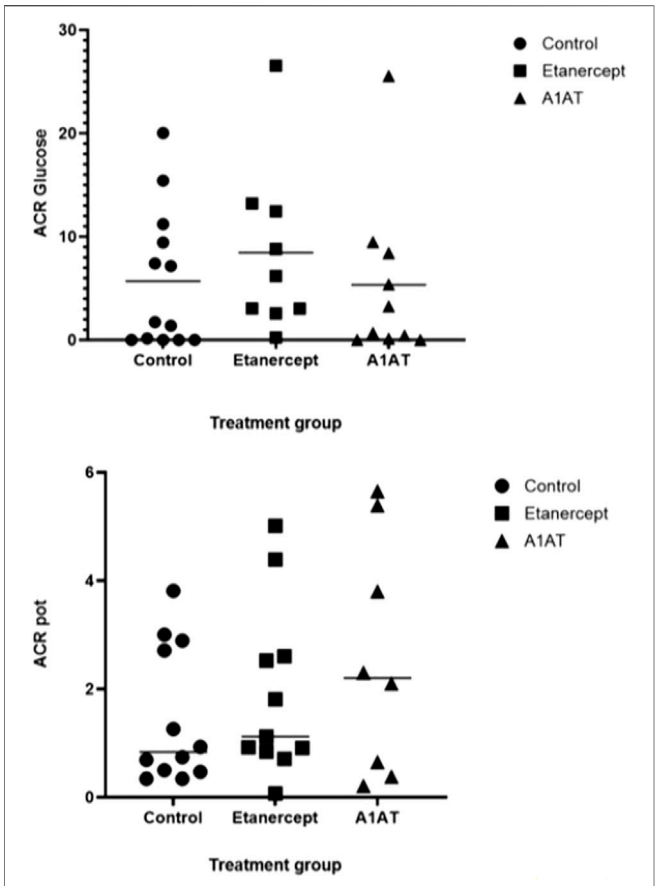


FIGURE 1 | ACR glu and ACR pot at 2 years post TPIAT.

Irvine, CA) were used for DNA purification and bisulfite treatment of the DNA. We measured the levels of INS DNA by droplet digital PCR targeting two methylation-sensitive sites of the human INS gene in positions +396 and +399 from the transcription start site (hg19_knownGene_uc021qcd.1 range = chr11:2181009–2182439). Each 25-uL reaction volume consisted of Droplet Digital PCR supermix (Bio-Rad Laboratories, Hercules, CA), 900 nM of primer, 250 nM of probe and 10-uL of sample. The mixture and droplet generation oil were loaded onto a droplet generator (Bio-Rad Laboratories), and the generated droplets were transferred to a 96-well PCR plate. The PCR reaction was run on a thermal cycler with a 10-min activation at 95°C, 40-cycle two-step amplification protocol (30 s at 95°C denaturation and 60 s at 58°C), and 10-min inactivation step at 98°C. The DNA content of the droplets was analyzed with a QX200 droplet reader (Bio-Rad Laboratories) and QuantaSoft analysis software (Bio-Rad Laboratories). Discrimination between droplets that did and did not contain the target (positive and negative, respectively) was achieved by applying a fluorescence amplitude threshold based on the amplitude read from the negative template control. For each sample, the ratio was calculated as the unmethylated counts divided by the sum of the unmethylated and methylated counts (U/[M + U])

Statistical Methods

Characteristics of the three treatment groups (**Table 1**) were compared using one-way ANOVA for characteristics on continuous scales (e.g., BMI) and Fisher's exact test for characteristics on categorical scales (e.g., etiology).

For group comparisons according to diabetes outcomes and islet cell function (**Table 2**), we present unadjusted analyses using Fisher's exact test (on insulin, yes/no) or one-way ANOVA (all other outcomes). We performed two sets of adjusted analyses, adjusting for IEQ/kg and for IEQ/kg and sex; the results did not change notably (data not shown). The adjusted analyses used logistic regression (on insulin, yes/no) or multiple linear regression (all other outcomes).

Analyses of cytokine profiles, sA1AT, and unmethylated insulin DNA included multiple time points per participant and used mixed linear models with the restricted-likelihood method, with fixed effects group, time (treated as a categorical factor), and their interaction; adjusted analyses added adjusters as fixed effects. The random effect was participant. For analyses of cytokine profiles, the outcome (dependent variable) was the common logarithm of the measured cytokine level. For the analysis of unmethylated insulin DNA, the outcome was the common logarithm of U/(U + M) + 0.026; the latter is the 2.5th percentile of non-zero U/(U + M) values and was added to all values so the log transformation could be applied to all values including zeroes.

All analyses used JMP (v. 16.1.0 Pro, SAS Institute Inc., Cary NC USA).

RESULTS

Participant Characteristics

Table 1 describes the study's participants. The age, BMI, and initial metabolic test results were comparable across the groups. However, the A1AT group had a significantly greater proportion of male participants (p = 0.004). Although the groups had comparable IEQ per kg body weight, the A1AT group had lower islet number per kg (not adjusted for islet size) (p = 0.045). The etanercept group was more likely to receive islet transplantation outside the liver (p = 0.003), which might be due to a trend towards higher volumes of infused tissue in this group (p = 0.08). When comparing only the intraportal IEQ/kg infused, all groups received a similar intraportal islet mass.

Diabetes Outcomes and Islet Cell Function 2 Years Post TPIAT

Insulin dose and glycemic control were similar in the three groups 2 years post-transplant (**Table 2**). Islet cell function assessed by MMTT, IVGTTT and GPAIS did not differ significantly between the three groups. The etanercept group had the best response to IVGTT (ACRglu and AIR glu) compared to control group and A1AT group (**Figure 1**) but this difference was not statistically significant (p = 0.72, p = 0.74 respectively).

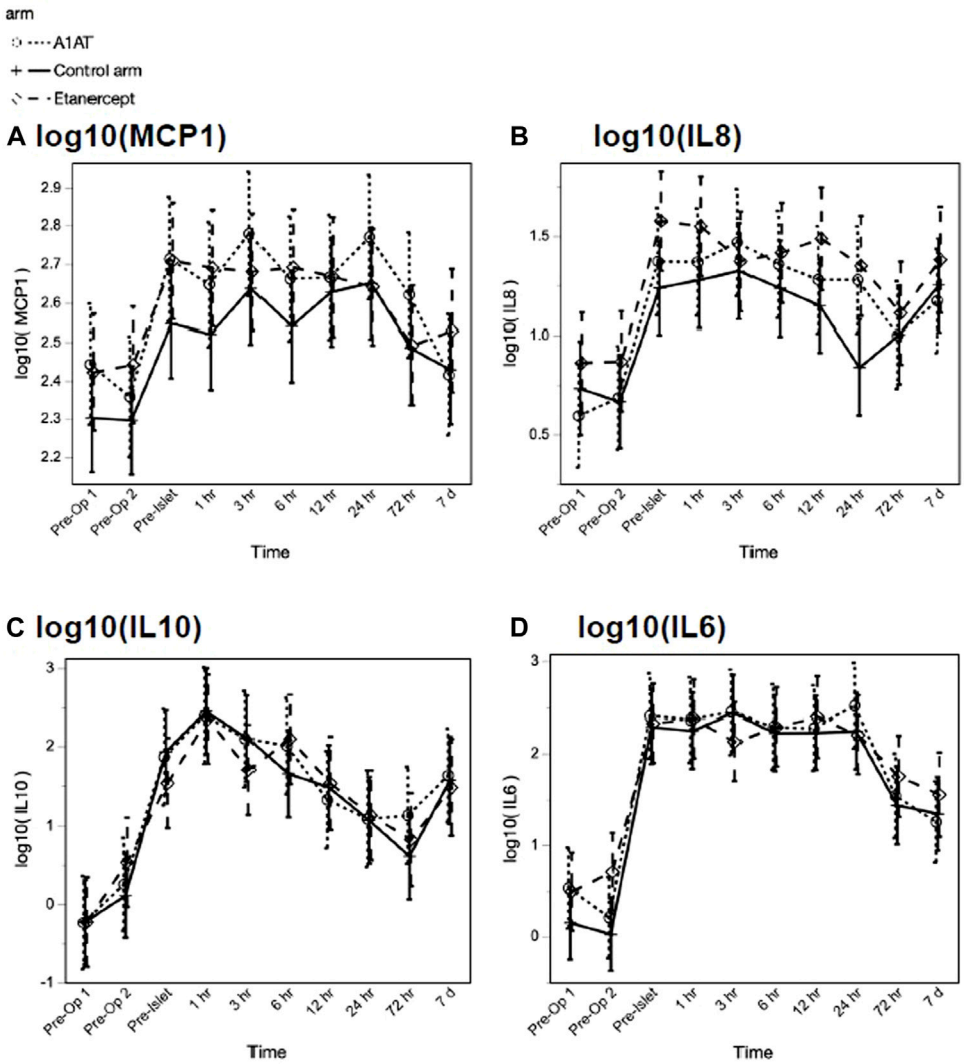


FIGURE 2 | MCP1 (panel **A**), IL8 (panel **B**), IL10 (panel **C**) and IL6 (panel **D**) levels at different time points in the three groups.

Cytokine Profiles

Among the inflammatory cytokines assayed, only TNFα differed significantly by treatment, with highest levels in the etanercept group ($p = 0.027$). Other measured cytokines did not differ significantly by treatment group in their trajectories after islet infusion. As expected, we observed increases in inflammatory cytokines related to TPIAT, particularly for IL-6, IL-8, IL-10, and MCP-1, which all increased during TPIAT surgery, though notably these elevations occurred even before islet infusion (**Figure 2**).

The TNFα level was significantly higher in the etanercept group, compared to the A1AT group and the control group ($p = 0.027$) and continued to increase significantly with time ($p < 0.0001$) over the first 7 days post-TPIAT (**Figure 3**).

Serum A1AT Level

Serum A1AT (sA1AT) levels increased significantly from pre-TPIAT baseline in all 3 groups (**Figure 4**)—including participants not treated pharmacologically with A1AT—with the peak level observed at 7 days post-TPIAT ($p < 0.001$). For all treatment groups, mean sA1AT was above the upper limit of normal, and remained above normal through 28 days post-TPIAT. Although the A1AT-treated group did not differ significantly from the other two groups when considering all times post-TPIAT ($p = 0.32$), the A1AT-treated group had a slightly higher peak at day 7 ($p = 0.08$ vs. control and $p = 0.006$ vs. etanercept).

Unmethylated Insulin DNA

To assess whether islet loss differed between groups, we compared the log-transformed ratio of unmethylated insulin DNA to total insulin DNA (U/(U + M)) over time post-transplant, adjusted for islet mass (IEQ/kg) transplanted (**Figure 5**), adjusted for islet mass (IEQ/kg) transplanted. As expected, U/(U + M) increased significantly after TPIAT ($p < 0.0001$), with the highest values from 15 min to 1 h after islet infusion, consistent with known early islet loss. Higher IEQ/kg

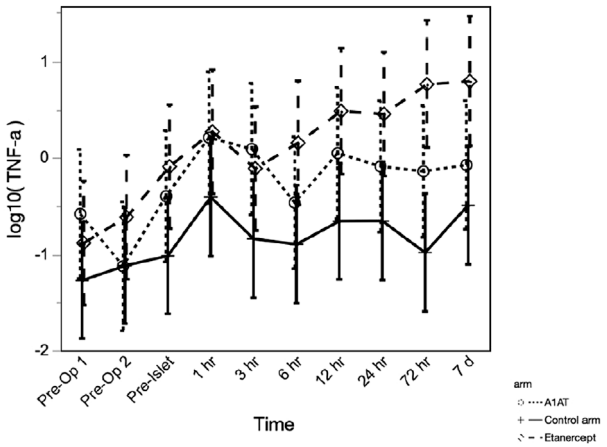


FIGURE 3 | TNF-α level at different time points in the three groups.

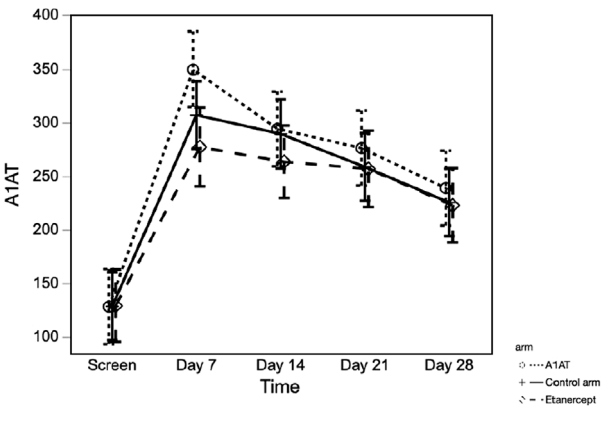


FIGURE 4 | Serum A1AT level at different time points in the three groups.

transplanted was associated with higher U/(U + M) (p = 0.013). When evaluating the trajectory of islet loss for the entire study period, from pre-TPIAT to 2 years post-TPIAT, the trajectories did not differ between groups (p = 0.66). However, a trend towards higher U/(U + M) was seen in the control group, vs. the etanercept and A1AT groups, from 6 h to day 28: in *post hoc* tests, controls were about 22% higher than participants treated with A1AT (p = 0.023) and 17% higher than those treated with etanercept (p = 0.067). This time frame is notable because it assesses islet loss during the drug treatment period, excluding the immediate islet loss that may be driven by islet damage during isolation.

C-Peptide Level in the Peri-Operative Period

Peri-operative C -peptide level was highest at 1 h after islet infusion, reflecting early islet loss, and continued to decline until day 28. There was no significant difference between the three groups in C-peptide levels (p = 0.48).

DISCUSSION

This is the first randomized trial of either etanercept or A1AT treatment in patients undergoing TPIAT. We previously reported better insulin secretion at 3 months in the etanercept-treated group. In this second stage of our study, we evaluated long-term insulin secretion and glycemic outcomes, and early mechanistic markers of inflammation and islet loss. Overall, there was no evidence of clinically meaningful efficacy of short-term treatment with either etanercept or A1AT. Treated groups and untreated controls had similar inflammatory profiles and similar metabolic outcomes at 2 years.

We observed upregulation of endogenous circulating sA1AT in the control and etanercept groups, which has not previously been reported. Although the group receiving A1AT treatment showed slightly higher sA1AT soon after surgery, the difference in magnitude was relatively small. The finding of increased sA1AT in the non-treatment groups was unexpected—our intention was to measure the pharmacological effect of treatment with A1AT therapy. A1AT is produced in the liver as an acute-phase reactant in response to inflammation and its level can remain elevated for >1 week depending on the underlying trigger [19]. We suspect that 'injury' from the surgical trauma and islet infusion triggered secretion of higher levels of A1AT. Because we did not measure sA1AT level intraoperatively before islet infusion, we cannot determine whether elevation was precipitated by major surgery, rather than by islet infusion. We speculate that we did not see a benefit of pharmacologic treatment with A1AT because drug treatment only minimally enhanced already high endogenous levels.

At the 2-year follow-up, the three groups exhibited similar outcomes for insulin dependence, insulin dosage, HbA1c levels, and CGM data. Overall insulin independence rates were similar to historical outcomes. Also, the three groups did not differ in islet function as assessed using MMTT (mixed-meal tolerance test), IVGTT (intravenous glucose tolerance test), and GPAIS (glucose-potentiated arginine induced insulin secretion) tests. This is similar to what we reported previously at the 1 year follow up. Note that this study was designed as a pilot, and thus may be underpowered to observe small differences. However, the absence of a strong signal of robust benefit suggests a reduced likelihood of significant advantages associated with either A1AT or etanercept alone.

Consistent with our clinical outcomes, cytokine levels did not show any clear effect of treatment on the inflammatory cascade triggered by TPIAT. Cytokine levels (IP-10, IL-1α, IL-1β, IL-6, IL-8, IL-10, MCP-1, TNF-α) significantly increased from baseline after TPIAT. In our cohort this was present during surgery even *before* islet infusion, suggesting that at least some of this inflammatory cascade is triggered by the stress of the TPIAT surgery itself. This raises the possibility that improved outcomes could be achieved by better addressing this surgical inflammation before islet infusion. However, we did administer the first dose of etanercept or A1AT before the surgical procedure, and this single dose was not effective in mitigating the inflammatory response. Generally, inflammatory cytokines continued to be elevated

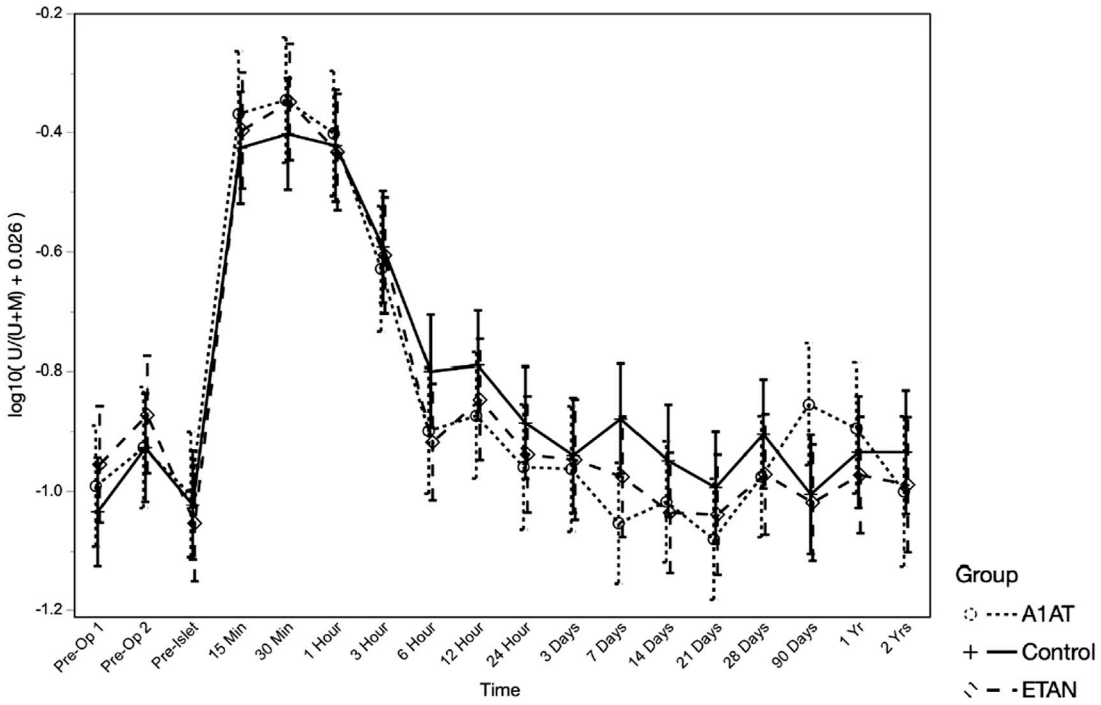


FIGURE 5 | Unmethylated insulin DNA ratio at different time points in the three groups.

7 days post-TPIAT. Comparing the three groups' cytokine profiles, the groups did not differ except in TNF-α, which was significantly higher in the etanercept group. This is likely a treatment effect of etanercept, which binds to TNF-α and prevents it from binding to its receptors. Elevated TNF-α levels have been reported in conditions treated with etanercept [20, 21].

These finding differ somewhat from prior work by Naziruddin et al, who reported that administering etanercept reduced IL-8, IL-6, and MCP-1 levels compared with controls [22]. However, that study was a retrospective, non-controlled study and in contrast, we have a randomized single-center study, which may avoid bias from time- or center-dependent effects. The same study found that combining IL-1 blockade with etanercept significantly suppressed elevation of IL-8, IL-6, and MCP-1 and led to better islet function as assessed by basal C-peptide, glucose and hemoglobin A1c. We did not attempt combination therapy in this pilot study, and multi-level blockade of inflammation might have better efficacy.

Our findings are also notably different from alloislet transplant studies in which *non-randomized* administration of etanercept is suggested to benefit long-term outcomes. There are, however, a few important distinctions between the allo- and autograft settings. First, although alloislet recipients do not undergo the major surgical procedure of pancreatectomy, in one small study directly comparing allo and autografts, the inflammatory response to alloislet infusion was much more pronounced, likely due to the immunologic contributions of HLA-mismatched tissue [23]. Second, alloislet recipients may have a pronounced TNF-alpha response to induction medications like anti-thymocyte globulin [24]. Lastly, the first dose of etancercept in the alloislet setting is administered intravenously. In our study we administered all doses, including the first dose, subcutaneously. This approach was chosen to mimic a protocol that could be used in the clinic, without need for an IND. Since all subsequent doses are administered subcutaneously in both our study and the alloislet setting, we would expect any impact from the different route of administering with the first dose to be limited to the first 3–7 days post-transplant. For these reasons, our results may not be directly extrapolated to the alloislet setting.

Our patients received etanercept intermittently between day 0 and day 21 or A1AT between day 0 and 28 after TPIAT. This selection of short-term treatments was based on the known limited duration of instant blood-mediated inflammatory reaction (IBMIR). The finding that etanercept resulted in better islet function 3 months post-TPIAT is promising and suggests that longer duration of treatment might have provided a more prolonged benefit. However, prolonging treatment duration could increase risks of side effects, as well as increasing financial costs. These considerations highlight the need to balance the potential benefits of longer treatment duration with the potential risks and resources required for such an approach.

Consistent with our prior reports, we did observe a robust rise in unmethylated INS DNA measures immediately after islet infusion, confirming that islet loss is universally occurring in these TPIAT recipients. Although the treatment groups did not differ overall, *post hoc* tests found an intriguing signal, from Hour 6 to Day 28, of lower levels of unmethylated INS DNA in the etanercept and A1AT groups

combined, when adjusted for IEQ/kg. This analysis should be interpreted with caution, as it was undertaken *post hoc* based on observed patterns. In the context of a pilot study, however, it suggests a potential treatment effect, and may support the idea of treatment prolonged beyond Day 28.

One limitation of the current trial is that we cannot definitively identify a mechanism to explain the *lack* of therapeutic benefit of either etanercept or alpha-1 antitrypsin. There may be other mechanistic measures—such as local intrahepatic inflammatory and injury measures—that were not obtainable and which might have directly addressed reasons for failure of these investigational drug therapies. However, we hypothesize that the failure of these agents to favorably impact the post-TPIAT inflammatory response explains the lack of efficacy. It is important to note that this study was a pilot trial, so its statistical power may be limited in detecting small effects. Some data were missing due to missed visits or technical constraints, such as instances where intravenous access was lost. Some participants did not return for in-person testing at 2 years, which was partly compounded by interruptions in research visits necessitated by the COVID-19 pandemic, as well as hesitancy of some participants to travel by plane during the pandemic even after we were able to resume visits. In these cases, we collected data that could be gathered remotely, mainly insulin use and HbA1c levels.

In summary, this pilot study's findings did not indicate a significant reduction in inflammation or improved islet engraftment with either etanercept or A1AT, as evidenced by similar cytokine profiles and markers of beta cell death among the three groups during the early post-transplant period. The etanercept group did, however, exhibit better islet function at 3 months post-TPIAT. Unfortunately, this improvement was not sustained at the 1-year and 2-year follow-ups. These results suggest that exploring different doses or extending the duration of etanercept treatment may lead to more prolonged effects that could potentially benefit patients undergoing TPIAT.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JH contributed to data analysis in addition to study design, data collection and interpretation of the results, drafting and revising the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by National Institute of Health grant number R01DK109914 (PI Bellin) and grant number NIDDK T32 DK065519. This research was also supported by the National Institutes of Health's National Center for Advancing Translational Sciences, grant UL1TR002494 as well as National Institute of Health grant number DK 057846 and grant number DK129523 and DoD grant number W81XWH-18-1-0687.

CONFLICT OF INTEREST

MB declares research support from Dexcom and Viacyte; consulting/DSMB membership with Vertex, Insulet, and consulting for Emerging Therapy Solutions. BJH holds equity in and serves as a paid executive officer and director of Diabetes Fee, Inc., a company that may commercially benefit from the results of this research. This interest has been reviewed and managed by the University of Minnesota in accordance with its Conflict of Interest policies.

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.

AUTHOR DISCLAIMER

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health's National Center for Advancing Translational Sciences.

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ORIGINAL RESEARCH
published: 16 January 2024
doi: 10.3389/ti.2024.12104

The BAR Score Predicts and Stratifies Outcomes Following Liver Retransplantation: Insights From a Retrospective Cohort Study

*Felix J. Krendl[1‡], Margot Fodor[1‡], Madita L. Buch[1], Jessica Singh[1], Hannah Esser[1], Benno Cardini[1], Thomas Resch[1], Manuel Maglione[1], Christian Margreiter[1], Lisa Schlosser[2†], Tobias Hell[2†], Benedikt Schaefer[3], Heinz Zoller[3], Herbert Tilg[3], Stefan Schneeberger[1] and Rupert Oberhuber[1]**

[1]Department of Visceral, Transplant and Thoracic Surgery, Center for Operative Medicine, Medical University of Innsbruck, Innsbruck, Austria, [2]Department of Mathematics, Innsbruck, Austria, [3]Department of Internal Medicine I, Gastroenterology, Hepatology, Endocrinology and Metabolism, Medical University of Innsbruck, Innsbruck, Austria

Liver retransplantation (reLT) yields poorer outcomes than primary liver transplantation, necessitating careful patient selection to avoid futile reLT. We conducted a retrospective analysis to assess reLT outcomes and identify associated risk factors. All adult patients who underwent a first reLT at the Medical University of Innsbruck from 2000 to 2021 (N = 111) were included. Graft- and patient survival were assessed via Kaplan-Meier plots and log-rank tests. Uni- and multivariate analyses were performed to identify independent predictors of graft loss. Five-year graft- and patient survival rates were 64.9% and 67.6%, respectively. The balance of risk (BAR) score was found to correlate with and be predictive of graft loss and patient death. The BAR score also predicted sepsis (AUC 0.676) and major complications (AUC 0.720). Multivariate Cox regression analysis identified sepsis [HR 5.179 (95% CI 2.575–10.417), $p < 0.001$] as the most significant independent risk factor for graft loss. At a cutoff of 18 points, the 5 year graft survival rate fell below 50%. The BAR score, a simple and easy to use score available at the time of organ acceptance, predicts and stratifies clinically relevant outcomes following reLT and may aid in clinical decision-making.

Keywords: risk factors, patient survival, graft survival, sepsis, futility

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***Correspondence**
Rupert Oberhuber,
rupert.oberhuber@i-med.ac.at

†Present address:
Lisa Schlosser,
Data Lab Hell GmbH, Zirl, Austria
Tobias Hell,
Data Lab Hell GmbH, Zirl, Austria

‡These authors share first authorship

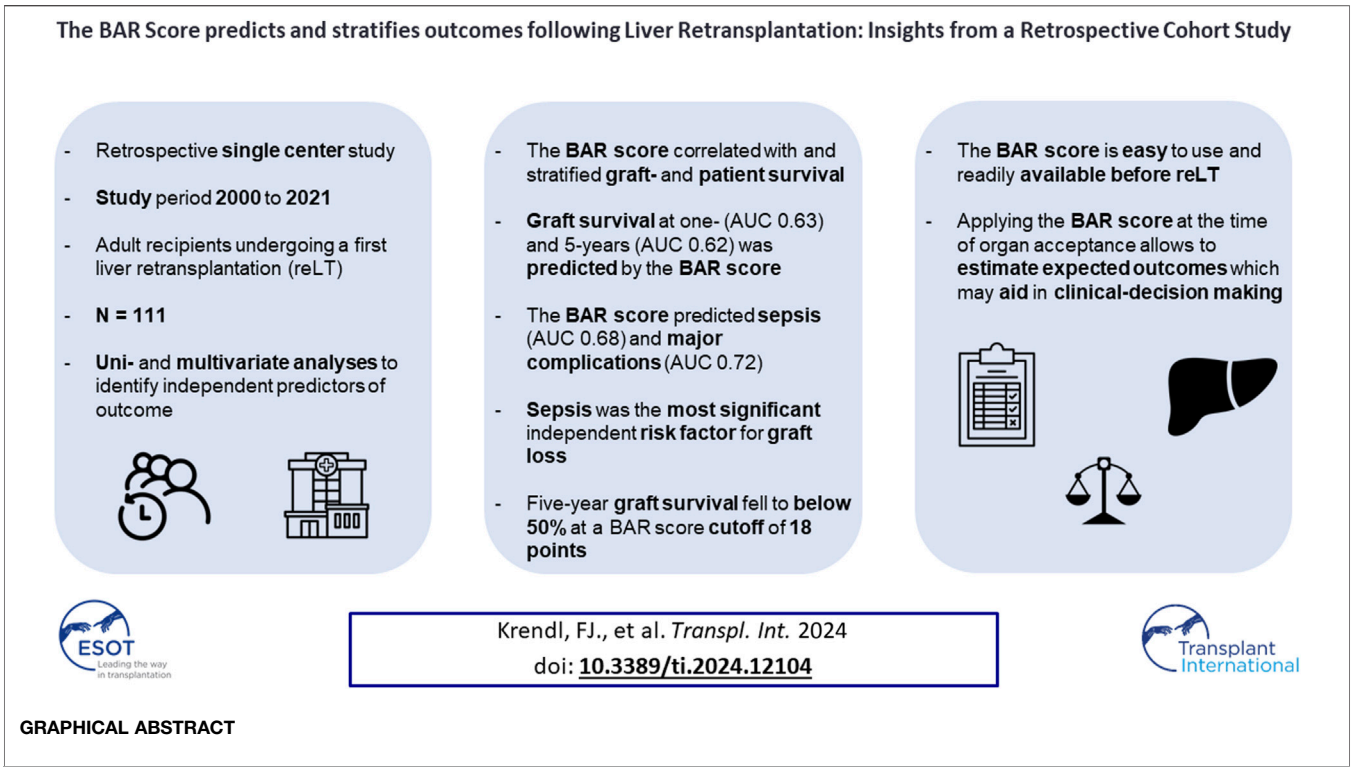
Received: 23 September 2023
Accepted: 04 January 2024
Published: 16 January 2024

Citation:
Krendl FJ, Fodor M, Buch ML, Singh J, Esser H, Cardini B, Resch T, Maglione M, Margreiter C, Schlosser L, Hell T, Schaefer B, Zoller H, Tilg H, Schneeberger S and Oberhuber R (2024) The BAR Score Predicts and Stratifies Outcomes Following Liver Retransplantation: Insights From a Retrospective Cohort Study. Transpl Int 37:12104. doi: 10.3389/ti.2024.12104

INTRODUCTION

Liver transplantation (LT) is a curative treatment option for selected patients with end-stage liver disease and patients with certain forms of primary or secondary malignancies of the liver [1, 2]. In case of graft failure, a liver retransplantation (reLT) is the only recourse. Despite surgical, immunological and perioperative advancements, reLT remains a challenging procedure which is associated with inferior outcomes compared to primary LT [3–7].

The MELD score has been implemented by Eurotransplant and UNOS because it is an accurate predictor of short-term mortality and provides objective criteria for organ allocation in the majority of patients with end-stage liver disease [8, 9]. Patient selection for transplant recipients who require reLT is based on less well validated criteria and poses challenges in MELD-based allocation systems. Furthermore, outcomes following transplantation are not taken into consideration by current allocation policies [10].



GRAPHICAL ABSTRACT

In a field plagued by a shortage of available organs the need for reLT takes a toll on the already limited organ pool. Ethical principles such as utility, beneficence and equity need to be taken into consideration with patients on the waiting list competing for organs. It is therefore important to identify risk factors associated with negative outcomes in order to avoid futile retransplantations and maximize transplant benefit. While best achievable outcomes for LT and reLT have been quite well defined for selected standard risk (i.e., benchmark) cases [3, 11], futility and rationing remain concepts that are ill-defined [12, 13]. Previously, 5 year survival rates of 50% and more have been suggested to constitute an acceptable outcome [14]. Several risk scores have been published in an attempt to stratify risk and predict outcomes following reLT [15–18]. Yet, most of these scores are based on old data or lack adequate prediction of risk and are therefore of limited clinical applicability [18], which might explain why, so far, none of the published risk scores has found its way into routine clinical practice.

The aim of this study was to 1) evaluate the incidence of reLT in a high-volume Eurotransplant center, 2) assess graft- and patient survival as well as other relevant post-transplant complications following reLT and 3) identify potential risk factors associated with worse outcomes in the setting of reLT.

PATIENTS AND METHODS

Study Population and Study Design

At the Medical University of Innsbruck, all adult patients who underwent a first deceased donor reLT between 1st January 2000 and 31st December 2021 were included in the study. Following discharge patients were routinely followed at our gastroenterology and hepatology outpatient clinic. Patient data were extracted from the electronic health records and pseudonymized. Data collection was performed from December 2022 until February 2023.

The study was conducted in accordance with both, the Declaration of Helsinki and Istanbul, and was approved by the Institutional Review Board of the Medical University of Innsbruck, Austria (EK1240/2022). The need for informed consent was waived by the ethics committee due to the retrospective nature of this study. The results were reported according to the STROBE guidelines [19].

Surgical Technique

At our center, the standard implantation technique involves a bicaval, cava-replacing approach, without veno-venous bypass. Should individual circumstances preclude a cava-replacing approach, we employ a cava-sparing piggyback technique. Only if, 1) the hemodynamics of the patient preclude a cava-replacing approach and 2) the anatomical situation prevents a safe cava-sparing hepatectomy would we consider performing a bypass.

Definitions

Graft Loss and Graft Dysfunction

Graft loss was defined as patient death or reLT (i.e., second reLT). Primary non-function (PNF) was defined as peak AST ≥3,000 IU/L plus at least one of the following criteria: INR ≥2.5, serum lactate ≥4 mmol/L and total bilirubin ≥10 mg/dL (values measured on postoperative day 3, biliary obstruction being

excluded) [20]. Early allograft dysfunction (EAD) was defined according to the Olthoff criteria [21].

Rejections

Rejection episodes were diagnosed based on clinical suspicion and confirmed with liver biopsy. If rejection was suspected, patients received an intravenous steroid pulse of 500 mg methylprednisolone for 3 days followed by an increase in maintenance immunosuppression.

Infectious Complications and Sepsis

Any documented infection requiring some form of antimicrobial treatment was recorded as infectious complication. Sepsis was defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, in accordance with the third international consensus definitions for sepsis and septic shock [22].

Biliary Complications

Biliary complications were classified as bile duct leaks, anastomotic stenosis (AS), non-anastomotic stenosis (NAS) and cholangitis. Multifocal pathologies affecting the macroscopic donor bile ducts (NAS, biliary cast syndrome and bile duct necrosis with intrahepatic leakage and biloma formation) in the absence of thrombosis or severe stenosis of the hepatic artery that could not be explained by recurrent disease (i.e., primary sclerosing cholangitis) were classified as post-transplant cholangiopathy [23].

Balance of Risk (BAR) Score

The BAR score incorporates six variables (MELD score, donor age, recipient age, CIT, retransplantation and the need for life support) available at the time of organ acceptance and ranges from 0 to 27 points. BAR score values have been calculated according to the publication by Dutkowski et al. [24] using the online BAR score calculator.[1]

Classification and Quantification of Complications

Postoperative complications were graded according to the Clavien-Dindo classification system [25]. Clavien-Dindo grade I and II were recorded as minor complications. Clavien-Dindo Grade IIIa complications were considered moderate complications, while grade IIIb or higher were defined as major complications. Complications were further quantified using the comprehensive complication index (CCI) within a time frame of 3 months and 1 year after transplantation [26, 27].

Outcomes

The primary outcomes were graft- and patient survival. Secondary outcomes included the incidence of post-transplant complications such as PNF, EAD, rejection episodes, infectious complications and sepsis, biliary and arterial complications as well as risk factors and their influence on graft loss and patient death.

[1]https://www.assessurgery.com/bar-score/bar-score-calculator/

Statistical Analysis

For descriptive analyses, categorical variables were summarized with the help of absolute numbers and relative (percentages) frequencies, continuous variables were summarized with means and standard deviation (SD) or medians and interquartile range (IQR) as appropriate. Comparative analysis of categorical variables was conducted using the Chi-square or Fisher's exact test (if one or more cells had an expected count of less than five). The Mann–Whitney U test was used to compare continuous, not normally distributed variables. Uni- and multivariate analyses were performed for the primary and secondary endpoints, starting with a univariate analysis of each variable. Any variable having a significant univariate test was selected as a candidate for the multivariate analysis [28]. Kaplan-Meier survival curves and the log-rank test were used to analyze and compare graft- and patient survival. Uni- and multivariate analysis for graft- and patient survival endpoints was performed with Cox proportional hazards regression analysis. Binary logistic regression analysis was used to assess the effects of clinical parameters on secondary outcomes. Potential associations between continuous variables were investigated with the help of bivariate correlation analysis using the Spearman correlation coefficient. Receiver operating characteristic (ROC) curves were plotted and areas under the curve (AUC) analyzed to evaluate the performance of binary classifiers. All p-values < 0.05 were considered statistically significant. Missing values were not imputed. Statistical analysis was conducted with SPSS (IBM SPSS Statistics for Mac, Version 27.0.1.0 Armonk, NY: IBM Corp.).

RESULTS

Recipient Characteristics

Overall, 1,290 adult LTs were performed during the study period. Out of these 1,290 LTs, 111 (8.6%) were first reLTs. Indications for reLT and recipient demographics are presented in **Table 1**. The median recipient age was 57 years (50–65); 24 recipients (21.6%) were female, 87 were male (78.4%). The median recipient BMI was 23.5 (21.1–27.0). The most common indications for reLT were biliary complications (36.9%) followed by recurrence of disease (21.6%) and HAT (17.1%). The median time from primary LT to reLT was 13 months (2.0–66.0). Twenty-five patients (22.5%) underwent high urgency (HU) reLT. The median MELD score at reLT was 20 (14–26). The median BAR score in our cohort was 12 points (9–16) and ranged from 4 to 26 points. The median length of hospital stay was 32 days (20–55), with the median follow-up being 39.4 months (11.8–89.5).

Donor Characteristics and Operative Factors

The median donor age was 46 years (32–54); 55 donors (49.5%) were female, 56 (50.5%) were male (**Table 2**). The median ET-DRI was 1.44 (1.25–1.73), with the median donor BMI being 24.8 (23.0–27.0). All donors were donation after brain death (DBD) donors. The anhepatic time, warm ischemia time (WIT) and cold ischemia time (CIT) were 57.0 min (48.0–66.0), 45.0 min

TABLE 1 | Recipient characteristics.

	All *N* = 111	Graft loss *n* = 52	Graft survival *n* = 59	*p*-value
Age (years)	57.0 (50.0–65.0)	56.0 (50.0–61.0)	59.0 (51.0–65.0)	0.11
Sex				0.08
- Female	24 (21.6)	15 (28.8)	9 (15.3)	
- Male	87 (78.4)	37 (71.2)	50 (84.7)	
BMI (kg/m^2)	23.5 (20.8–27.0)	23.5 (20.7–26.8)	22.9 (21.1–27.0)	0.82
MELD score	20.0 (14.0–26.0)	21.50 (17.0–28.0)	17.0 (12.0–24.0)	**0.01**
BAR score	12.0 (9.0–16.0)	12.5 (11.0–16.0)	10.0 (8.0–16.0)	**0.04**
Indication for reLT				
- Biliary complications	41 (36.9)	21 (40.4)	20 (33.9)	0.48
- Disease recurrence	24 (21.6)	15 (28.8)	9 (15.3)	0.08
- HAT	19 (17.1)	6 (11.5)	13 (22.0)	0.14
- PNF	4 (3.6)	1 (1.9)	3 (5.1)	0.70
- Sepsis	1 (0.9)	1 (1.9)	0 (0.0)	0.95
- Rejection	9 (8.1)	2 (3.8)	7 (11.9)	0.23
- Other	10 (9.0)	4 (7.7)	6 (10.2)	0.90
- Not reported	3 (2.7)	2 (3.8)	1 (1.7)	0.91
Time to reLT (days)	406 (78–2010)	370 (79–2091)	406 (63–1,090)	0.79
AB induction (yes/no)	49 (45.0)	27 (54.0)	22 (37.3)	0.08
- IL2	44 (40.4)	24 (48.0)	20 (33.9)	0.14
- ATG	4 (3.7)	2 (4.0)	2 (3.4)	1.000
- Alemtuzumab	1 (0.9)	1 (2.0)	0 (0.0)	0.93
- Missing	2	0	2	
ABO blood group				
- A	50 (45.0)	21 (40.4)	29 (49.2)	0.35
- B	9 (8.1)	3 (5.8)	6 (10.2)	0.62
- 0	41 (36.9)	21 (40.4)	20 (33.9)	0.48
- AB	11 (9.9)	7 (13.5)	4 (6.8)	0.24
CMV mismatch				
- D+/R-	21 (19.6)	12 (24.0)	9 (15.8)	0.27
- D-/R+	39 (36.4)	17 (34.0)	22 (38.6)	0.62
- D+/R+	39 (36.4)	18 (36.0)	21 (36.8)	0.93
- D-/R-	8 (7.5)	3 (6.0)	5 (8.8)	0.86
- Missing	4	2	2	
Median follow-up (months)	39.4 (11.8–89.5)	6.0 (1.3–72.8)	67.0 (23.0–138.0)	

Values are presented as medians or absolute numbers with IQRs, and percentages in parentheses. Significant p-values are highlighted in bold. AB, antibody; ATG, anti-thymocyte globulin; BAR, balance of risk; BMI, body mass index; CMV, cytomegalovirus; MELD, model for end-stage liver disease; HAT, hepatic artery thrombosis; IL2, interleukin 2; IQR, interquartile range; PNF, primary non-function; reLT, liver retransplantation.

(37.0–56.0) and 8.1 h (6.5–9.5) respectively. The median operating time was 7.6 h (6.0–8.9). Cava-replacing LT was performed in 93.7% (104 of 111) of cases with piggy-back transplantation being performed in 6.3% of cases (7 of 111). An arterial jump graft was used in 24.3% of cases (27 of 111) and a bilioenteric anastomosis was carried out in 36.0% of cases (40 of 111). In 14.4% of cases (16 of 111) the liver graft had undergone normothermic machine perfusion before implantation.

Complications Following reLT

Six patients (5.4%) developed primary non-function (PNF) following reLT. The EAD rate was 35.1% (39 of 111). Ten recipients (9.0%) had a rejection episode; 78 patients (70.3%) developed infectious complications. Sepsis occurred in 22 patients (19.8%). Overall, 11 patients (9.9%) developed an arterial complication; HAT occurred in four patients (3.6%) with arterial stenosis (n = 4, 3.6%), dissection (n = 1, 0.9%) and pseudoaneurysm (n = 2, 1.8%) being responsible for the other arterial complications.

Out of 111 patients, 54 (48.6%) developed a biliary complication. Seventeen patients (15.3%) had one or more cholangitis episode. Bile duct leaks occurred in 19.8% (22 of 111), anastomotic strictures in 24.3% (27 of 111), non-anastomotic strictures in 10.8% (12 of 111) and post-transplant cholangiopathy in 17.1% (19 of 111) of the recipients (**Table 3**). Patients with biliary complications tended to have a higher graft loss rate compared to patients without biliary complications, however the difference was not statistically significant [27.0% (30 of 111) vs. 20.7% (23 of 111), p = 0.11].

Graft Survival Analysis

The overall graft failure rate (patient death or reLT) was 46.8% (52 of 111) over the observation period of 22 years. Out of these

TABLE 2 | Donor characteristics and operative data.

	All *N* = 111	Graft loss *n* = 52	Graft survival *n* = 59	*p*-value
Age (years)	46.0 (32.0–54.0)	49.0 (34.0–54.8)	42.0 (30.0–54.0)	0.16
Sex				0.07
- Female	55 (49.6)	21 (40.4)	34 (57.6)	
- Male	56 (50.5)	31 (59.6)	25 (42.4)	
BMI (kg/m^2)	24.8 (22.9–29.0)	25.4 (23.0–28.0)	24.2 (23.0–26.0)	0.19
COD				0.58
- Trauma	35 (31.5)	17 (32.7)	18 (30.5)	
- Anoxia	7 (6.3)	4 (7.7)	3 (5.1)	
- CVA	65 (58.6)	30 (59.3)	35 (57.7)	
- Other	4 (3.6)	1 (1.9)	3 (5.1)	
ECD	74 (66.7)	32 (61.5)	37 (62.7)	0.90
DCD	0 (0.0)	0 (0.0)	0 (0.0)	
DBD	111 (100.0)	52 (100.0)	59 (100.0)	
NMP	16 (14.4)	4 (7.7)	12 (20.3)	0.06
Preservation				0.64
- UW	37 (33.6)	16 (31.4)	21 (35.6)	
- HTK	73 (66.4)	35 (68.6)	38 (64.4)	
- Missing	1	1	0	
reLT era				0.06
- 2000–2010	41 (36.9)	24 (46.2)	17 (28.8)	
- 2011–2021	70 (63.1)	28 (53.8)	42 (71.2)	
Duration reLT (hours)	7.6 (6.0–8.9)	7.6 (5.9–9.4)	7.6 (6.3–8.7)	0.83
Anhepatic time (minutes)	57.0 (48.0–66.0)	56.0 (45.3–70.0)	57.0 (50.0–66.0)	0.79
WIT (minutes)	45.0 (37.0–56.0)	45.0 (37.0–55.0)	44.0 (37.8–58.0)	0.84
CIT (hours)	8.1 (6.5–9.5)	8.7 (6.8–10.6)	7.7 (6.3–9.3)	**0.02**
ET-DRI	1.44 (1.25–1.73)	1.48 (1.34–1.71)	1.41 (1.13–1.82)	0.34

Values are presented as medians or absolute numbers with IQRs, and percentages in parentheses. Significant p*-values are highlighted in bold. BMI, body mass index; COD, cause of death; CVA, cerebrovascular accident; ECD, extended criteria donor; ET-DRI, Eurotransplant donor risk index; HTK, histidine-tryptophan-ketoglutarate. IQR, interquartile range; SAB, subarachnoid hemorrhage; UW, University of Wisconsin; WIT, warm ischemia time.*

TABLE 3 | Clinical outcomes and complications.

	All *N* = 111	Graft loss *n* = 52	Graft survival *n* = 59	*p*-value
PNF	6 (5.4)	6 (11.5)	0 (0.0)	**0.02**
EAD	39 (35.5)	20 (39.2)	19 (32.2)	0.44
Rejection	10 (9.1)	7 (13.5)	3 (5.1)	0.23
Infectious complications	78 (72.2)	38 (74.5)	40 (69.0)	0.52
Sepsis	22 (19.8)	19 (38.0)	3 (5.1)	<**0.001**
Biliary complications	54 (48.6)	30 (57.7)	24 (40.7)	0.07
- Cholangitis	17 (15.3)	12 (23.1)	5 (8.5)	**0.03**
- Bile duct leaks	22 (19.8)	12 (23.1)	10 (16.9)	0.42
- AS	27 (24.3)	17 (32.7)	10 (16.9)	0.05
- NAS	12 (10.8)	8 (15.4)	4 (6.8)	0.15
- Post-Tx Cholangiopathy	19 (17.1)	11 (21.2)	8 (13.6)	0.29
Arterial complications	11 (9.9)	8 (15.4)	3 (5.1)	0.07
- Stenosis	4 (3.6)	3 (5.8)	1 (1.7)	0.52
- Thrombosis	4 (3.6)	3 (5.8)	1 (1.7)	0.52
- Dissection	1 (0.9)	1 (1.9)	0 (0.0)	0.95
- Pseudoaneurysm	2 (1.8)	1 (1.9)	1 (1.7)	1.000
Major complication (at discharge)	91 (82)	47 (92.2)	44 (77.2)	**0.03**
CCI				
- 3 months	54.2 (39.7–86.5)	68.1 (51.0–100)	47.3 (26.2–69.0)	<**0.001**
- 12 months	63.9 (42.4–100)	100 (63.8–100)	54.2 (33.7–75.7)	<**0.001**
Reoperation	69 (62.2)	37 (71.2)	32 (54.2)	0.07
- Reoperation ≤30 days	64 (57.7)	35 (67.3)	29 (49.2)	0.05
Hospital stay (days)	32 (20–55)	38 (20–59)	29 (22–46)	0.55

Values are presented as medians or absolute numbers with IQRs, and percentages in parentheses. Significant p*-values are highlighted in bold. AS, anastomotic stricture; CCI, comprehensive complication index; EAD, early allograft dysfunction; NAS, non-anastomotic stricture; PNF, primary non function; Post-Tx, post-transplant.*

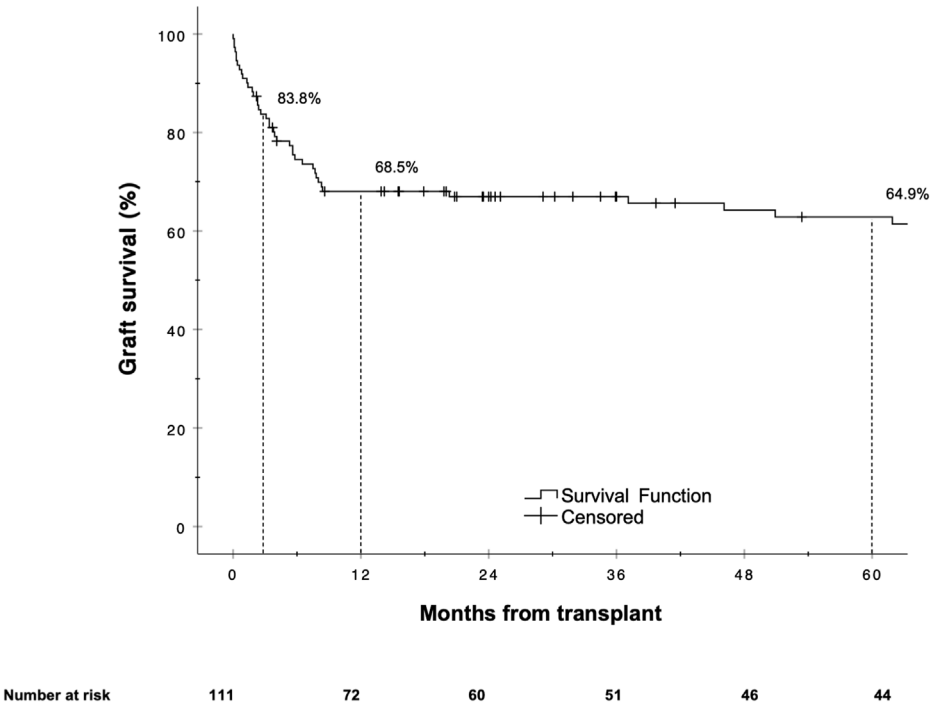


FIGURE 1 | Estimated Kaplan-Meier graft survival for the reLT cohort.

52 patients, seven underwent a second reLT and 45 died with their second graft. The most common cause of graft failure was sepsis (34.6%) followed by recurrence of disease (17.3%), vascular complications (15.4%) and post-transplant malignancies (9.6%). Kaplan Meier estimates for 90 days, 1 and 5 year graft survival are shown in **Figure 1**. Graft survival was significantly associated with the BAR score in univariate analysis. ROC curve analysis showed the BAR score to be predictive of overall [AUC 0.613 (95% CI 0.508–0.719), p = 0.04], 1 [AUC 0.630 (95% CI 0.518–0.742), p = 0.03] and 5 year graft loss [AUC 0.616 (95% CI 0.506–0.725), p = 0.045] but not 90 days graft loss [AUC 0.640 (95% CI 0.477–0.803), p = 0.06] (**Figure 2**).

Different BAR score values were analyzed to find optimal cutoffs which best stratify risk of graft failure at different time points following reLT. Cutoffs were based on the maximum Youden-index [29].

A BAR score cutoff of 11 points (BAR score <11 points vs. ≥11 points) provided the best separation of risk. At this cutoff the positive predictive value (PPV) for graft failure at 1 and 5 years was 40.6% and 43.5% respectively, while the negative predictive value (NPV) was 83.3% and 78.6% respectively. Patients with a BAR score ≥11 points had an increased hazard of graft loss at 1 [HR 2.784 (95% CI 1.215–6.381), p = 0.02] and 5 years [HR 2.396 (95% CI 1.136–5.055), p = 0.02] compared to patients with a BAR score <11 points. At a cutoff of 18 points the 5 year graft survival rate fell to 46.7% (**Figure 3**).

Univariate analysis revealed MELD score, donor age, CIT, BAR score, cholangitis, major complication (CD > IIIa), sepsis, reoperation within 30 days and PNF to be risk factors for graft loss.

Considering these factors for multivariate Cox regression analysis, donor age, PNF and sepsis remained as independent risk factors for graft loss (**Supplementary Table S1**). When excluding the MELD score, donor age and CIT (all parameters are included in the BAR score) as well as PNF (PNF invariably leading to graft loss, **Table 3**) from the multivariate Cox regression, only sepsis [HR 5.179 (95% CI 2.575–10.417, p < 0.001] remained as independent significant risk factor for graft loss (**Table 4**).

Patient Survival Analysis

The overall mortality rate was 45% (50 of 111). The in-hospital mortality rate was 16.2% (18 of 111).

The Kaplan-Meier estimates for 90 days, 1 and 5 year patient survival are shown in **Figure 4**. Similar to graft survival, patient survival was significantly associated with the BAR score in univariate analysis. ROC curve analysis showed the BAR score to be predictive of overall [AUC 0.628 (95% CI 0.523–0.733), p = 0.02], 1 [AUC 0.637 (95% CI 0.524–0.750), p = 0.02] and 5 year patient death [AUC 0.620 (95% CI 0.510–0.731), p = 0.04] but not 90 days mortality [AUC 0.644 (95% CI 0.473–0.816), p = 0.06] (**Figure 5**).

The BAR score cutoff with the best separation of risk for patient death was the same as for graft survival (11 points). The PPV for patient death at 1 and 5 years was 37.7% and 40.6% respectively. The NPV at 1 and 5 years was 85.7% and 81.0% respectively.

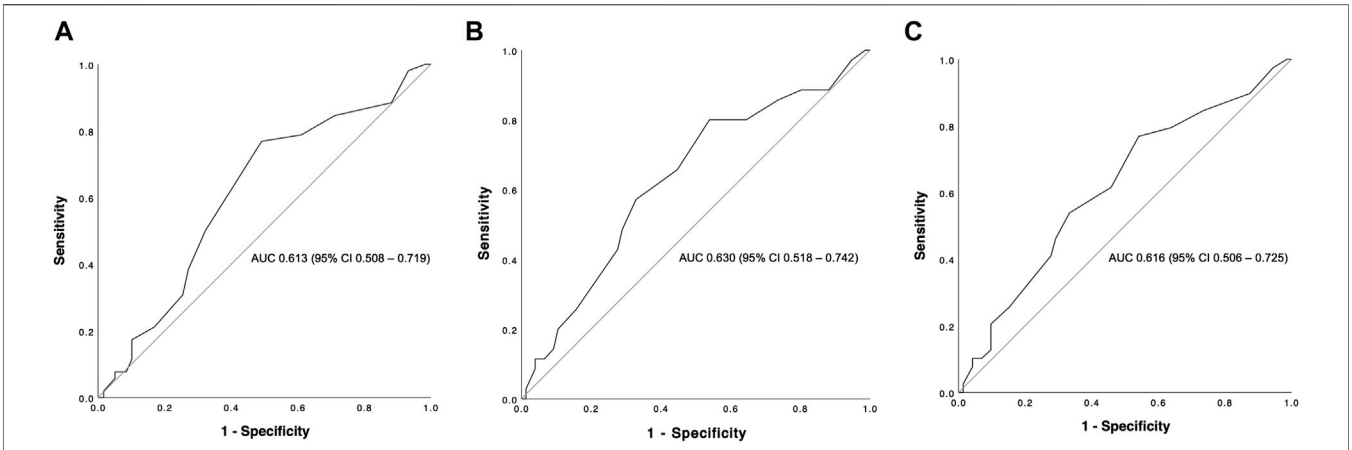


FIGURE 2 | ROC curve analysis depicting the predictive capability of the BAR score for overall **(A)**, 1 **(B)** and 5 year **(C)** graft survival. The BAR score performed best at predicting 1 year graft survival (AUC 0.630).

The hazard ratios for 1 and 5 year mortality at a BAR score cutoff of 11 were [HR 2.963 (95% CI 1.218–7.205), $p = 0.02$] and [HR 2.474 (95% CI 1.126–5.435), $p = 0.02$] respectively. At a BAR score cutoff of 18 points 5 year patient survival dropped to 53.3% (**Figure 6**).

Univariate analysis revealed the MELD score, donor age, BAR score, cholangitis, major complications (CD > IIIa), PNF, sepsis, arterial complications, and reoperation within 30 days as risk factors for patient death. Considering these factors for multivariate Cox regression analysis the most significant independent risk factors for patient death were PNF, sepsis and donor age (**Supplementary Table S2**). In a separate model where the MELD score and donor age (both included in the BAR score) have been excluded from the multivariate Cox regression analysis, PNF [HR 29.987 (95% CI 7.514–119.664), $p < 0.001$], and sepsis [HR 3.755 (95% CI 1.819–7.751), $p < 0.001$] remained as the only independent risk factors for patient death (**Table 5**).

BAR Score

In our analysis, the BAR score not only correlated significantly with graft- and patient survival but also with sepsis [OR 1.146 (CI 95% 1.035–1.269), $p = 0.01$], major complications (CD > IIIa) at discharge [OR 1.236 (CI 95% 1.064–1.437), $p = 0.01$] and the duration of the hospital stay (Spearman's r = 0.329, $p < 0.001$) as well as CCI at 3 (Spearman's r = 0.318, $p < 0.001$) and 12 months (Spearman's r = 0.272, $p = 0.004$). The BAR score was highly predictive of the incidence of major complications (CD > IIIa) with an AUC of 0.720 (95% CI 0.613–0.828, $p = 0.004$) and the occurrence of sepsis [AUC 0.676 (CI 95% 0.548–0.804), $p = 0.01$]. The BAR score correlated with the ET-DRI (r = 0.213, $p < 0.02$) (the scores share two variables: donor age and CIT). However, in comparison to the BAR score the ET-DRI was not predictive of patient- [AUC 0.522 (CI 95% 0.405–0.639), $p = 0.72$] or graft survival [AUC 0.568 (CI 95% 0.461–0.676), $p = 0.21$].

In response to the strong correlation of the BAR score with major complications and sepsis we performed additional Cox regression analysis excluding these two parameters from the multivariate model to avoid any interference, after which the BAR score remained as a significant independent risk factor for graft loss and patient death (**Supplementary Tables S3, S4**).

Sepsis and PNF

In our analysis, sepsis and PNF were the strongest independent predictors of graft failure and patient death in the multivariate Cox regression models. Univariate binary logistic regression analysis revealed EAD [OR 2.769 (CI 95% 1.063–7.211), $p = 0.04$], reoperation within 30 days [OR 3.030 (CI 95% 1.027–8.945), $p = 0.045$], BAR score [OR 1.146 (CI 95% 1.035–1.269), $p = 0.01$] and MELD score [OR 1.092 (CI 95% 1.029–1.159), $p = 0.003$] to correlate with sepsis. Considering EAD, reoperation within 30 days and the BAR score for multivariate binary logistic regression analysis only the BAR score remained significantly associated with sepsis [OR 1.122 (CI 95% 1.011–1.254), $p = 0.03$]. No single parameter was found to correlate with PNF.

DISCUSSION

This study, evaluating outcomes following reLT over the course of a 22 years period, found the BAR score to correlate with and be predictive of graft loss and patient death as well as the occurrence of sepsis and major complications. Furthermore, the BAR score positively correlated with the CCI at 3 and 12 months as well as the duration of hospital stay. The incidence of reLT over the duration of the study period was 8.6% and is in line with those reported at other transplant centers [6].

Multivariate Cox regression analysis revealed sepsis and PNF to be the strongest independent risk factors of graft failure and patient death. The overall morbidity and mortality were high with more than 80% of recipients developing a major complication and a 5 year patient survival below 70%, underscoring the high-risks associated with reLT.

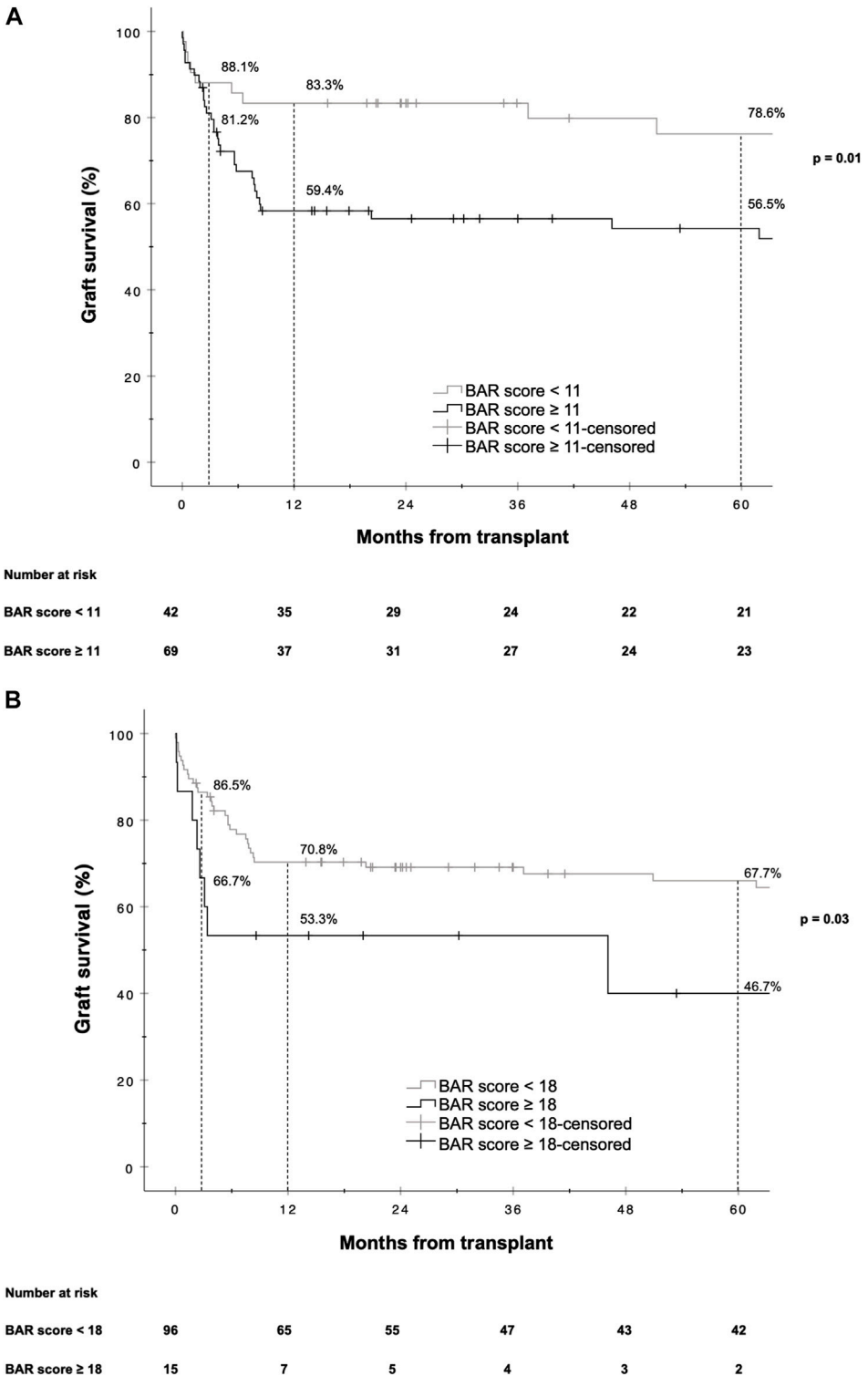


FIGURE 3 | Kaplan-Meier survival curves showing graft survival for reLT recipients with a BAR score <11 points vs. ≥11 points **(A)** and <18 points vs. ≥18 points **(B)**. The difference in graft survival at 1 and 5 years following reLT was highest at a BAR score cutoff of 11 points. At a BAR score cutoff of 18 points 5 year graft survival fell to below 46.7%.

TABLE 4 | Graft survival–Multivariate adjusted Cox proportional hazards regression analysis[a].

	HR	95% CI	*p*-value
BAR score	1.019	0.952–1.091	0.59
Cholangitis	1.934	0.959–3.901	0.07
Major complication	1.490	0.453–4.907	0.51
Sepsis	5.179	2.575–10.417	**<0.001**
Reoperation within 30 days	1.279	0.619–2.640	0.51

BAR, balance of risk; CI, confidence interval; HR, hazard ratio.
[a]*The MELD score, donor age and CIT (all included in the BAR score) as well as PNF have been excluded.*

Previously, an expected 5 year patient survival of 50% or more has been demanded to justify LT [30, 31].

Schlegel et al. have defined futility as in-hospital or 90 days mortality [32]. At a recent consensus meeting the expert panel recommended patient- and graft survival at 1 year after LT to define futility [33]. In the context of reLT an expected 1 year patient survival of 50% and more as well as an expected 5 year graft survival above 50% have been suggested as minimum thresholds to define acceptable outcomes [14]. While this is an arbitrary cutoff it also lacks clinical feasibility since outcome projections in reLT are ill-defined.

In our cohort, the hazard of graft loss and patient death was highest in the first months following reLT with survival curves running almost parallel after the first year (**Figures 3**, **6**). In line with this observation, the BAR score performed best at predicting risk of patient death at 1 year (AUC 0.637) and 1 year graft loss (AUC 0.630) (**Figures 2**, **5**).

The AUCs reported for the BAR score in our cohort were higher compared to those of previously published risk models for reLT and similar to the AUC for the model published in 2011 by Hong et al. (AUC 0.64) as well as the recently published Liver Retransplant Risk Score by Brüggenwirth et al. (time-dependent AUC for graft loss at 1 year 0.623) [17, 18]. The Liver Retransplant Risk Score was developed from a large dataset of the European Liver Transplant Registry (ELTR) and externally validated, however levels of pretransplant bilirubin, creatinine and INR were missing in more than 50% of cases with MELD score and CIT missing in 48% and 38% of cases respectively.

The Liver Retransplant Risk Score uses similar parameters as the BAR score (donor and recipient age, CIT, MELD score, life support before reLT), substituting the need for life support prior to reLT with hospitalization before reLT, and adds two new variables: indication for reLT and time to reLT. Both factors were analyzed in the present study but not significantly associated with neither graft- nor patient survival (**Supplementary Tables S5, S6**). Consistent with our observations, other authors also found the indication for reLT as well as the time interval from LT to reLT not to be associated with graft- or patient survival [4, 34].

Similar to our observation, Brüggenwirth et al. discovered that the discriminating power of the Liver Retransplant Risk Score is most prominent in the first 6 to 12 months following reLT with survival curves running parallel thereafter [18]. Correspondingly,

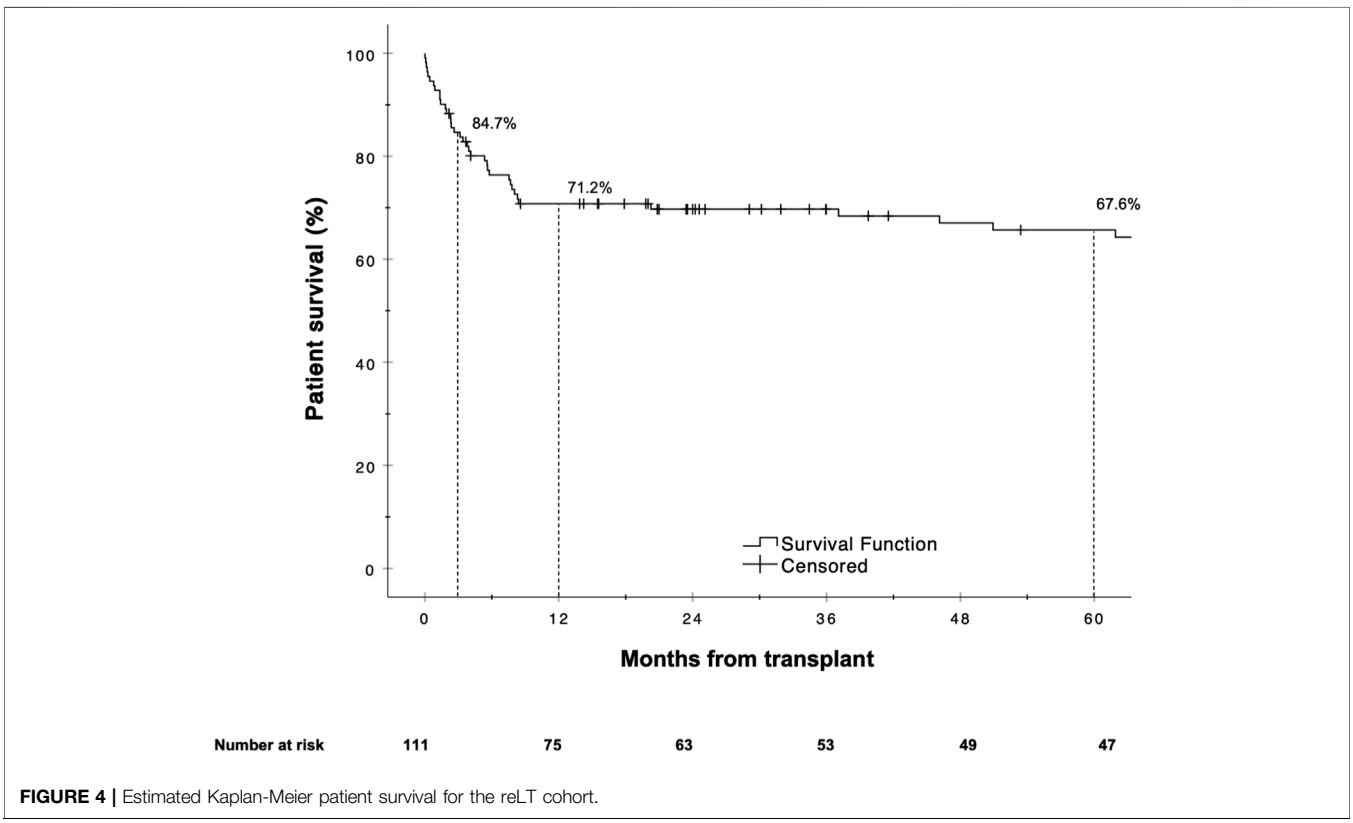


FIGURE 4 | Estimated Kaplan-Meier patient survival for the reLT cohort.

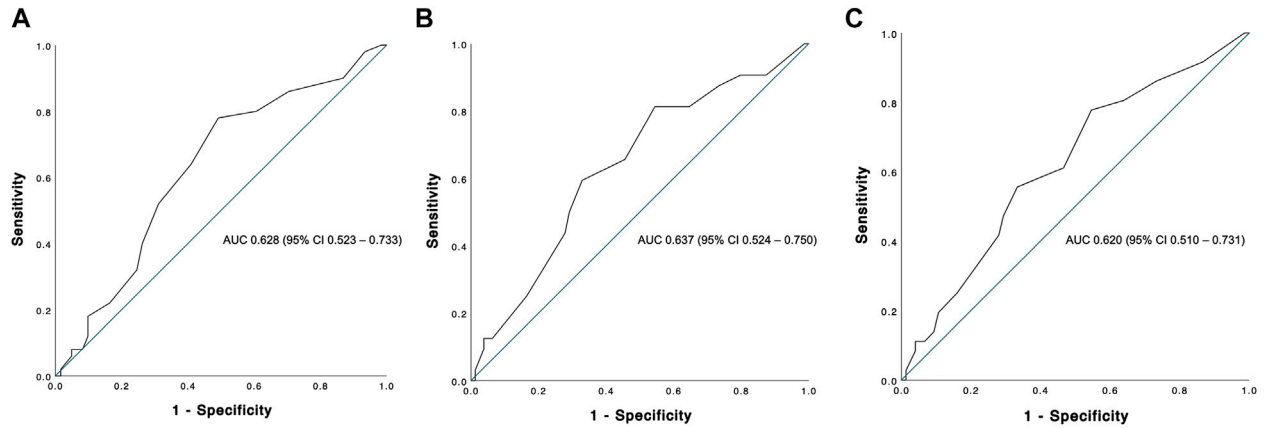


FIGURE 5 | ROC curve analysis depicting the predictive capability of the BAR score for overall **(A)**, 1 **(B)** and 5 years **(C)** patient survival. The BAR score performed best at predicting 1 year patient survival (AUC 0.637).

Yoon et al. also observed the most significant decline in survival during the first year following reLT [4].

In addition to its predictive value for graft- and patient survival, we observed the BAR score to exhibit a moderate positive correlation with the duration of hospital stay (r = 0.329, $p < 0.001$) and the CCI at 3 and 12 months (r = 0.272, $p = 0.004$). Furthermore, the BAR score showed good prediction of the incidence of major complications (AUC 0.720, $p = 0.004$). These findings are consistent with those previously reported by Schlegel et al. and Boecker et al. [32, 35]. In our cohort, the BAR score also predicted the incidence of sepsis–among the strongest independent risk factors of graft loss and patient death–with reasonable accuracy (AUC 0.676, $p = 0.01$).

An ideal predictive score is simple and easy to use, incorporates relevant donor and recipient factors and is available at the time of organ acceptance. The BAR score fulfils all these criteria. Among risk scores which are based on data available at the time of organ acceptance, the BAR score performs best and its robustness in predicting post-transplant outcomes in various settings has been shown in multiple studies including ours [36–38].

Various BAR score cutoffs have been suggested in the past. Boecker et al. found a cutoff of 14 points to best predict risk when analyzing 90 days patient- and graft survival following LT. However, 5 year patient survival was only moderately stratified at this cutoff (76% vs. 69%) [35].

Martínez et al. reported a cutoff of 15 points to best discriminate risk of 3 months, 1 and 5 year mortality [39], while Zakareya et al. determined that a cutoff of 10 points is best at predicting risk of patient death at 3 months, 1 and 5 years [40]. In our analysis a BAR score cutoff of 11 points exhibited the highest discriminating power in terms of graft loss and patient death at 1 and 5 years. Dutkowski et al. proposed a BAR score cutoff of 18 points as they observed that 5 year survival rates start to decline exponentially beyond this point [24]. In line with this observation, we found that 5 year graft survival dropped to below 50% for recipients with a BAR score ≥18 points (**Figure 3**).

With different definitions of futility and rationing in use, the transplant community often refers to a 5 year survival rate of 50% or higher as the threshold for an acceptable outcome [14]. However, futility and rationing will mean different things to different people in different contexts. The local waitlist dynamics as well as the availability of a potential live donor program will certainly impact the decision-making process. Given these complexities, it is difficult to recommend a definitive BAR score cutoff although a score around 18 points seems to mark a transition zone where outcomes are declining below what is considered acceptable. Consequently, optimizing donor-recipient combinations needs to be at the forefront of medical decision-making when accepting organs. In our study, good quality grafts–signified by the low median ET-DRI (1.44)–were used.

In the end, maximizing transplant benefit (i.e., the life years gained with LT as opposed to remaining on the waiting list) for the individual patient must be the main goal. To achieve this goal, transplant programs must be conscientious of their local circumstances including waitlist dynamics, recipient risk profiles and organ availability.

Limitations and Strengths

The present study has several limitations, which are mostly related to its retrospective study design. Although the overall number of LTs performed at the Medical University of Innsbruck was quite high, the sample size was limited since the proportion of reLTs was below 10%. The small sample size may have limited the statistical power especially for outcomes with low event rates. Furthermore, even though the BAR score has been shown to have the best predictive capability of all risk scores which are based on data available at the time of organ acceptance, better scores with AUCs well above 0.7 for relevant clinical outcome measures would be desirable.

Strengths of our study include the prospectively maintained LT database at our center and the high data granularity with little

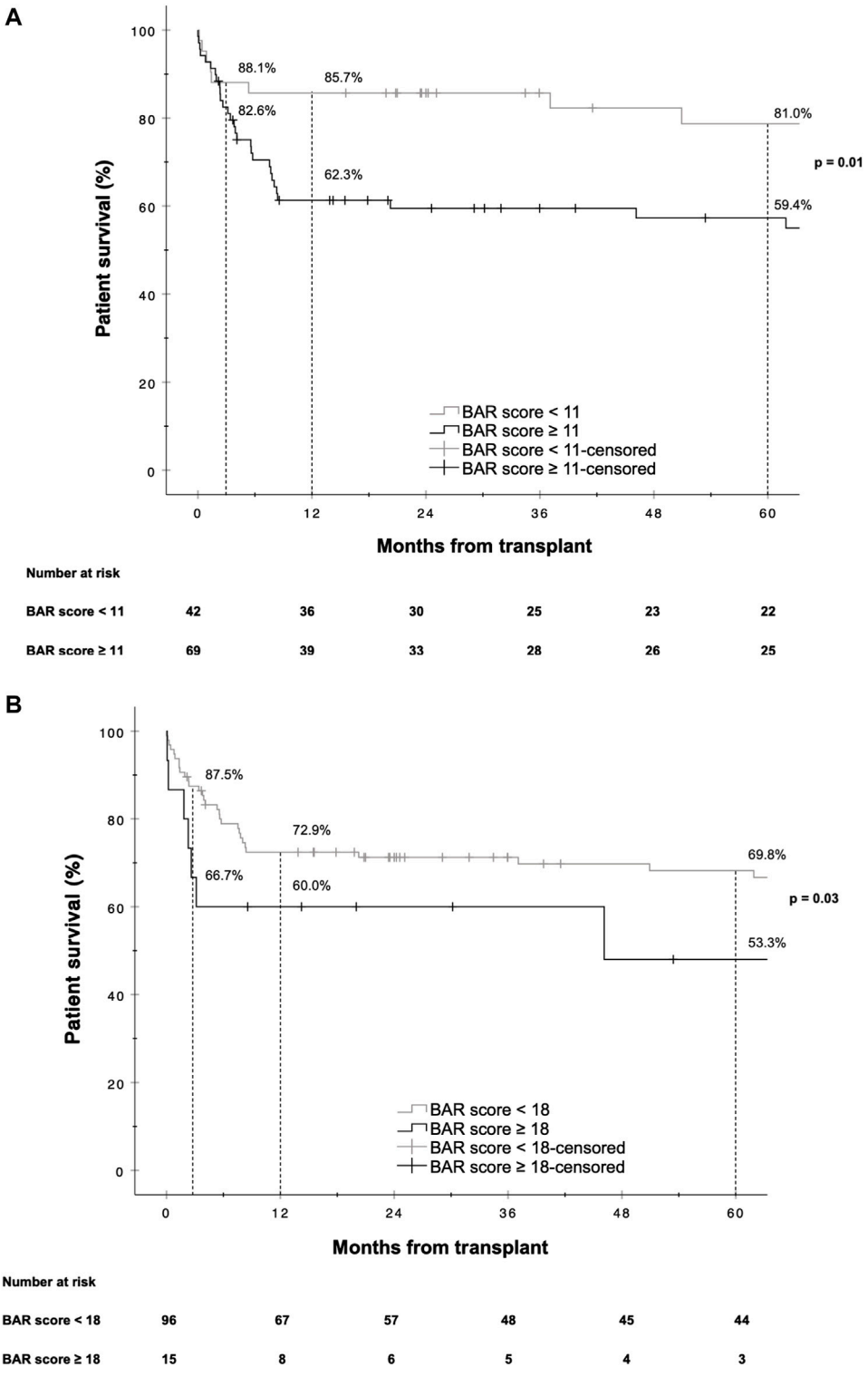


FIGURE 6 | Kaplan-Meier survival curves showing patient survival for reLT recipients with a BAR score <11 points vs. ≥11 points **(A)** and <18 points vs. ≥18 points **(B)**. The difference in graft survival at 1 and 5 years following reLT was highest at a BAR score cutoff of 11 points. At a BAR score cutoff of 18 points 5 year graft survival fell to below 53.3%.

TABLE 5 | Patient survival–multivariate adjusted Cox proportional hazards regression analysis[a].

	HR	95% CI	*p*-value
BAR score	1.050	0.981–1.125	0.16
Cholangitis	1.558	0.729–3.328	0.25
Major complication	1.427	0.430–4.737	0.56
PNF	29.987	7.514–119.664	**<0.001**
Sepsis	3.755	1.819–7.751	**<0.001**
Arterial complication	1.601	0.656–3.907	0.30
Reoperation within 30 days	1.002	0.473–2.120	0.996

BAR, balance of risk; CI, confidence interval; HR, hazard ratio; PNF, primary non-function.

[a]*The MELD, score and donor age (both included in the BAR, score) have been excluded.*

to no missing data in comparison to large registry studies. Moreover, the clinical management—from recipient evaluation to donor organ selection, surgical procedures and post-transplant care–was fairly homogenous at our center despite the long observation period. Still, multi-center studies evaluating clinical risk scores in the setting of reLT are needed.

CONCLUSION

The BAR score, a simple and readily available score available at the time of organ acceptance, is predictive of graft- and patient survival as well as duration of hospital stay, occurrence of sepsis and major complications following reLT. A cutoff of 11 points demonstrated the best discriminating power in terms of graft loss and patient death (i.e., the difference in survival between groups was highest at this cutoff). The hazards of graft loss and patient death were highest in the first year following reLT. In line with this observation, the BAR score performed best at predicting the 1 year risk of graft loss and patient death, comparing favorably to previously published reLT risk scores. For recipients with a BAR score ≥18 points, 5 year patient- and graft survival rates dropped to 50% and below. Sepsis and PNF were the strongest independent risk factors of graft loss and patient death. The occurrence of sepsis was predicted by the BAR score. In summary, the BAR score may serve as a predictive tool, allowing clinicians to estimate expected outcomes thereby facilitating clinical decision-making.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request. Requests to access these datasets should be directed to rupert.oberhuber@i-med.ac.at.

ETHICS STATEMENT

The studies involving humans were approved by the Institutional Review Board (Ethics commission) of the Medical University of Innsbruck, Austria (EK1240/2022). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

FK: study design, data acquisition, data analysis, interpretation of the data, drafting and revising the manuscript; MF: data acquisition, interpretation of the data, drafting and revising the manuscript; MB: data acquisition and revising of the manuscript; JS: data acquisition and revising the manuscript; HE: data acquisition and revising the manuscript; BC: interpretation of the data, drafting and revising the manuscript; TR: interpretation of the data, drafting and revising the manuscript; MM: data acquisition, interpretation of the data, drafting and revising the manuscript; CM: interpretation of the data, drafting and revising the manuscript; LS: study design, data analysis and revising the manuscript; TH: study design, data analysis and revising the manuscript; BS: interpretation of the data and revising the manuscript; HZ: interpretation of the data and revising the manuscript; HT: interpretation of the data and revising the manuscript; SS: interpretation of the data, drafting and revising the manuscript; RO: study design, data analysis, interpretation of the data, drafting and revising the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12104/full#supplementary-material

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GLOSSARY

AUC	area under the curve
Aza	azathioprine
AS	anastomotic stenosis
BAR	balance of risk
BMI	body mass index
CCI	comprehensive complication index
CIT	cold ischemia time
CsA	cyclosporine a
DBD	donation after brain death
DCD	donation after cardiocirculatory death
EAD	early allograft dysfunction
ECD	extended criteria donor
EK	ethikkommission (institutional review board)
ET-DRI	eurotransplant donor risk index
HAT	hepatic artery thrombosis
HR	hazard ratio
HTK	histidine-tryptophan-ketoglutarate
IL2	interleukin 2
INR	international normalized ratio
IQR	interquartile range
LT	liver transplantation
MELD	model for end-stage liver disease
MMF	mycophenolate mofetil
MPA	mycophenolic acid
PNF	primary non-function
reLT	liver retransplantation
SD	standard deviation
STROBE	strengthening the reporting of observational studies in epidemiology
Tac	tacrolimus
UNOS	united network for organ sharing
UW	university of wisconsin
WIT	warm ischemia time

ESOT | Transplant International

ORIGINAL RESEARCH
published: 12 January 2024
doi: 10.3389/ti.2023.11551

A European Multi-Center Analysis of Extracorporeal Photopheresis as Therapy for Chronic Lung Allograft Dysfunction

Alberto Benazzo[1], Cecilia Bagnera[2], Fabio Ius[3,4], Claudia Del Fante[5], Jens Gottlieb[3,6], Konrad Hoetzenecker[1], Federica Meloni[2], Peter Jaksch[1] and Mark Greer[3,6]*

[1]Department of Thoracic Surgery, Medical University of Vienna, Vienna, Austria, [2]Malattie dell'Apparato Respiratorio, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, [3]Department of Respiratory Medicine, Hannover Medical School, Hannover, Germany, [4]Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany, [5]Servizio Immunoematologia e Medicina Trasfusionale, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy, [6]German Centre for Lung Research, Biomedical Research in End-Stage and Obstructive Lung Disease Hannover, Hannover, Germany

Extracorporeal photopheresis (ECP) is used by few lung transplant centers to treat chronic lung allograft dysfunction (CLAD). Although reported results suggest a beneficial effect on CLAD progression, evidence is limited to single center experiences. The aim of this study is to analyze outcomes of ECP in a large multicenter European cohort. The primary endpoint was patient survival after initiation of ECP. This study included 631 patients, 87% suffered from bronchiolitis obliterans syndrome (BOS), and 13% had restrictive allograft syndrome (RAS). Long-term stabilization was achieved in 42%, improvement in 9%, and no response in 26%. Within the first 12 months of therapy, 23% of patients died. Patients' survival after initiation of ECP at 5 years was 56% in stable, 70% in responders, and 35% in non-responders ($p = 0.001$). In multivariable Cox regression, both stabilization (HR: 0.48, CI: 0.27–0.86, $p = 0.013$) and response (HR: 0.11, CI: 0.04–0.35, $p < 0.001$) to ECP were associated with survival. Absolute FEV1 at baseline was also protective (HR: 0.09, CI: 0.01–0.94, $p = 0.046$). RAS phenotype was the only risk factor for mortality (HR: 2.11, 1.16–3.83, $p = 0.006$). This study provides long-term outcomes of ECP use in CLAD patients in the largest published cohort to date. Two-thirds of the cohort had a sustained response to ECP with excellent long-term results.

Keywords: lung transplantation, extracorporeal photopheresis, CLAD, FEV1, lung function

OPEN ACCESS

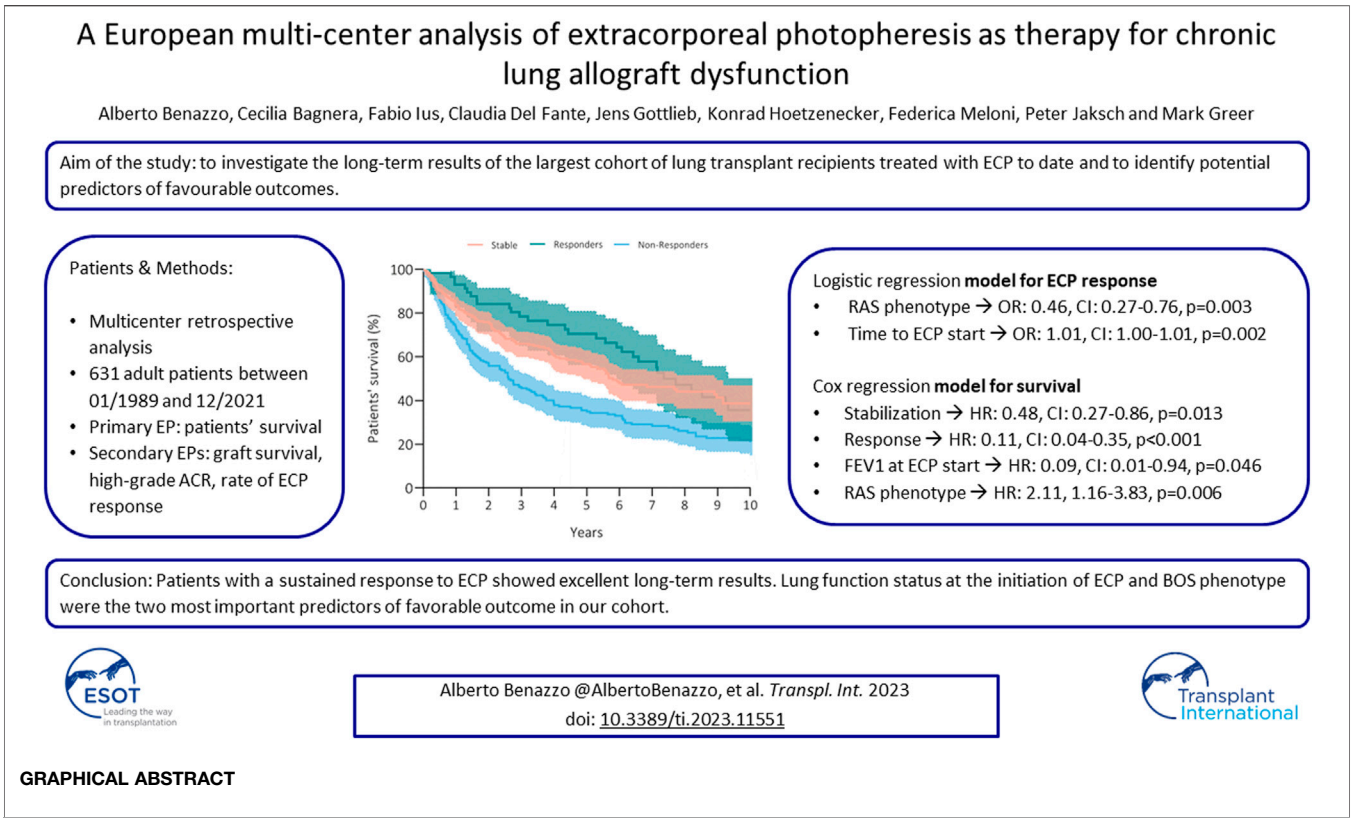
Correspondence:
Alberto Benazzo
alberto.benazzo@meduniwien.ac.at

Received: *07 May 2023*
Accepted: *07 December 2023*
Published: *12 January 2024*

Citation:
Benazzo A, Bagnera C, Ius F, Del Fante C, Gottlieb J, Hoetzenecker K, Meloni F, Jaksch P and Greer M (2024) A European Multi-Center Analysis of Extracorporeal Photopheresis as Therapy for Chronic Lung Allograft Dysfunction. Transpl Int 36:11551. doi: 10.3389/ti.2023.11551

INTRODUCTION

Chronic lung allograft dysfunction (CLAD) remains the major long-term cause of graft loss, affecting up to 60% of recipients within 5 years after lung transplantation (LTx) [1]. Although significant improvements have been implemented in the diagnosis and management of CLAD, effective treatment options are still lacking. Over the last two decades, extracorporeal photopheresis (ECP) has been increasingly used, to stabilize the deterioration of lung function besides other possible strategies, such as immunosuppression augmentation or administration of azithromycin [2–5]. ECP is an extracorporeal therapy, combining leukapheresis with photoactivation. It consists in the incubation of mononuclear cells with 8-methoxypsoralen (8-MOP) and subsequent activation of 8-MOP with ultraviolet A radiation. The cells are then reinfused into the patient. 8-MOP is a



GRAPHICAL ABSTRACT

biologically inert substance, but in the presence of UVA light it cross-links DNA by forming covalent bonds with pyrimidine bases and causes cell apoptosis [6]. ECP has been firstly developed for treatment of cutaneous T cell lymphomas and later used in a variety of other indications including graft-versus-host disease and organ transplantation [7]. Up to date, only a limited number of LTx programs use ECP as a treatment for CLAD. To date published evidence is limited to single center retrospective analyses. According to available evidence, approximately 60%–70% of treated recipients profit from ECP, while in the rest of the treated patients lung function continues to deteriorate. In the current analysis, we examine the long-term outcomes of the largest cohort of lung transplant recipients treated with ECP to date.

METHODS

This is a retrospective multicenter analysis, including all lung transplant recipients transplanted between January 1989 and December 2021 and treated with ECP over the same time period in three European centers: Medical University of Vienna, Hannover Medical School Hannover and IRCCS Policlinico San Matteo. The primary endpoint was patient survival. Secondary endpoints were rate of ECP response, rate of high grade acute cellular rejection (ACR) and graft survival. Inclusion criteria were all patients ≥18 years, commencing ECP for progressing CLAD. This study has been approved by the Ethical Committe and was conducted according the declaration of Helsinki. The study was registered to *clinicaltrials.gov* with the number NCT04792294.

Spirometry was performed and interpreted according to ATS/ERS guidelines [8]. Values collected for the analysis were Forced Expired Volume in 1s (FEV1), Forced Vital Capacity (FVC) and Total Lung Capacity (TLC). Individual patient spirometry baselines were calculated based on the most recent ISHLT recommendations, with the mean value of the 2 best postoperative measurements obtained >3 weeks apart [9]. Diagnosis of CLAD was established by two independent physicians according to the consensus report of the ISHLT(9). CLAD was confirmed if FEV1 decline of ≥20% persisted for at least 3 months after exclusion or treatment of possible secondary causes, e.g., infections, acute rejection or extrapulmonary causes. Spirometry, TLC measurements and CT appearance were used to define CLAD phenotypes [9]. All transbronchial biopsies between transplantation and initiation of CLAD were included in the analysis and were classified according to ISHLT criteria [10]. A high-grade ACR was considered as A≥2, while high-grade LB was considered as B≥2.

Patient diagnosed with definite CLAD received a trial with azithromycin or montelukast for at least 3 months depending on center-specific clinical practice. In case of further deterioration, recipients started ECP. ECP was performed either on-line or off-line. On-line ECP was performed using the Therakos® CELLEX® Photopheresis System (Therakos UK Ltd., a Mallinckrodt Pharmaceuticals company), which is a closed-loop sterile

system. The procedure has been described in detail elsewhere [11]. During ECP, peripheral blood mononuclear cells is separated from the whole blood in a Latham centrifuge (Latham International, Chesterton, UK) at 2,700 RPM. The collected cells (buffy-coat bag) is treated with 8-methoxypsoralen solution (UVADEX®, Therakos, Mallinckrodt Pharmaceuticals) and exposed extracorporeally to ultraviolet A light (1–2 J/m2) before reinfusion to the patient. During each treatment, four to six collection cycles are performed or 1,500 mL blood is processed, depending on the patient's hematocrit level. Initially a 2 day treatment cycle was performed every second week for the first two to 6 months, according to institutional preferences. Then, a 2 day treatment cycle was performed once a month. When ECP was performed using the off-line technique, PBMCs were collected from the patient using a cell separator device, processing 1.5–2 blood volumes. Hemocytometric analysis was performed on the product at the end of each collection (quality control). Then, cells were irradiated (UV-A at 2 J/cmq; Macogenic, Macopharm a, France) after the dilution with saline solution and the addition of 8-methoxypsoralen (at 200 ng/mL concentration). Finally, the photoactivated PBMCs were immediately reinfused into the patient [12].

Responders were defined as patients with >10% improvement in FEV1 compared with the value at the time ECP treatment was started. Stable patients were defined as patients with ≤10% improvement or ≤10% worsening of FEV1 compared with the value at the time of initiation of ECP treatment. Non-responders were defined as patients who had a decline of >10% after ECP treatment. Interim response was evaluated at 3 and 6 months and long-term response was evaluated at completion of ECP or at the time of data analysis in the patients currently on ECP for >6 months. The rate of lung function decline was defined as a decrease in FEV1 in ml between two time points: positive values indicate a decrease in ml per month, whereas negative values indicate an increase in ml per month.

Statistical Analysis

Categorical variables were reported as absolute and relative frequencies (%), continuous variables as median (interquartile range, IQR) or mean (± standard deviation). Relative frequencies were calculated based on the number of patients alive in follow-up at the respective timepoint. Chi-square tests, Fisher exact tests, Mann-Whitney U-tests, or ANOVA were used to compare variables as applicable. Survival curves were generated with the Kaplan-Meier method and compared by log-rank tests. Univariate and multivariable Cox regression were performed to find risk factors for mortality. Variables were included in a multivariable Cox regression when they reached the level of significance in the univariate analysis. Univariate and multivariable logistic regression were performed to find predictors of response (defined as stable and responders) to ECP. Variables were included in a multivariable logistic regression when they reached the level of significance in the univariate analysis. Data was analyzed using SPSS version 26.0 software or R 3.4.2 and graphics were designed with GraphPad Prism 6.

TABLE 1 | Patients' characteristics.

Patients' characteristics (N = 613)		
Female (n, %)		291, 48%
Age at LuTx (median, IQR)		49 (35–56)
High-risk CMV mismatch (n, %)		131, 23%
Underlying diagnosis	COPD (n, %)	225, 37%
	Fibrosis (n, %)	155, 25%
	iPAH (n, %)	57, 10%
	CF (n, %)	106, 17%
	CLAD (n, %)	31, 5%
	Others (n, %)	39, 6%
Type of Tx	DLuTX (n, %)	524, 86%
	SLuTX (n, %)	67, 11%
	HLuTx (n, %)	21 (3%)
FEV1 baseline (L/min) (median, IQR)		2.7 (2.1–3.9)
TLC baseline (L) (median, IQR)		5.5 (4.7–6.5)
High-grade ACR (n, %)		88, 18%
High-grade LB (n, %)		74, 15%
CLAD phenotypes	BOS (n, %)	513, 87%
	RAS (n, %)	78, 13%
Time to CLAD (months) (median, IQR)		34 (18–64)
Azithromycin (n, %)		553 (90%)
Montelukast (n, %)		221 (36%)
FEV1 at ECP start (L/min) (median, IQR)		1.4 (1.1–1.9)
TLC at ECP start (L) (median, IQR)		5.2 (4.1–6.2)
FEV1 at ECP (% baseline) (median, IQR)		56 (44–67)
Time to ECP (months) (median, IQR)		46 (26–88)
ECP cycles (median, IQR)		15 (11–25)
Response to 3 months of ECP	Stable (n, %)	319 (61%)
	Responder (n, %)	43 (8%)
	Non-Responder (n, %)	130 (25%)
	Death within 3 months (n, %)	32 (6%)
Response to 6 months of ECP	Stable (n, %)	294 (52%)
	Responder (n, %)	57 (10%)
	Non-Responder (n, %)	138 (24%)
	Death within 6 months (n, %)	79 (14%)
Long-term response to ECP	Stable (n, %)	252 (42%)
	Responder (n, %)	55 (9%)
	Non-Responder (n, %)	160 (26%)
	Death within 12 months (n, %)	138 (23%)

Abbreviations. N, numbers; IQR, interquartile range; SD, standard deviation; ECP, extracorporeal photopheresis; LuTx, lung transplantation; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; iPAH, idiopathic pulmonary arterial hypertension; CF, cystic fibrosis; CLAD, chronic lung allograft dysfunction; ReTx, retransplantation; DLuTx, double lung transplantation; SLuTx, single lung transplantation; HLuTx, heart-lung transplantation; ACR, acute cellular rejection; LB, lymphocytic bronchiolitis; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; ECP, extracorporeal photopheresis.

RESULTS

Patients' Demographics

This multicenter analysis included 631 patients from three European centers. Forty-eight percent (n = 291) were female and the mean age was 49 years (IQR: 35–56). The underlying diagnosis was COPD in 37% (n = 225) of patients, fibrosis in

TABLE 2 | Demographics per group.

		Stable (*n* = 252)	Responders (*n* = 55)	Non-Responders (*n* = 160)	Death within 12 months (*n* = 138)	*p*-value
Age at LuTx (median, IQR)		49 (34–56)	50 (39–56)	47 (30–54)	53 (37–59)	.030
High-risk CMV mismatch (n, %)		57 (23%)	14 (26%)	32 (21%)	38 (28%)	.579
Underlying diagnosis	COPD (n, %)	94 (37%)	23 (42%)	53 (33%)	53 (38%)	.943
	Fibrosis (n, %)	61 (24%)	14 (26%)	44 (28%)	35 (25%)	
	iPAH (n, %)	25 (10%)	4 (8%)	18 (11%)	9 (7%)	
	CF (n, %)	43 (17%)	6 (10%)	30 (19%)	24 (17%)	
	CLAD (n, %)	11 (5%)	4 (7%)	7 (4%)	9 (7%)	
	Others (n, %)	18 (7%)	4 (7%)	8 (5%)	8 (6%)	
Type of Tx	DLuTX (n, %)	213 (85%)	47 (85%)	131 (83%)	125 (91%)	.299
	SLuTX (n, %)	28 (11%)	8 (15%)	21 (13%)	10 (7%)	
	HLuTx (n, %)	11 (4%)	0	7 (4%)	3 (2%)	
FEV1 baseline (L/min) (median, IQR)		2.8 (2.2–3.4)	2.5 (2–3.2)	2.8 (2.3–3.4)	2.7 (2.2–3.1)	.101
TLC baseline (L) (median, IQR)		5.8 (4.7–6.9)	5.7 (4.8–6.5)	5.3 (4.7–6.4)	5.3 (4.4–6.2)	.056
Higher-grade ACR (n, %)		36 (17%)	3 (7%)	27 (22%)	22 (19%)	.181
Higher-grade LB (n, %)		27 (13%)	3 (7%)	23 (19%)	20 (17%)	.213
CLAD phenotypes	BOS (n, %)	**218 (92%)**	**49 (93%)**	**126 (81%)**	**113 (83%)**	**.005**
	RAS (n, %)	**20 (8%)**	**4 (7%)**	**30 (19%)**	**23 (17%)**	
Time to CLAD (months) (median, IQR)		35 (21–73)	37 (17–64)	34 (18–63)	30 (15–52)	.245
Azithromycin (n, %)		232 (93%)	51 (93%)	138 (87%)	125 (91%)	.208
Montelukast (n, %)		95 (38%)	20 (37%)	59 (37%)	47 (34%)	.914
FEV1 at ECP start (L/min) (median, IQR)		**1.6 (1.2–2)**	**1.2 (0.9–1.6)**	**1.5 (1.2–1.9)**	**1.3 (1–1.7)**	**<.001**
TLC at ECP start (L) (median, IQR)		5.3 (4.4–6.5)	6.1 (5.3–7.1)	4.8 (3.8–6)	5.1 (3.8–6)	.138
FEV1 at ECP (% baseline) (median, IQR)		60 (48–70)	52 (42–58)	55 (45–68)	49 (39–62)	.074
Time to ECP (months) (median, IQR)		**56 (33–101)**	**44 (24–92)**	**41 (24–78)**	**39 (22–76)**	**<.001**
Rate of FEV1 decline before ECP (mL/month) (median, IQR)		**18 (10–35)**	**24 (12–57)**	**28 (14–51)**	**29 (13–60)**	**<.001**
Rate of FEV1 decline in 3 months of ECP (mL/month) (median, IQR)		**10 (-27–43)**	**–113 (-160–-37)**	**57 (10–120)**	**72 (23–137)**	**<.001**
Rate of FEV1 decline in 6 months of ECP (mL/month) (median, IQR)		**4 (-10–23)**	**–65 (-107–-8)**	**36 (16–77)**	**43 (20–82)**	**<.001**

The rate of FEV1 decline was calculated as the difference in ml between two time points per month: positive values indicate a decline in ml per month, while negative values indicate an increase in ml per month. Abbreviations. N, numbers; IQR, interquartile range; SD, standard deviation; LuTx, lung transplantation; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; iPAH, idiopathic pulmonary arterial hypertension; CF, cystic fibrosis; CLAD, chronic lung allograft dysfunction; ReTx, retransplantation; DLuTx, double lung transplantation; SLuTx, single lung transplantation; HLuTx, heart-lung transplantation; ACR, acute cellular rejection; LB, lymphocytic bronchiolitis; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; ECP, extracorporeal photopheresis.
Bold values are the significant results.

25% (*n* = 155), iPAH in 10% (*n* = 57), and CF in 17% (*n* = 106). Twenty-three percent (*n* = 131) of patients had high-risk CMV mismatch, and 86% (*n* = 524) underwent bilateral lung transplantation. The median baseline FEV1 was 2.7 (2.1–3.9) L/min and the median baseline TLC was 5.5 L (4.7–6.5). Eighty-eight patients (18%) had high-grade ACR, and 74 (15%) had high-grade LB. Eighty-seven percent of patients treated with ECP had BOS patients, and only a minority (78, 13%) had RAS at the time of ECP initiation. The median time to CLAD after transplantation was 34 (18–64) months. Before initiation of ECP, 90% (*n* = 553) of patients had been treated with azithromycin and 36% (*n* = 221) with montelukast. Full demographic data can be found in **Table 1**.

Extracorporeal Photopheresis

Recipients with CLAD started ECP after a median of 34 months (18–64) after lung transplantation. The median FEV1 at the start of ECP was 1.4 L/min (1.1–1.9) and the median TLC was 5.2 L (4.1–6.2). ECP was performed for a median of 15 cycles (11–25). Response rate decreased at 3, 6 months and at the end of ECP. After 3 months of ECP, 61% showed stabilization of lung function, 8% showed an improvement and 25% showed a further worsening. Within the first 3 months of therapy, 6% of patients died. After 6 months of ECP, 52% exhibited stabilization of lung function, 10% improvement and 24% a worsening. Within the first 6 months of therapy, 14% of patients died. Long-term stabilization was achieved in 42%, improvement in 9%, and no response in 26%. Within the first 12 months of therapy, 23% of patients died. Long-term stable patients and responders were predominantly BOS patients (p = 0.005, **Table 2**) while non-responders were mostly RAS (p = 0.005, **Table 2**) and a shorter time to ECP start ($p < 0.001$, **Table 2**). A logistic regression was performed to find predictors of response to ECP (**Table 3**). Interestingly, in the multivariable regression model, RAS phenotype (OR: 0.46, CI: 0.27–0.76, p = 0.003) represented the only risk-factor for failed response while longer time to initiation of ECP (OR: 1.01, CI: 1.00–1.01, p = 0.002) seems to be predictive of a favorable response.

TABLE 3 | Logistic regression for response to ECP.

		OR (CI)	*p*-value	Adjusted OR (CI)	*p*-value
Age at LuTx		1.00 (0.99–1.02)	.574		
High-risk CMV mismatch		1.06 (0.71–1.58)	.771		
Underlying diagnosis	COPD	Reference			
	Fibrosis	0.74 (0.48–1.33)	.166		
	iPAH	0.93 (0.50–1.72)	.821		
	CF	0.88 (0.54–1.44)	.615		
	CLAD	1.09 (0.49–2.43)	.839		
	Others	0.99 (0.48–2.06)	.990		
Type of Tx	DLuTX	Reference			
	SLuTX	0.89 (0.53–1.51)	.673		
	HLuTx	1.13 (0.45–2.86)	.791		
FEV1 baseline (L/min)		1.01 (0.83–1.22)	.937		
TLC baseline (L)		0.99 (0.99–1.00)	.713		
Higher-grade ACR		0.86 (0.53–1.39)	.543		
Higher-grade LB		0.62 (0.37–1.02)	.062		
CLAD phenotypes	BOS	Reference		Reference	
	RAS	**0.45 (0.27–0.73)**	**<0.001**	**0.46 (0.27–0.76)**	**.003**
Time to CLAD (months)		1.00 (0.99–1.01)	.119		
Azithromycin		**1.77 (1.01–3.12)**	**.049**	1.47 (0.81–2.66)	.207
Montelukast		1.05 (0.74–1.49)	.778		
FEV1 at ECP start (L/min)		1.08 (0.83–1.41)	.562		
TLC at ECP start (L)		1.36 (0.80–1.71)	.100		
FEV1 at ECP (% baseline)		1.02 (0.99–1.01)	.668		
Time to ECP (months)		**1.01 (1.00–1.10)**	**<0.001**	**1.01 (1.00–1.01)**	**.002**

Bold values are the significant results.

Outcomes

Three hundred fifty patients (57%) died, and the most common causes of death were CLAD in 47% (n = 164, 27% from the whole cohort) and sepsis in 19% (n = 66, 11% from the whole cohort) of recipients (**Table 4**). Patients' survival rates after initiation of ECP were at 5 years: 56% in stable, 70% in responders and 35% in non-responders; at 10 years: 39% in stable, 36% in responders and 23% in non-responders (p = 0.001) (**Figure 1**). Fifty-three patients (9%) received retransplantation. Graft survival rates after initiation of ECP were at 5 years: 53% in stable, 68% in responders and 30% in non-responders; at 10 years: 35% in stable, 31% in responders and 20% in non-responders (p = 0.001) (**Figure 2**).

Cox regression was performed to examine the effect of response to extracorporeal photopheresis on patient survival after adjusting for confounding factors (**Table 5**). Multivariable regression showed that long-term stabilization (HR: 0.48, CI: 0.27–0.86, p = 0.013) or response (HR: 0.11, CI: 0.04–0.35, p < 0.001) to ECP were associated with survival. Interestingly, absolute FEV1 at baseline ECP was also protective (HR: 0.09, CI: 0.01–0.94, p = 0.046). RAS phenotype was the only risk factor for mortality (HR: 2.11, 1.16–3.83, p = 0.006).

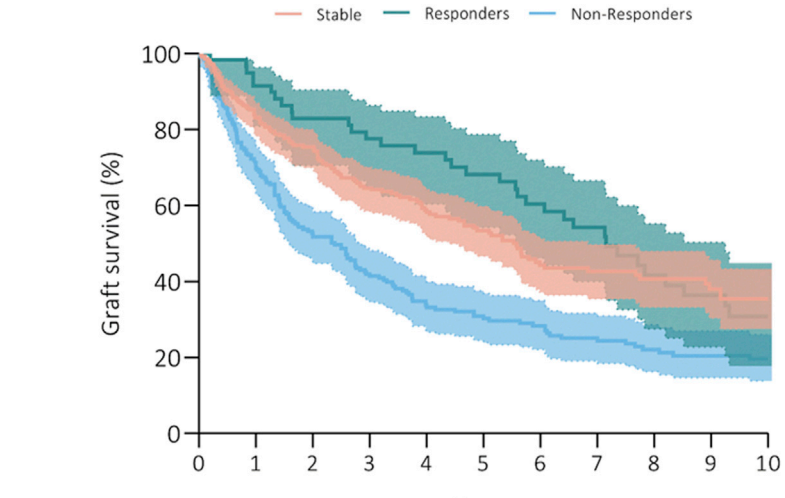
TABLE 4 | Outcomes.

Outcomes		
ReTx (n, %)		**53 (9%)**
Death (n, %)	All	350 (57%)
	CLAD (n, %)	164 (47%)
	Sepsis (n, %)	66 (19%)
	Malignancy (n, %)	22 (6%)
	Others (n, %)	98 (28%)
Graft survival (months) (median, IQR)		98 (53–152)

Abbreviations. N, numbers; IQR, interquartile range; ReTx, retransplantation; CLAD, chronic lung allograft dysfunction.

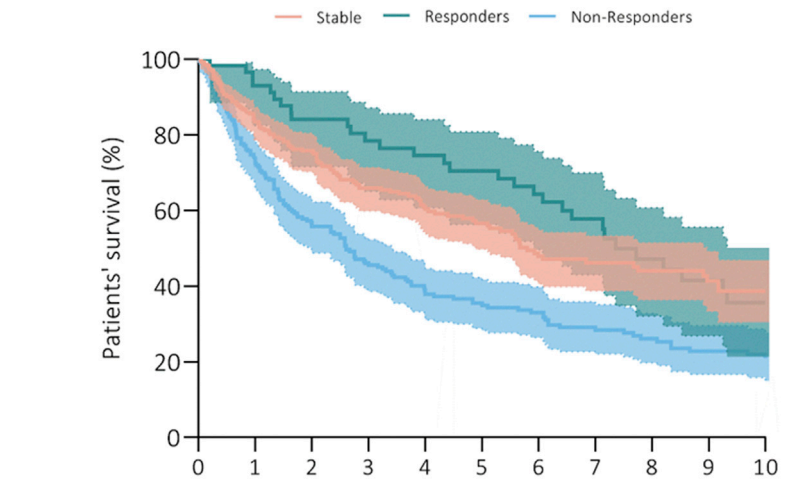
DISCUSSION

Chronic lung allograft dysfunction remains the leading cause of morbidity and mortality after lung transplantation. According to the international benchmarks, median survival after the diagnosis of CLAD ranges between 3 and 5 years. Curative treatments have not been established yet, however, different therapeutic interventions can slow down the progression of allograft dysfunction. Extracorporeal photopheresis is an immunomodulatory therapy, which targets T-cell mediated injury and improves mortality and morbidity in a range of T-cell mediated diseases as well as graft-versus-host disease [13]. With the same rationale, ECP was introduced in solid organ transplantation as a salvage therapy for a range of indications. The current study, including more than 600 patients, presents the largest experience with ECP in a CLAD population to date. The herein reported results show that 63% of CLAD patients experienced a stabilization or



Subjects at risk											
Stable	314	236	191	143	105	84	61	46	36	30	26
Responders	61	56	49	44	41	36	32	23	17	14	11
Non-Responders	217	149	103	80	62	51	45	37	28	25	22

FIGURE 1 | Kaplan Meier's curve showing patients' survival after initiation of ECP. Curves have been compared with log-rank test.



Subjects at risk											
Stable	314	230	186	142	105	86	64	47	38	32	28
Responders	61	55	48	42	39	35	32	23	18	15	13
Non-Responders	217	156	112	89	70	59	52	42	33	28	23

FIGURE 2 | Kaplan Meier's curve showing overall graft survival after initiation of ECP. Curves have been compared with log-rank test.

improvement of the allograft function after ECP initiation, which was associated with a survival benefit.

To date, ECP treatment has been used as second-line therapy for CLAD after lung transplantation. However, efficacy data is based only on small single-center studies. Greer et al. found that RAS patients, as well as patients whose lung function deteriorated rapidly, had lower response rates and worse long-term outcomes [4]. Similarly, in another analysis, only BOS was associated with better outcomes [3]. A prospective study published by the Vienna group confirmed the results of previous retrospective analyses, showing a 61% response rate and improved survival in the responder population [5].

TABLE 5 | Cox Regression for patient survival.

			HR (CI)	*p*-value	Adjusted HR (CI)	*p*-value
Age at LuTx			**1.03 (1.02–1.04)**	**<.001**	1.02 (0.99–1.05)	.214
High-risk CMV mismatch			1.18 (0.92–1.53)	.198		
Underlying diagnosis	COPD		Reference			
	Fibrosis		1.03 (0.79–1.34)	.828		
	iPAH		**0.51 (0.34–0.77)**	**<.001**	0.90 (0.24–3.38)	.886
	CF		0.72 (0.53–1.01)	.054		
	CLAD		0.88 (0.54–1.45)	.617		
	Others		0.91 (0.57–1.44)	.684		
Type of Tx	DLuTX		Reference			
	SLuTX		0.81 (0.59–1.12)	.199		
	HLuTx		0.50 (0.27–1.11)	.124		
FEV1 baseline (L/min)			**0.87 (0.76–0.98)**	**.023**	1.92 (0.65–5.62)	.237
TLC baseline (L)			1.00 (0.99–1.01)	.560		
Higher-grade ACR			0.91 (0.66–1.26)	.577		
Higher-grade LB			1.35 (0.98–1.86)	.064		
CLAD phenotypes	BOS		Reference		Reference	
	RAS		**2.01 (1.53–2.63)**	**<0.001**	**2.11 (1.16–3.83)**	**.015**
Time to CLAD (months)			**0.99 (0.98–0.99)**	**<0.001**	0.99 (0.97–1.01)	.379
Azithromycin			0.71 (0.51–1.00)	.051		
Montelukast			0.94 (0.76–1.18)	.610		
FEV1 at ECP start (L/min)			**0.64 (0.53–0.76)**	**<0.001**	**0.09 (0.01–0.94)**	**.046**
TLC at ECP start (L)			**0.88 (0.78–0.99)**	**.029**	1.09 (0.95–1.27)	.227
FEV1 at ECP (% baseline)			**0.99 (0.98–0.99)**	**<0.001**	1.04 (0.98–1.11)	.187
Time to ECP (months)			**0.99 (0.98–0.99)**	**<0.001**	0.99 (0.97–1.01)	.064
Response to ECP at end of ECP		Stable	0.50 (0.40–0.63)	**<0.001**	**0.48 (0.27–0.86)**	**.013**
		Responder	0.48 (0.33–0.71)	**<0.001**	**0.11 (0.04–0.35)**	**<0.001**
		Non-Responder	Reference		Reference	

Bold values are the significant results.

Recently, the Hannover group proposed an innovative approach to assessing CLAD patient outcomes using a temporal characterization of allograft function [14]. In this study, the authors not only reported a response rate to ECP comparable to previously published studies, but also suggested that grafts with lower performance at the beginning of ECP were more likely to be associated with worse outcomes [14]. The current analysis includes 631 patients from three European centers with a long-standing experience with ECP. Long-term stabilization of graft function could be achieved in 53% of the cohort, 10% showed an improvement while the remaining 37% fail to respond to ECP. These rates confirm previously published experience. Our data showed that the BOS phenotype was associated with a higher response rate and improved survival, while RAS phenotype was associated with lower response rate and higher mortality. Interestingly, absolute TLC at initiation of ECP did not seem to be a risk factor. Thus, the results of our analysis suggests that the unresponsiveness of this subpopulation is related more to the restrictive phenotype *per se* and its underlying pathophysiology than to the reduction of lung volumes. This finding is not completely novel. Indeed, the majority of previous series could show the same difference in response between the phenotypes [3, 5, 14], however, the mechanistic reason remains elusive. RAS is characterized by a more intense allogeneic inflammatory response followed by diffuse fibrotic processes in various anatomic compartments [15]. The most widely accepted hypothesis is that a severe and fulminant immune response is triggered by an acute event such as ACR, AMR, or viral infection, which initiates extensive pro-fibrotic events involving airways, pleura, septum, alveoli, and vessels [15]. On the other side, BOS is mostly a chronic airway-centered disease. External exposures, airway-specific autoantibodies, a type 17 immune response, and early ischemic injury to the airway epithelium can chronically affect lung allografts via the airway [15]. It is reasonable to speculate that the slowly evolving immunomodulatory effect of ECP is more effective in the subclinical injury typical of BOS. In addition, there is a hypothesis that ECP is less effective in modulating endothelial activation and fibrogenic mechanisms characteristic of RAS. Although RAS appears to be associated with CLAD progression and nonresponse to ECP, this alone can hardly explain the 37% nonresponse rate. Therefore, the mechanisms of action of ECP need to be further elucidated to understand its application and limitations in CLAD.

An important finding of the current study is that the absolute FEV1 value at the initiation of ECP is an independent predictor of survival in our cohort but unrelated to ECP treatment response. This is a novel finding is new and suggests that the use of baseline lung function estimates may be misleading in the design of clinical trials intended to assess functional response to new

CLAD therapies. Indeed, the risk of baseline estimates is that they tend to overestimate lung allograft function, thereby discriminating against patients at higher risk for worse outcomes. As early as 2007, Burton et al warned that the use of an estimated baseline FEV1 represents a statistical bias and disadvantages recipients with lower baseline values [16]. Applied to the current topic, this means that patients with lower absolute FEV1 values are classified as having a more severe CLAD grade, while also having poorer functional reserve. As a result, they deteriorate more rapidly and, in most cases, end in fatal respiratory failure before they can experience a benefit from the started therapy. The prospective nonrandomized study conducted by the EPI Study Group is the best example of this limitation in the context of ECP(17). Because of the study design, patients with low FEV1 values and more rapid deterioration were more likely to undergo ECP and have a fatal outcome [17]. On the other hand, however, ECP was associated with a 93% reduction in FEV1 decline, and none of the fatal outcomes were related to ECP(17). Instead, 92% of mortality cases were due to end-stage lung failure. Similar results were observed in the recent work from Hannover, which showed that absolute FEV1 at the onset of ECP had the greatest impact on patient and graft survival [14]. Taken together, this underscores that the absolute FEV1 at the initiation of ECP may be the most important confounding bias in evaluating outcomes over time and, in parallel, this finding suggests that ECP should be initiated at earlier stages rather than used as rescue therapy when functional reserve has reached a dangerous level.

Another important observation of this study is that a longer interval to initiation of ECP is associated with better outcomes. This is clearly a surrogate measure of the severity of CLAD. Patients with a shorter time to initiation of ECP were those whose condition deteriorated rapidly and who had a more fulminant course. In these patients, the ECP effect may never have manifested. Similar findings were already observed in smaller single-center series [3, 5]. Moreover, it is already known that the effects of ECP are not apparent for at least 4–5 months in GvHD patients and over 12 months in scleroderma patients. Therefore, in conjunction with the previously discussed findings, possible use of ECP could be considered to increase the efficacy of this therapy in CLAD patients.

We are aware that this study is not free of limitations. First, because of the retrospective nature of the study, there is a possibility that the data were miscoded. In addition, we cannot exclude the possibility that the indication for ECP became more liberal over time because of increasing clinical experience. Another limitation arises from the multicenter nature of the study, as clinical practice might differ among the centers. Another limitation of our retrospective multicenter analysis is that data on AMR and DSAs are not included. Because the pathogenic role of AMR and DSA in lung transplantation is relatively recent and the analysis covers a period of more than 20 years, these data are available only for patients treated in the last 5 years. Finally, the three centers use different ECP systems, which could potentially affect the results.

Despite these limitations, this study provides the long-term outcomes of ECP application in CLAD patients in the largest published cohort to date. Two thirds of the cohort had a sustained response to ECP, showing excellent long-term results in CLAD patients compared to international benchmarks of untreated patients. Lung function status at the initiation of ECP and BOS phenotype were the two most important predictors of favorable outcome in our cohort. Both the excellent results and the new evidence support this therapy and suggest that early initiation of ECP may be beneficial in terms of both response and survival. Further studies are needed to elucidate the exact mechanisms of action and thus improve its application.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The dataset are property of the Medical University of Vienna, Hannover Medical School and IRCCS Policlinico San Matteo. Access to the dataset can be provided after formal approval of the legal departments of the three involved centers and the of the first and last authors. Requests to access these datasets should be directed to rechtsabteilung@meduniwien.ac.at.

ETHICS STATEMENT

The studies involving humans were approved by the ethic committee of Medical University of Vienna. The studies were conducted in accordance with the local legislation and institutional requirements. Due to the retrospective nature of the study, written informed consent was waived according to the ethics committee.

AUTHOR CONTRIBUTIONS

Designed research/study: AB, FM, PJ, MG. Performed research/study: AB, FM, PJ, MG. Collected data: AB, CB, MG. Analyzed data: AB, MG. Wrote paper: AB, CB, FI, CD, JG, KH, FM, PJ, MG.

FUNDING

The authors declare that this study received funding from Mallinckrodt Pharmaceuticals. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

CONFLICT OF INTEREST

Authors AB, PJ, and MG received speakers' fee within the last five years from Mallinckrodt Pharmaceuticals.

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
published: 24 January 2024
doi: 10.3389/ti.2024.12061

COVID-19 Outcomes in Lung Transplant Recipients Following Pre-Exposure Prophylaxis With Tixagevimab-Cilgavimab During the Omicron BA.5 Surge: A Single Center Analysis

Saartje Demolder[1], Veronique Schaevers[2], Katrien Lagrou[3,4†], Paul De Munter[4,5†], Hanne Beeckmans[6†], Geert M. Verleden[1,6†], Laurent Godinas[1,6†], Lieven J. Dupont[1,6†], Pascal Van Bleyenbergh[1], Natalie Lorent[1,6†] and Robin Vos[1,6†]*

[1]Department of Respiratory Diseases, University Hospitals Leuven, Leuven, Belgium, [2]Lung Transplant Team, University Hospitals Leuven, Leuven, Belgium, [3]Department of Laboratory Medicine and National Reference Center for Mycosis, University Hospitals Leuven, Leuven, Belgium, [4]Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium, [5]Department of General Internal Medicine, University Hospitals Leuven, Leuven, Belgium, [6]Department of Chronic Diseases, Metabolism and Ageing (CHROMETA), Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), KU Leuven, Leuven, Belgium

Lung transplant (LTx) recipients are at high risk for COVID-19 related morbidity and mortality. Data regarding pre-exposure prophylaxis (PrEP) with tixagevimab-cilgavimab in this population are scarce. We therefore evaluated COVID-19 breakthrough infections and COVID-19 related complications after PrEP in a retrospective single-center study, including 264 LTx recipients who received PrEP between June 2022 and December 2022, when Omicron BA.5 was the dominant circulating SARS-CoV-2 variant. PrEP was indicated for fully vaccinated patients with poor seroconversion (anti-S <260 BAU/mL). COVID-19 breakthrough infection after PrEP occurred in 11.0% within the first 3 months, increasing to 17.4% within 6 months. Hospitalization rate rose from 27.6% to 52.9% (p = 0.046), while ICU admissions and COVID-19 mortality remained low, respectively occurring in 6.5% and 4.3% of patients with breakthrough infection within 6 months. COVID-19 breakthrough infection and associated hospitalization remained an important problem during the Omicron BA.5 surge in fully vaccinated LTx recipients with deficient seroconversion, despite PrEP with tixagevimab-cilgavimab. However, ICU admissions and COVID-19 mortality were low. Waning of neutralizing effects of PrEP and changing

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***Correspondence**
Robin Vos,
robin.vos@uzleuven.be

†ORCID:
Katrien Lagrou
orcid.org/0000-0001-8668-1350
Paul De Munter
orcid.org/0000-0002-5944-3530
Hanne Beeckmans
orcid.org/0000-0002-6176-0404
Geert M. Verleden
orcid.org/0000-0003-3048-2429
Laurent Godinas
orcid.org/0000-0003-2214-5879
Lieven J. Dupont
orcid.org/0000-0003-3961-1522
Natalie Lorent
orcid.org/0000-0002-2653-5885
Robin Vos
orcid.org/0000-0002-3468-9251

Received: 17 September 2023
Accepted: 09 January 2024
Published: 24 January 2024

Citation:
Demolder S, Schaevers V, Lagrou K, De Munter P, Beeckmans H, Verleden GM, Godinas L, Dupont LJ, Van Bleyenbergh P, Lorent N and Vos R (2024) COVID-19 Outcomes in Lung Transplant Recipients Following Pre-Exposure Prophylaxis With Tixagevimab-Cilgavimab During the Omicron BA.5 Surge: A Single Center Analysis. Transpl Int 37:12061. doi: 10.3389/ti.2024.12061

Abbreviations: AR, arrythmia; AZA, azathioprine; BAU, Binding Antibody Units; BMI, body mass index; BOS, bronchiolitis obliterans syndrome; CAPA, COVID-19 associated pulmonary aspergillosis; CLAD, chronic lung allograft syndrome; CKD, chronic kidney disease (defined as pre-existing severe chronic renal insufficiency stage 4, with eGFR <30 mL/min/1.73 m^2); CS, corticosteroids; CSA, cyclosporine A; DM, diabetes mellitus; FEV_1, Forced Expiratory Volume in one second; HD, heart disease; HF, non-ischemic heart failure with or without preserved ejection fraction; HFNO, high flow nasal oxygen; ICU, intensive care unit; IHD, ischemic heart disease; IQR, interquartile range; LTx, lung transplant; MMF, mycophenolate mofetil; MV, mechanical ventilation; NIV, non-invasive ventilation; PrEP, pre-exposure prophylaxis; RAS, restrictive allograft syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation; SOT, solid organ transplant; TAC, tacrolimus; UK, unknown.

circulating SARS-CoV-2 variants may explain increases in COVID-19 infections and hospitalizations over time after PrEP, highlighting the need for novel, long-term effective PrEP strategies in these high-risk patients.

Keywords: COVID-19, lung transplantation, pre-exposure prophylaxis, tixagevimab-cilgavimab, outcome predictors

INTRODUCTION

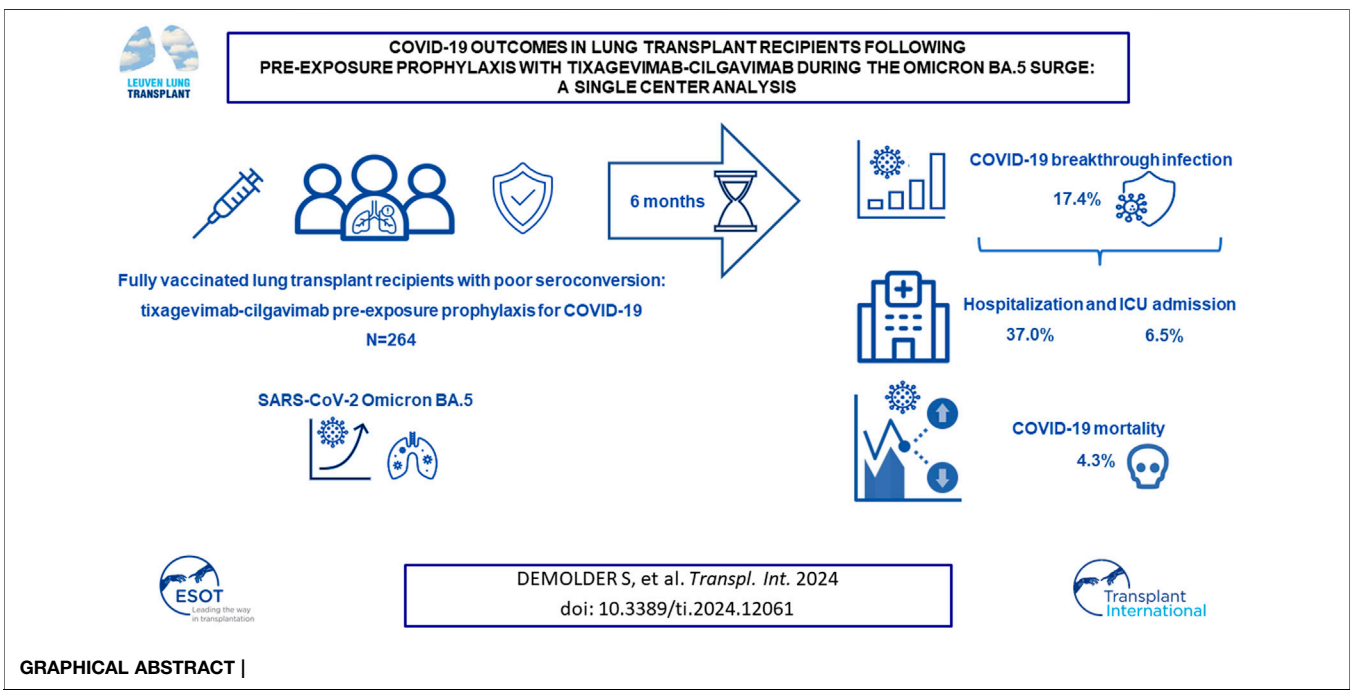
The incidence of COVID-19 infections in solid organ transplant (SOT) recipients is high in the Omicron era, especially in lung transplant (LTx) recipients, which are particularly at risk for COVID-19 related complications (i.e., hospitalization, severe disease, intensive care unit (ICU)-admission, respiratory failure, and death) [1–6] due to suboptimal or ineffective antibody responses following prior vaccination [7, 8]. As we previously reported, LTx recipients in our center demonstrated poor antibody seroconversion rates of only 47% after the third ("booster") vaccine dose, and the lowest antibody titers compared with other SOT recipients, resulting in the highest rates of severe breakthrough infection (10.5%) and death (2.5%) [9]. These poorer outcomes in the LTx population highlight the importance of LTx-specific studies and further research in LTx regarding effective prevention and treatment options.

Tixagevimab-cilgavimab (Evusheld®) is a long-acting dual monoclonal antibody against the SARS-CoV-2 spike protein, which has been available for pre-exposure prophylaxis (PrEP)—in adjunction to vaccination - in severely immunocompromised patients in Belgium since May 2022, as it retained activity against some circulating SARS-CoV-2 Omicron variants. Emerging international evidence of prophylactic treatment with tixagevimab-cilgavimab suggest efficacy for COVID-19 related complications in SOT recipients, but data in LTx recipients are scarce [10–16].

PATIENTS AND METHODS

Study Population

We conducted a retrospective single-center study of lung transplant recipients receiving tixagevimab-cilgavimab PrEP during the Omicron period. We included all consecutive LTx recipients who received tixagevimab-cilgavimab PrEP in our institution between 10th June 2022 and 13th December 2022. As per national recommendations [17], the indication for tixagevimab-cilgavimab PrEP in immunocompromised patients was: SARS-CoV-2 anti-Spike antibody titers <260 Binding Antibody Units (BAU)/mL (AdviseDx SARS-CoV-2 IgG II assay, Abbott, IL, United States) assessed >14 days after the second COVID-19 booster vaccine (i.e., fully vaccinated patients with insufficient seroconversion, considered at risk for severe COVID-19). Tixagevimab-cilgavimab was provided by the National Health Authorities to eligible immunocompromised patients, in whom this antibody titer cut-off was mandatory and thus country-specific. PrEP was administered as a single dose (tixagevimab 150 mg/cilgavimab 150 mg, two separate consecutive intramuscular injections). The dominant circulating SARS-



GRAPHICAL ABSTRACT |

CoV-2 variant in Belgium during the study period was Omicron BA.5. In our center, PrEP was routinely offered to all patients with the above indication by their treating transplant physician at their outpatient follow-up visits, provided they had no symptoms suggestive of COVID-19, and subsequently administered upon informed consent. Patients were subsequently monitored for 1 h after PrEP for possible serious adverse events, which required reporting to the National Health Authorities in case these occurred.

Data Collection

Data on demographics, reports of positive SARS-CoV-2 PCR, and clinical outcomes of interest were extracted from the patients' electronic medical records. Demographics included age, sex, body mass index (BMI), time and type of transplant, type of immunosuppressive regimen and comorbidities, including diabetes mellitus, chronic kidney disease and heart disease. Pre-existing chronic kidney disease was defined as severe chronic renal insufficiency stage 4, with eGFR <30 mL/min/1.73 m^2. Pre-existing heart disease was defined as ischemic heart disease (IHD), non-ischemic heart failure with or without reduced ejection fraction (HF), or arrythmia (AR).

SARS-CoV-2 positivity was defined by a positive PCR test; rapid antigen tests were not included, due to possible reporting bias. All patients with new clinical symptoms suggestive for COVID-19 were instructed to undergo COVID-19 PCR testing, either by their general practitioner or at the transplant center, which allowed to clinically assess the patient's symptoms at each new COVID-19 diagnosis, and refer/admit to hospitalization, if deemed necessary. The PCR results were prospectively documented in the patient's medical records and a centralized database.

Prevalence of symptomatic COVID-19 breakthrough infections, hospitalization, ICU admission and all-cause mortality were assessed up to 6 months after tixagevimab-cilgavimab administration. Patients with a recently confirmed COVID-19 infection prior to (<3 weeks) ($n = 6$) or post (<5 days) PrEP ($n = 0$) were excluded for analysis. We allocated the term *mild* disease to patients who solely required ambulatory care, *moderate* disease to those who were hospitalized and *severe* disease to those in need of intensive care unit management.

Statistical Analysis

Patient characteristics and variables of interest/endpoints were summarized using descriptive statistics, and results are expressed as total value, proportions, mean (standard deviation) or median (interquartile range), wherever appropriate. Proportions were compared using Chi-square testing. Groups were compared using paired t-tests, unpaired Mann-Whitney tests, or Wilcoxon matched-pairs signed rank tests for repeated measures. Correlation analyses were performed using Spearman rank testing. A p-value <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism 10.0 (Dotmatics, Boston, MA, United States).

Ethics Approval and Consent to Participate

At listing for LTx, all patients provide signed informed consent to use their clinical data for scientific research purposes by affiliated researchers of University Hospitals Leuven. Tixagevimab-cilgavimab PrEP was standard of care and administered after oral consent of the LTx recipient following therapeutic proposal by their treating physician. The institutional Ethics Review Board waived approval for the current retrospective, observational study (MP024291, S68119).

RESULTS

Study Population

A total of 285 LTx recipients (40.3% of our total LTx population of 708 patients) were eligible for tixagevimab-cilgavimab PrEP. The flow chart of included and excluded patients is given in **Figure 1**. The 264 included patients received PrEP between 10th June 2022 and 13th December 2022, at which moment Omicron BQ.1 (a subvariant of BA.5) replaced BA.5 (BF) as the main circulating Omicron variant in Belgium (**Figures 2**, **3**), and consequently administration of tixagevimab-cilgavimab PrEP was no longer recommended by the National Health Authorities because of reduced neutralizing efficacy against this BQ.1 subvariant [19, 20]. Median time between LTx and PrEP was 79.8 (43.0–138.5) months, and time between last SARS-CoV-2 booster vaccine and PrEP 173 (145–202.5) days. No serious adverse events (i.e., serious hypersensitivity reactions, including anaphylaxis) were seen immediately following PrEP administration, in addition, none of the included patients experienced a cardiovascular serious adverse event (i.e., myocardial infarction or stroke) during the 6-month post-PrEP study period.

Patient demographics are summarized in **Table 1**. Most patients were female (51.5%), and median age was 64 (55–68) years. 254 patients (96.2%) underwent lung transplantation only, 5 patients combined heart-lung transplantation, 3 others combined liver-lung transplantation, 1 patient a kidney-lung transplantation and another a liver-kidney-lung transplantation. Most patients (n = 235, 89.0%) were on a tacrolimus-based immunosuppressive regimen. None of the included patients was treated with a m-TOR inhibitor, had nor had received lymphocyte-depleting treatment (e.g., total lymphoid irradiation, antithymocyte globulin, or rituximab) for progressive chronic lung allograft dysfunction (CLAD) within 4–6 weeks prior to PrEP administration (as patients were required to be clinically stable to safely allow PrEP administration). Median BMI in our study population was in the normal range (23.4 (20.7–26.8), chronic lung allograft dysfunction (CLAD) was present in 27.7% (N = 73) of patients, 30.7% suffered from diabetes mellitus (n = 81), 17.8% from chronic renal insufficiency (n = 47) and 26.5% from pre-existing heart disease (n = 70).

Median SARS-CoV-2 anti-Spike antibody titer prior to PrEP was 13.2 (3.0–91.3) BAU/mL in the 264 included patents (i.e., <260 BAU/mL to be eligible for PrEP). In comparison, median SARS-CoV-2 anti-Spike antibody titer was 2243.0 (716.3–4785.0) BAU/mL in the 319 patients not eligible for PrEP ($p < 0.0001$).

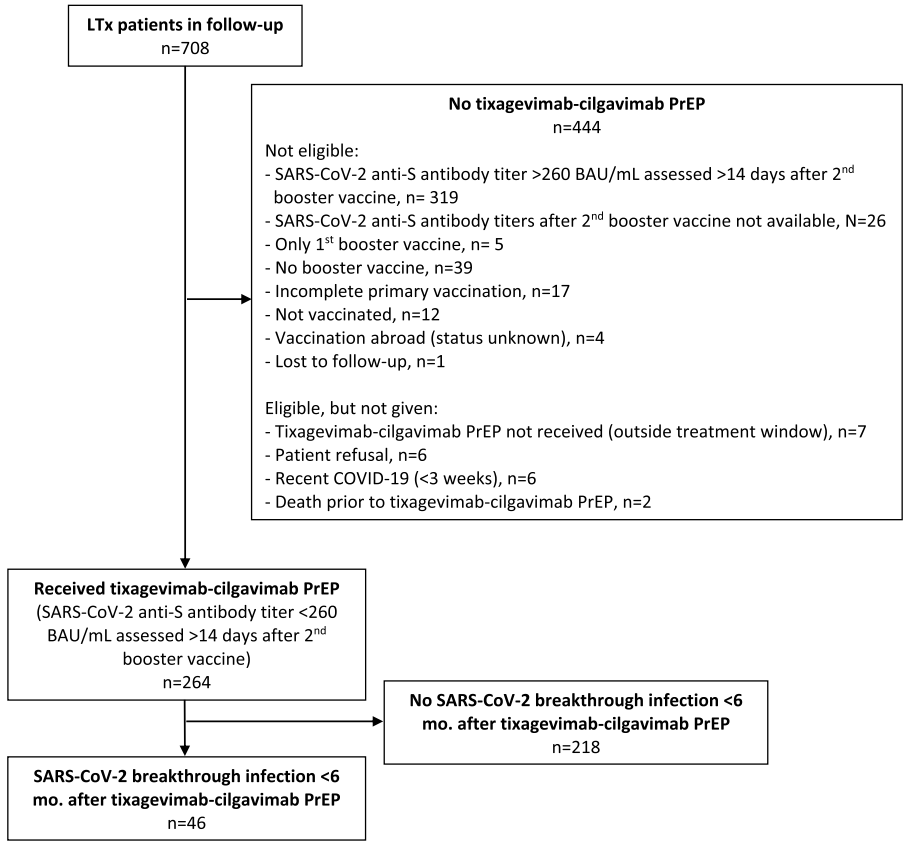


FIGURE 1 | Flow chart. Legend: Abbreviations: BAU, Binding Antibody Unit; LTx, lung transplantation; PrEP, pre-exposure prophylaxis.

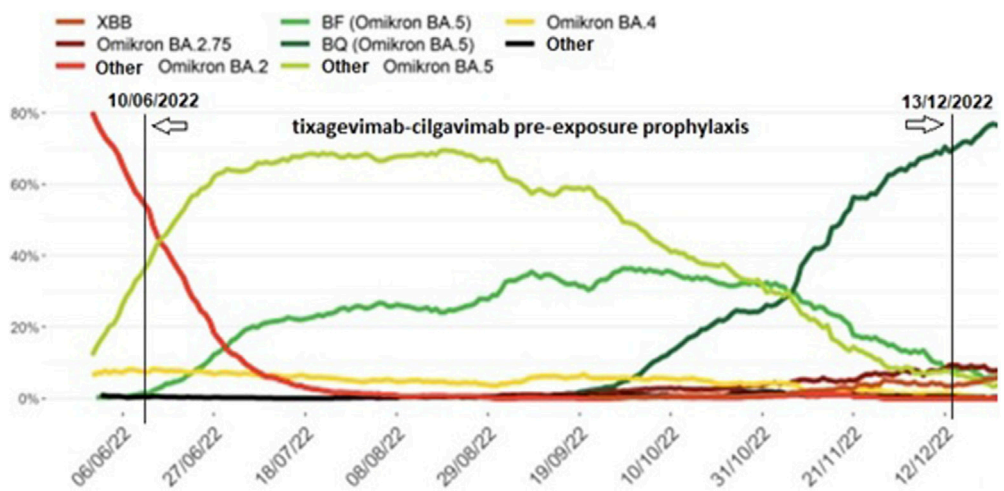


FIGURE 2 | Prevalence of SARS-CoV-2 Omicron subvariants identified during baseline surveillance in Belgium from June 2022 until January 2023, 7-day moving average. Legend: Different SARS-CoV-2 Omicron subvariants (coloured lines) during the tixagevimab-cilgavimab pre-exposure prophylaxis administration period in our patient cohort (10th June 2022 to 13th December 2022, vertical lines) (adapted from [18]).

COVID-19 Outcomes at 3 Months Follow-Up

A total of 29 (11.0%) patients had confirmed COVID-19 within 3 months after PrEP, of whom 8 (27.6%) were hospitalized, one of whom was admitted to ICU (3.4%). There was one COVID-19 related death (3.4%), being the ICU-hospitalized patient. Median time between PrEP and breakthrough infection was 43 (28–50) days.

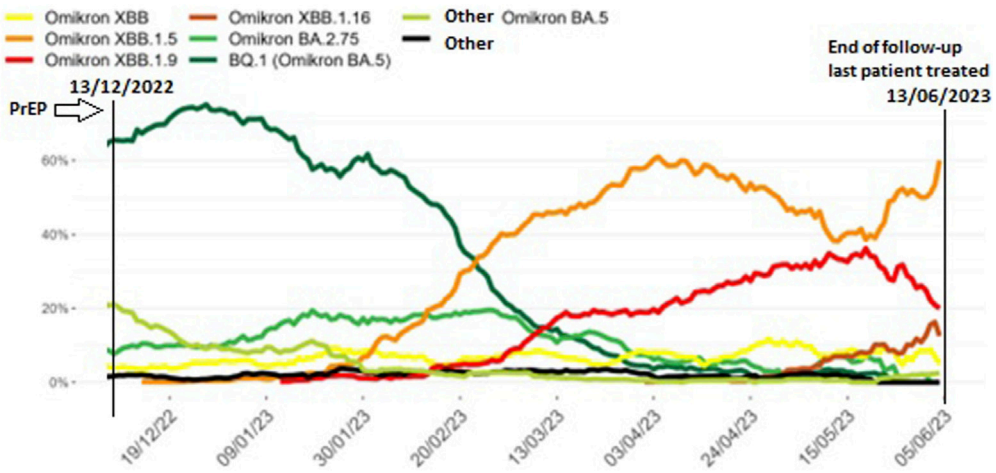


FIGURE 3 | Prevalence of SARS-CoV-2 Omicron subvariants identified during baseline surveillance in Belgium from November 2022 until June 2023, 7-day moving average. Legend: Different SARS-CoV-2 Omicron subvariants (coloured lines) in the 6 months after the tixagevimab-cilgavimab pre-exposure prophylaxis (PrEP) administration period in our patient cohort (13th December 2022 to 13th June 2023, vertical lines) (adapted from [19]).

TABLE 1 | Demographics of all study patients.

Demographic variable	All study patients (*n* = 264)
Age, y, median (IQR)	64 (55–68)
Male sex, *n* (%)	128 (48.5%)
Female sex, *n* (%)	136 (51.5%)
Single organ (lung only) transplant, *n* (%)	254 (96.2%)
Heart-lung transplant, *n* (%)	5 (1.9%)
Liver-lung transplant, *n* (%)	3 (1.1%)
Kidney-lung transplant, *n* (%)	1 (0.4%)
Liver-kidney-lung transplant, *n* (%)	1 (0.4%)
Tacrolimus-based immunosuppressant regimen, *n* (%)	235 (89.0%)
Cyclosporine A-based immunosuppressant regimen, *n* (%)	29 (11.0%)
Chronic lung allograft dysfunction, *n* (%)	73 (27.7%)
Bronchiolitis Obliterans Syndrome	55 (75.3%)
Mixed	6 (8.2%)
Restrictive Allograft Syndrome	12 (16.4%)
BMI, median (IQR)	23.4 (20.7–26.8)
Diabetes mellitus, *n* (%)	81 (30.7%)
Chronic renal insufficiency (CKD stage 4), *n* (%)	47 (17.8%)
Heart disease, *n* (%)	70 (26.5%)
Ischemic heart disease	15 (5.7%)
Non-ischemic heart failure with or without preserved ejection fraction	13 (4.9%)
Arrythmia	42 (15.9%)
Months between LTx and PrEP, median (IQR)	79.8 (43.0–138.5)
SARS-CoV-2 anti-Spike antibody titers prior to PrEP, BAU/mL (IQR)	13.2 (3.0–91.3)

Abbreviations: BAU, binding antibody unit; BMI, body mass index; CKD, chronic kidney disease; LTx, lung transplantation; PrEP, pre-exposure prophylaxis.

Relevant clinical variables and immunosuppressant regimen in the patients with or without breakthrough infection within 3 months after PrEP are summarized in **Table 2**; and did not differ between both groups. All, but one, COVID-19 patients were single organ (lung only) transplant recipients. There was one COVID-19 patient who had received a combined heart-lung transplantation, this patient only suffered from mild disease (i.e., not hospitalized).

COVID-19 Outcomes at 6 Months Follow-Up

Another 17 patients had confirmed COVID-19 within 3–6 months after PrEP, resulting in a total of number of 46 patients (17.4% of all PrEP patients) with COVID-19 breakthrough infection within 6 months after PrEP (**Figure 4**). Demographics of all lung transplant recipients with COVID-19 within 6 months after tixagevimab-cilgavimab PrEP, according to time to COVID-19 breakthrough infection since PrEP (i.e., <3 months and 3–6 months), are summarized in **Table 3**. There were no significant differences between patients infected <3 months vs. 3–6 months after PrEP.

Main outcomes in the 46 COVID-19 patients, according to time of breakthrough infection after PrEP, are summarized in **Table 4**. Median time to breakthrough infection was 54 (36–124) days.

Of these patients, 17 (37%) were hospitalized and 3 (6.5%) were admitted to ICU. Mortality in the COVID-19 group (4.3%) was comparable to the total study group (5.3%) ($p = 0.37$). In total 11 out of 46 COVID-19 patients had pre-existing CLAD (8 BOS, 1 Mixed, 2 RAS). Of these, 1 patient demonstrated CLAD (BOS) progression with a FEV_1 decline of >10% during the 6 months study period, 1 patient (RAS) died due to COVID-19, and in the 9 other patients FEV_1 remained stable pre-COVID-19 vs. 6 months post-COVID-19 [FEV_1 1.66 (1.18–2.15) L vs. 1.72 (1.05–1.96) L, $p = 0.74$].

Relevant clinical variables according to disease severity (mild vs. moderate to severe) in these 46 COVID-19 patients are summarized in **Table 5**. Patients presenting with mild disease were on average sooner infected after PrEP compared to patients requiring hospitalization: 47 (34–99) vs. 114 (61–121) days ($p = 0.006$). Patients with pre-existing heart disease tended to be more hospitalized (47.1% vs. 27.6%, $p = 0.09$), but no increased hospitalization

TABLE 2 | Clinical variables in the lung transplant recipients with or without COVID-19 within 3 months after tixagevimab-cilgavimab PrEP.

Clinical variable	No COVID-19 (*n* = 235)	COVID-19 infected (*n* = 29)	*p*-value
Age, median (IQR)	64 (55–68)	65 (56–68)	0.87
Gender, Male/Female, *n*	114/121 (48.5/51.5%)	14/15 (48.3/51.7%)	0.98
BMI, median (IQR)	23.2 (20.5–26.6)	24.1 (21.4–29.2)	0.21
Diabetes mellitus *n* (% of total)	74 (31.5%)	7 (24.1%)	0.42
Chronic renal insufficiency, *n* (% of total)	39 (16.6%)	8 (27.6%)	0.14
Heart disease, *n* (% of total)	59 (25.1)	11 (37.9%)	0.14
Ischemic Heart Disease	15 (6.4%)	0 (0.0%)	
Heart Failure	11 (4.7%)	2 (6.9%)	
Arrythmia	33 (14.0%)	9 (31.0%)	
Chronic lung allograft dysfunction, *n* (% of total)	62 (26.4%)	11 (37.9%)	0.19
Bronchiolitis Obliterans Syndrome	47 (19.1%)	8 (27.6%)	
Mixed	5 (2.1%)	1 (3.4%)	
Restrictive Allograft Syndrome	10 (4.2%)	2 (6.9%)	
Immunosuppressant regimen, *n* (% of total)			0.82
TAC/MMF/CS	131 (55.7%)	13 (44.8%)	
TAC/AZA/CS	45 (19.1%)	6 (20.7%)	
CSA/MMF/CS	17 (7.2%)	2 (6.9%)	
CSA/AZA/CS	4 (1.7%)	1 (3.4%)	
TAC/CS	32 (13.6%)	7 (24.1%)	
TAC/AZA	1 (0.4%)	0 (0.0%)	
CSA/CS, *n* (%)	5 (2.1%)	0 (0.0%)	
Months of follow-up since transplant, median (IQR)	79.9 (44.3–138.2)	78.6 (34.3–152.8)	0.82
Days between transplant and PrEP, median (IQR)	2,214 (1,051–3,926)	2,095 (743–4,348)	0.83
Days between last booster vaccine and PrEP, median (IQR)	181.5 (166.0–200.5)	173.0 (143.5–202.5)	0.33

Abbreviations: AZA, azathioprine; BMI, body mass index; COVID-19, Coronavirus Disease 2019; CS, corticosteroids; CSA, cyclosporine A; MMF, mycophenolate mofetil; PReP, pre-exposure prophylaxis; TAC,tacrolimus.

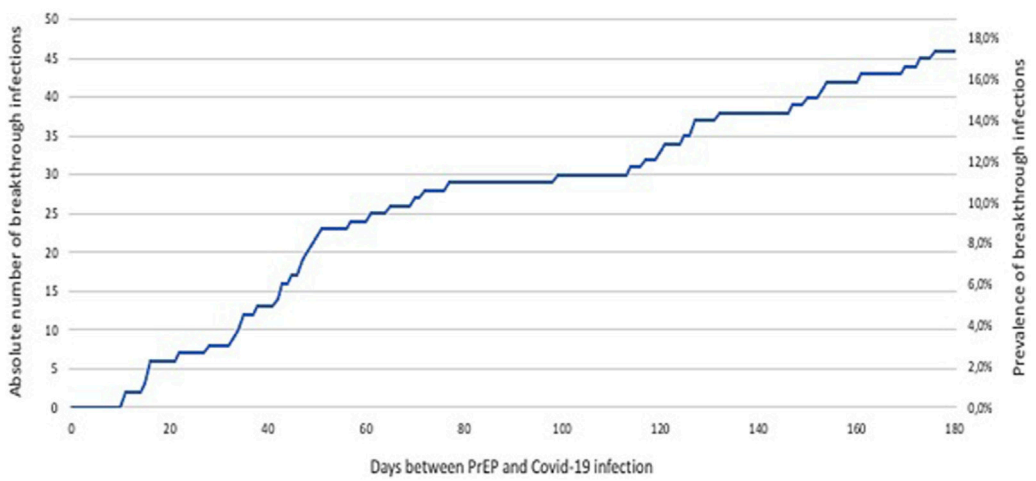


FIGURE 4 | Evolution of COVID-19 breakthrough infections in lung transplant recipients during 6 months follow-up after tixagevimab-cilgavimab PrEP. Legend: Abbreviations: PrEP, pre-exposure prophylaxis.

risk was seen for concurrent CLAD, diabetes mellitus or chronic renal insufficiency, nor for type of immunosuppressive regimen.

Furthermore, only one COVID-19 patient had received lymphoid-depleting treatment with total lymphoid irradiation for allograft rejection within the 6 months prior to PrEP, none had received antithymocyte globulin treatment. Only four COVID-19 patients received remdesivir, all were hospitalized, and one died in the ICU (**Table 3**). None of the infected patients received nirmatrelvir/ritonavir for COVID-19 treatment in our programme.

Notably, SARS-CoV-2 anti-Spike antibody titer pre-PrEP was lower in COVID-19 patients ($n = 46$) compared to non-infected patients ($n = 218$): 3.1 (3.0–49.8) vs. 18.5 (3.0–98.3) BAU/mL ($p = 0.039$). Likewise, in a subgroup of patients ($n = 36$, 13.6%) in whom SARS-CoV-2 anti-Spike antibody titer was measured post-PrEP, a significantly lower antibody titer was seen in COVID-19 patients ($n = 10$) compared to non-infected patients ($n = 26$):

TABLE 3 | Characteristics of lung transplant recipients with COVID-19 within 6 months after tixagevimab-cilgavimab PrEP.

Nr	Days between PrEP and COVID-19 positive	Sex	Age (y)	BMI	DM	CKD	HD	CLAD (type)	Lymphoid-depleting treatment pre-PrEP (type)	Days between LTx and booster vaccine	Days between LTx and PrEP	Days between booster and PrEP	Anti-S Abs pre-PrEP (BAU/mL)	Hospitalization (duration, days)	ICU	All-cause mortality	COVID-19 mortality	High flow nasal oxygen	MV or NIV	Dialysis	CAPA	Remdesivir	Nirmatrelvir/Ritonavir
<3 Months																							
1	11	M	65	28.1	No	No	Yes (AR)	No	No	942	1,117	175	3.0	Yes (3)	No	No	No	No	No	No	No	No	No
2	11	F	52	31.3	No	No	No	**Yes (BOS)**	No	3,647	3,850	203	223.3	No	No	No	No	No	No	No	No	No	No
3	15	F	68	27.5	No	**Yes**	No	No	No	1,368	1,421	53	8.9	No	No	No	No	No	No	No	No	No	No
4	16	F	58	31.2	No	No	No	**Yes (RAS)**	No	1,929	2,095	166	24.9	No	No	No	No	No	No	No	No	No	No
5	16	F	63	15.8	No	**Yes**	No	No	No	4,196	4,383	187	3.0	No	No	No	No	No	No	No	No	No	No
6	16	F	75	24.7	No	No	No	No	No	3,175	3,395	220	110.1	No	No	No	No	No	No	No	No	No	No
7	22	F	66	34.2	No	No	No	No	No	566	732	166	3.0	No	No	No	No	No	No	No	No	No	No
8	28	F	62	22.0	No	**Yes**	No	No	No	594	761	167	3.0	No	No	No	No	No	No	No	No	No	No
9	33	M	57	21.4	No	No	No	**Yes (RAS)**	No	2,600	2,830	230	3.0	**Yes (40)**	**Yes**	**Yes**	**Yes**	**Yes**	No	No	No	**Yes**	No
10	34	M	69	28.1	No	No	**Yes (AR)**	**Yes (BOS)**	No	1,848	2,036	188	170.5	No	No	No	No	No	No	No	No	No	No
11	35	F	65	21.4	No	**Yes**	No	No	No	614	736	122	3.0	No	No	No	No	No	No	No	No	No	No
12	35	F	45	21.8	**Yes**	No	**Yes (AR)**	No	No	6,587	6,768	181	3.0	No	No	No	No	No	No	No	No	No	No
13	38	M	71	19.6	No	**Yes**	**Yes (HF, AR)**	No	No	5,589	5,767	178	3.0	No	No	No	No	No	No	No	No	No	No
14	42	F	45	24.0	**Yes**	**Yes**	No	**Yes (BOS)**	No	7,366	7,553	187	3.0	**Yes (11)**	No	No	No	No	No	No	**Yes**	No	No
15	43	M	66	29.7	No	**Yes**	No	No	No	655	840	185	3.0	No	No	No	No	No	No	No	No	No	No
16	43	M	51	24.5	**Yes**	No	No	No	No	7,672	7,865	193	171.6	No	No	No	No	No	No	No	No	No	No
17	45	M	74	23.4	No	No	**yes (AR)**	No	No	4,182	4,313	131	3.0	No	No	No	No	No	No	No	No	No	No
18	47	M	57	30.7	No	No	No	No	No	543	732	189	3.0	No	No	No	No	No	No	No	No	No	No
19	47	M	69	23.8	No	No	No	No	No	4,216	4,493	277	52.6	**Yes (3)**	No	No	No	No	No	No	No	No	No
20	48	M	57	19.6	**Yes**	No	No	No	No	524	688	164	5.0	No	No	No	No	No	No	No	No	No	No
21	49	F	46	31.6	**Yes**	No	No	No	No	584	750	166	3.8	No	No	No	No	No	No	No	No	No	No
22	50	F	66	15.5	**Yes**	No	**Yes (AR)**	**No**	No	–97	259	356	177.9	No	No	No	No	No	No	No	No	No	No
23	51	F	67	17.1	No	No	No	**Yes (BOS)**	**Yes (TLI)**	uk	3,205	uk	3.0	No	No	No	No	No	No	No	No	No	No
24	57	F	55	35.5	No	No	No	No	No	512	670	158	235.6	No	No	No	No	No	No	No	No	No	No
25	61	M	39	20.2	No	No	No	No	No	295	364	69	3.0	**Yes (5)**	No	No	No	No	No	No	No	No	No
26	65	M	68	28.7	**Yes**	**Yes**	**Yes (AR)**	**Yes (Mixed)**	No	2,675	2,907	232	7.7	**Yes (11)**	No	No	No	No	No	No	No	No	No
27	70	F	68	23.3	No	No	No	No	No	813	995	182	3.0	No	No	No	No	No	No	No	No	No	No
28	72	M	67	24.1	No	No	**Yes (HF, AR)**	No	No	2,128	2,295	167	3.0	**Yes (6)**	No	No	No	No	No	No	No	No	No
29	77	M	72	25.1	No	No	**Yes (AR)**	**Yes (BOS)**	No	5,216	5,461	245	3.0	**Yes (3)**	No	No	No	No	No	No	No	No	No
3–6 Months																							
30	99	M	63	30.1	**Yes**	No	**Yes (HF)**	No	No	445	661	216	237.0	No	No	No	No	No	No	No	No	No	No
31	114	V	68	19.3	No	No	No	No	No	973	1,210	237	3.9	**Yes (7)**	No	No	No	No	No	No	No	**Yes**	No
32	117	M	66	27.0	No	No	No	No	No	621	735	114	240.7	No	No	No	No	No	No	No	No	No	No
33	120	V	71	28.1	**Yes**	No	**Yes (IHD)**	No	No	1,967	2,106	139	3.0	**Yes (10)**	No	No	No	No	No	No	No	No	No
34	121	V	69	15.8	No	No	No	**Yes (BOS)**	No	7,952	8,125	173	45.8	**Yes (21)**	No	No	No	No	No	No	No	No	No
35	125	M	67	22.3	**Yes**	No	**Yes (AR)**	No	No	883	1142	259	29.5	**Yes (10)**	No	No	No	No	No	No	No	**Yes**	No

(Continued on following page)

TABLE 3 | (*Continued*) Characteristics of lung transplant recipients with COVID-19 within 6 months after tixagevimab-cilgavimab PrEP.

Nr	Days between PrEP and COVID-19 positive	Sex	Age (y)	BMI	DM	CKD	HD	CLAD (type)	Lymphoid-depleting treatment pre-PrEP (type)	Days between LTx and booster vaccine	Days between LTx and PrEP	Days between booster and PrEP	Anti-S Abs pre-PrEP (BAU/mL)	Hospitalization (duration, days)	ICU	All-cause mortality	COVID-19 mortality	High flow nasal oxygen	MV or NIV	Dialysis	CAPA	Remdesivir	Nirmatrelvir/Ritonavir
36	127	M	54	25.6	No	No	yes (AR)	yes (BOS)	No	2,974	3,118	144	3.0	No	No	No	No	No	No	No	No	No	No
37	127	V	66	26.9	No	**Yes**	No	No	No	381	552	171	4.6	**Yes (4)**	No	No	No	No	No	No	No	No	No
38	132	V	50	18.8	No	**Yes**	**Yes** (AR)	No	No	4,378	4,512	134	3.0	**Yes (19)**	**Yes**	No	No	**Yes**	**Yes** (NIV)	No	No	No	No
39	147	M	68	23.0	No	No	**Yes** (AR)	No	No	3,324	3,516	192	3.0	No	No	No	No	No	No	No	No	No	No
40	150	M	64	26.4	No	No	**Yes** (IHD)	**Yes** (BOS)	No	633	804	171	3.1	**Yes (5)**	No	No	No	No	No	No	No	No	No
41	153	V	70	17.5	No	**Yes**	No	No	No	3,296	3,477	181	23.3	No	No	No	No	No	No	No	No	No	No
42	154	V	52	20.0	**Yes**	No	No	No	No	3,629	3,707	78	3.0	No	No	No	No	No	No	No	No	No	No
43	161	V	44	15.8	No	No	No	No	No	103	268	165	95.0	**Yes (18)**	No	No	No	No	No	No	**Yes**	**Yes**	No
44	170	M	73	24.0	No	**Yes**	No	No	No	2,695	2,826	131	88.4	**Yes (32)**	**Yes**	**Yes**	**Yes**	**Yes** (MV)	No	No	No	No	No
45	173	M	62	34.4	**Yes**	No	No	No	No	548	738	190	3.0	No	No	No	No	No	No	No	No	No	No
46	176	V	61	24.6	No	No	No	No	No	403	557	154	48.8	No	No	No	No	No	No	No	No	No	No

Overview of the lung transplant recipients with COVID-19 within 6 months after tixagevimab-cilgavimab PrEP, according to time to COVID-19 breakthrough infection since PrEP (i.e., <3 months and 3–6 months). Abbreviations: AR, arrythmia; BMI, body mass index; BOS, bronchiolitis obliterans syndrome; CAPA, COVID-19 associated pulmonary aspergillosis; CLAD, chronic lung allograft dysfunction; CKD, chronic kidney disease defined as pre -existing chronic renal insufficiency stage 4, with eGFR <30 mL/; DM, diabetes mellitus; HD, pre-existing heart disease; HF, non-ischemic heart failure with or without preserved ejection fraction; ICU, intensive care unit; IHD, ischemic heart disease; LTx, lung transplantation; MV, mechanical ventilation; NIV, non-invasive ventilation; PrEP, pre-exposure prophylaxis; RAS, restrictive allograft syndrome; TLI, total lymphoid irradiation; UK, unknown.

Bold values visually represent the respctive parameter being present (YES), rather than absent (NO).

1261.6 (857.7–1835.1) vs. 2201.7 (1380.4–3405.7) BAU/mL (p = 0.0185) (**Table 6**).

In the COVID-19 patients, anti-Spike antibody titer pre-PrEP was similar in patients infected <3 months compared to those infected after 3–6 months: 3.0 (3.0–27.2) vs. 4.6 (3.0–91.7) (p = 0.16). Also, anti-Spike antibody titer pre-PrEP was similar in patients with mild COVID-19 compared to those with moderate to severe COVID-19: 3.0 (3.0–140.3) vs. 3.1 (3.0–37.7) BAU/mL (p = 0.65) (**Table 4**).

Relevant COVID-19 related outcome variables in the 17 *hospitalized* COVID-19 patients are summarized in **Table 7**. Overall, hospitalization duration was short (mean 12.2 ± 10.7 days), and respiratory support requiring non-invasive ventilation (n = 1), intubation with mechanical ventilation (n = 1), or extracorporeal support (n = 1) was rarely needed. COVID-19 associated pulmonary aspergillosis was diagnosed in two patients.

DISCUSSION

PrEP with tixagevimab-cilgavimab in at risk patients with poor seroconversion following prior vaccination may provide protection against severe COVID-19. However, in our lung transplant cohort, 11.0% of lung transplant recipients developed breakthrough SARS-CoV-2 infection/COVID-19 within the initial 3 months post-PrEP, which increased to 17.4% within 6 months. Notably, 27.6% of the patients with breakthrough infection within the first 3 months required hospitalization, while this number increased to 52.9% for those with breakthrough infection during the subsequent 3 months of follow-up. On the other hand, ICU admissions (3.4%) and COVID-19 related mortality (3.4%) only rarely occurred in COVID-19 patients during the first 3 months post-PrEP, whereas ICU admissions non-significantly increased (11.8%) and the number of COVID-related deaths remained similar (5.9%) in the subsequent 3 months of follow-up. Our study demonstrates that COVID-19 breakthrough infections and associated hospitalizations remained an important problem during the Omicron BA.5 surge, despite PrEP with tixagevimab-cilgavimab. Yet, overall ICU admissions (1% post-PrEP) and COVID-19 related mortality (2% post-PrEP) were very low. The latter finding concurs with the observed low mortality rate in an earlier study of SARS-CoV-2 Omicron-variant breakthrough infections in lung transplant patients without PrEP [1].

Al Jurdi et al. reported on 222 SOT recipients (kidney, lung, liver or multi-visceral) who received tixagevimab-cilgavimab PrEP between December 2021 and April 2022, and 222 vaccine-matched solid organ transplant recipients who did not receive PrEP. Breakthrough SARS-CoV-2 infection occurred in 5% of SOT recipients (11 patients) who received tixagevimab/cilgavimab and in 14% (32 patients) of SOT recipients in the control group (p < 0.001). This study included 80 lung transplant recipients, of whom 7.5% (6 patients) developed COVID-19 after PrEP during the 4-month follow-up period, and 72 lung transplant recipients without PrEP, in whom 22.2%

TABLE 4 | Main outcomes of lung transplant recipients with COVID-19 breakthrough infection, according to time of follow-up after tixagevimab-cilgavimab PrEP.

Patients with COVID-19 after PrEP	0–6 months follow-up (*n* = 46)	0–3 months follow-up (*n* = 29)	3–6 months follow-up (*n* = 17)	*p*-value (0–3 vs. 3–6 months)
Hospitalization, *n* (%)	17 (37.0%)	8 (27.6%)	9 (52.9%)	0.046
ICU admission, *n* (%)	3 (6.5%)	1 (3.4%)	2 (11.8%)	0.14
COVID-19 related mortality, *n* (%)	2 (4.3%)	1 (3.4%)	1 (5.9%)	0.35

Abbreviations: ICU, intensive care unit; PrEP, pre-exposure prophylaxis.

TABLE 5 | Characteristics according to disease severity in lung transplant recipients with COVID-19 breakthrough infection during 6 months follow-up after tixagevimab-cilgavimab PrEP.

	All COVID-19 infected patients (*n* = 46)	Mild COVID-19 (ambulatory) (*n* = 29)	Moderate to severe COVID-19 (hospitalization) (*n* = 17)
Age, y, mean (SD)	62 (9.0%)	62 (7.9%)	62 (10.8%)
Male sex, *n* (%)	22 (47.8%)	12 (41.0%)	10 (59.0%)
Female sex, *n* (%)	24 (52.2%)	17 (59.0%)	7 (41.0%)
Days between PrEP and infection, median (IQR)	54 (36–124)	47 (34–99)	114 (61–121)*
Single organ (lung) transplant, *n* (%)	45 (97.8%)	28 (96.6%)	17 (100.0%)
Heart-lung transplant, *n* (%)	1 (2.2%)	1 (3.4%)	0 (0.0%)
Liver-lung transplant, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Kidney-lung transplant, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Liver-kidney-lung transplant, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Chronic lung allograft dysfunction, *n* (%)	11 (23.9%)	5 (17.2%)	6 (35.3%)
Diabetes mellitus, *n* (%)	12 (26.1%)	8 (27.6%)	4 (23.5%)
Chronic renal insufficiency (CKD stage 4), *n* (%)	12 (26.1%)	7 (24.1%)	5 (29.4%)
Heart disease, *n* (%)	16 (34.8%)	8 (27.6%)	8 (47.1%)
Tacrolimus-based immunosuppression, *n* (%)	40 (87.0%)	25 (86.2%)	15 (88.2%)
Cyclosporine A-based immunosuppression, *n* (%)	6 (13.0%)	4 (13.8%)	2 (11.8%)
Mycophenolate as cell cycle inhibitor, *n* (%)	26 (56.5%)	15 (51.7%)	11 (64.7%)
SARS-CoV-2 anti-Spike Antibody titers pre-PrEP, BAU/mL (IQR)	3.1 (3.0–49.8)	3.0 (3.0–140.3)	3.1 (3.0–37.7)
All-cause mortality, *n* (%)	3 (6.5%)	0 (0.0%)	3 (17.6%)**
COVID-19 related mortality, *n* (%)	2 (4.3%)	0 (0.0%)	2 (11.8%)

Abbreviations: BAU, binding antibody unit; PrEP, pre-exposure prophylaxis.
*p = *0.006 vs. mild COVID-19,* **p = *0.0139 vs. mild COVID-19.*

(16 patients) developed COVID-19 [13]. Of note, the number of prior vaccines varied considerably in these patients, different to our study group in which all patients were fully vaccinated (i.e., two boosters).

Gottlieb et al. analysed their cohort of 419 lung transplant recipients that received PrEP between February and October 2022, with a median follow-up of 209 days. Of these, 19% (77 patients) developed SARS-CoV-2 breakthrough infection, of which 13% (10 patients) were hospitalized and 0.7% (1 patient) died. Notably there was no difference in severity of COVID-19 was observed with the control group that did not receive PrEP, but this could possibly be explained by the fact that both groups were not matched, and patients receiving PrEP were older, had more severe renal insufficiency, shorter time to transplant and lower SARS-CoV-2 antibody titers. Included patients had antibody titers of less than 260 BAU/mL after full vaccination or were included as per decision of their treating physician. Furthermore, most patients also had received double dose PrEP in most cases [15].

Most recently, Sindu et al. reported a 11.8% SARS-CoV-2 breakthrough infection rate and 20.8% hospitalization rate in 203 lung transplant recipients that had received PrEP between December 2021 and August 2022, in comparison to 16.6% and 43.1% in the control group. COVID-19-related mortality was high in both these propensity-score-matched groups (11.8%) [16].

Rates of SARS-CoV-2 breakthrough infections and hospitalization during the first 3 months of follow-up in our cohort are comparable with the studies performed by Al Jurdi et al. and Sindu et al, which took place in the same timeframe, although we—fortunately—note a lower percentage of COVID-19 related mortality. When comparing our overall results (June 2022 to December 2022, 6-month follow-up) to Gottlieb et al (February 2022 to October 2022, median 6.9-month follow-up), we found a similar SARS-CoV-2 breakthrough infection rate but noted a higher hospitalization rate.

When looking at epidemiological data for Belgium between November 2022 and January 2023, BQ.1, a sub-variant of BA.5, became the most dominant circulating SARS-CoV-2 variant [19, 20]. As stated by the Belgian Health Authorities at the end of November, tixagevimab-cilgavimab was deemed ineffective to neutralize the BQ.1 Omicron variant of SARS-CoV-2,

TABLE 6 | SARS-CoV-2 anti-Spike antibody titers post-PrEP.

	All patients (*n* = 36)	Non-infected patients (*n* = 26)	COVID-19 infected patients (*n* = 10)
SARS-CoV-2 anti-Spike Antibody titers post-PrEP, BAU/mL (IQR)	1742.5 (1100.6–2934.1)	2201.7 (1380.4–3405.7)	1261.6 (857.7–1835.1)*
Time between PrEP and measurement of SARS-CoV-2 anti-Spike Antibody titers post-PrEP, days (IQR)	70.0 (30.8–96.0)	63.0 (25.5–94.0)	80.5 (46.3–104.8)

*SARS-CoV-2 anti-Spike antibody titers post-PrEP were only available in 36/264 (13.6%) of included patients: in 10/46 patients (21.7%) with COVID-19 breakthrough infection and in 26/218 (11.9%) non-infected patients. *p = 0.0185 versus non-infected patients (time between PrEP and Antibody measurement was similar in both groups, p = 0.315). Abbreviations: BAU, binding antibody unit; PrEP, pre-exposure prophylaxis.*

TABLE 7 | Characteristics of hospitalized lung transplant recipients with COVID-19 breakthrough infection, according to time of follow-up after tixagevimab-cilgavimab PrEP.

Patients with *moderate to severe* disease (hospitalized)	0–6 months follow-up (*n* = 17)	0–3 months follow-up (*n* = 8)	3–6 months follow-up (*n* = 9)
Days of hospitalization, mean (SD)	12.2 (10.7)	10.3 (12.5)	14.0 (9.2)
ICU hospitalization, *n* (%)	3 (17.6%)	1 (12.5%)	2 (22.2%)
At most HFNO, *n* (%)	1 (5.9%)	1 (12.5%)	0 (0.0%)
At most NIV, *n* (%)	1 (5.9%)	0 (0.0%)	1 (11.1%)
Intubation, *n* (%)	1 (5.9%)	0 (0.0%)	1 (11.1%)
ECMO, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Dialysis, *n* (%)	1 (5.9%)	0 (0.0%)	1 (11.1%)
CAPA, *n* (%)	2 (11.8%)	1 (12.5%)	1 (11.1%)
COVID-19 related myocarditis, *n* (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
COVID-19 related mortality, *n* (%)	2 (11.8%)	1 (12.5%)	1 (11.1%)

Abbreviations: CAPA, COVID-19 associated pulmonary aspergillosis; ECMO, extracorporeal membrane oxygenation; HFNO, high flow nasal oxygen; ICU, intensive care unit; NIV, non-invasive ventilation; PrEP, pre-exposure prophylaxis.

necessitating termination of the PrEP program [20, 21]. Although we have no data available on the actual variants in our patient group, this epidemiologic evolution could explain the difference in hospitalization rate we noted between the first 3 months of follow-up and the subsequent 3 months, and the difference in hospitalizations compared to the Gottlieb et al. cohort [15]. Single dosage instead of double dosage, as well as inclusion of patients with a higher antibody count prior to PrEP may have further added to the difference in hospitalization rate compared to the Gottlieb et al. study. Also, the neutralizing effect of tixagevimab-cilgavimab PrEP for Omicron BA.5 has been reported to wane by 3 months post-injection, which might also have contributed to the surge in moderate to severe disease during the last 3 months of follow-up in our cohort [22].

We note several limitations to our study. First, its retrospective and observational design. Second, the incidence of COVID-19 in our study group may be underestimated as we relied on patients getting tested when experiencing symptoms and reporting back on PCR results when diagnosed outside our hospital. However, centralized documentation of a positive SARS-CoV-2 PCR test allowed cross-checking of breakthrough infections for the current study yet genotyping of SARS-CoV-2 was not systematically performed. Furthermore, our study lacked a control group because almost all patients who were eligible for PrEP agreed to treatment, and COVID-19 infections in patients without PrEP were not prospectively collected for analysis during the study period. However, the clinical demographics of the included patients are overall representative of our total lung transplant cohort (i.e., about 25%–30% of patients with CLAD, 30% with diabetes, 15%–20% with chronic kidney disease stage 4, and 25%–30% with heart disease; and about 90% on a tacrolimus-based immunosuppression). None of the included patients was treated with a m-TOR inhibitor, nor received TLI, rATG or rituximab for progressive CLAD in the weeks prior to PrEP administration (as patients needed to be clinically stable to safely allow PrEP administration).

In conclusion, our results add real-world evidence on COVID-19 breakthrough infections after PrEP with tixagevimab-cilgavimab in fully vaccinated LTx recipients with deficient seroconversion; and demonstrates a similar rate of infection after PrEP during the Omicron BA.5 surge as reported in other studies. However, COVID-19 associated hospitalization remained an important problem, despite PrEP with tixagevimab-cilgavimab, whereas severe COVID-19 necessitating ICU admission and COVID-19 mortality were low. Waning of the neutralizing effects of PrEP and changing circulating SARS-CoV-2 variants might possibly explain the increase in SARS-CoV-2 breakthrough infections and hospitalizations after PrEP, and highlights the need for novel, long-term effective PrEP strategies in these high-risk 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

At listing for LTx, all patients provide signed informed consent to use their clinical data for scientific research purposes by affiliated researchers of University Hospitals Leuven. Tixagevimab-cilgavimab PrEP was standard of care and administered after oral consent of the LTx recipient following therapeutic proposal by their treating physician. The institutional Ethics Review Board waived approval for the current retrospective, observational study (MP024291, S68119), which was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

AUTHOR CONTRIBUTIONS

SD: data collection, data curation, analysis, final draft preparation, review, and editing. VS: methodology, data collection, review, and editing. KL: methodology, data collection, review, and editing. PD: methodology, data collection, review, and editing. HB: methodology, data collection, review, and editing. GV: methodology, data collection, review, and editing. LG: methodology, data collection, review, and editing. PV: methodology, data collection, review, and editing. LD: methodology, data collection, review, and editing. NL: methodology, data collection, review, and editing. RV: conceptualization, methodology, data collection, data curation, final draft preparation, review, and editing, funding. All authors contributed to the article and approved the submitted version.

FUNDING

RV is supported by the Research Foundation-Flanders (FWO) as senior clinical researcher (1803521N) and by a research grant (G060322N). RV is supported by an academic research grant from AstraZeneca, but the study was conducted without Industry sponsorship.

CONFLICT OF INTEREST

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

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BRIEF RESEARCH REPORT
published: 26 January 2024
doi: 10.3389/ti.2024.12395

Late-Onset Exudative Pleural Effusions Without Concomitant Airway Obstruction or Lung Parenchymal Abnormalities: A Novel Presentation of Chronic Lung Allograft Dysfunction

*Devika Sindu[1], Sandhya Bansal[1], Bhuvin Buddhdev[1,2], Kendra McAnally[1,2], Hesham Mohamed[1,2], Rajat Walia[1,2], Thalachallour Mohanakumar[1] and Sofya Tokman[1,2]**

[1]Norton Thoracic Institute, Phoenix, AZ, United States, [2]School of Medicine, Creighton University, Phoenix, AZ, United States

Restrictive allograft syndrome (RAS) is an aggressive variant of CLAD characterized by progressive restrictive ventilatory decline and persistent pleuro-parenchymal changes that can be seen on chest CT. We identified four lung transplant recipients with a progressive restrictive ventilatory defect due to lymphocyte-predominant exudative pleural effusions, but no pleuro-parenchymal abnormalities typical of RAS. Using molecular analysis, we also found increased levels of previously described immune markers of RAS, including NFkB, 20S proteasome, lipocalin, TNFα, and TGFβ, within the circulating small extracellular vesicles of the remaining living lung transplant recipient. Despite the absence of lung parenchymal changes, these patients had a poor prognosis with rapid deterioration in allograft function and no response to pleural-based interventions such as thoracentesis, decortication, and pleurodesis. We hypothesize that these cases represent a distinct CLAD phenotype characterized by progressive restriction due to pleural inflammation, lymphocyte-predominant pleural effusion, resultant compressive atelectasis, and eventual respiratory failure in the absence of lung parenchymal involvement.

Keywords: chronic lung allograft dysfunction, exudative pleural effusion, lung transplant, small extracellular vesicles, new CLAD phenotype

OPEN ACCESS

***Correspondence**
Sofya Tokman,
sofya.tokman@dignityhealth.org

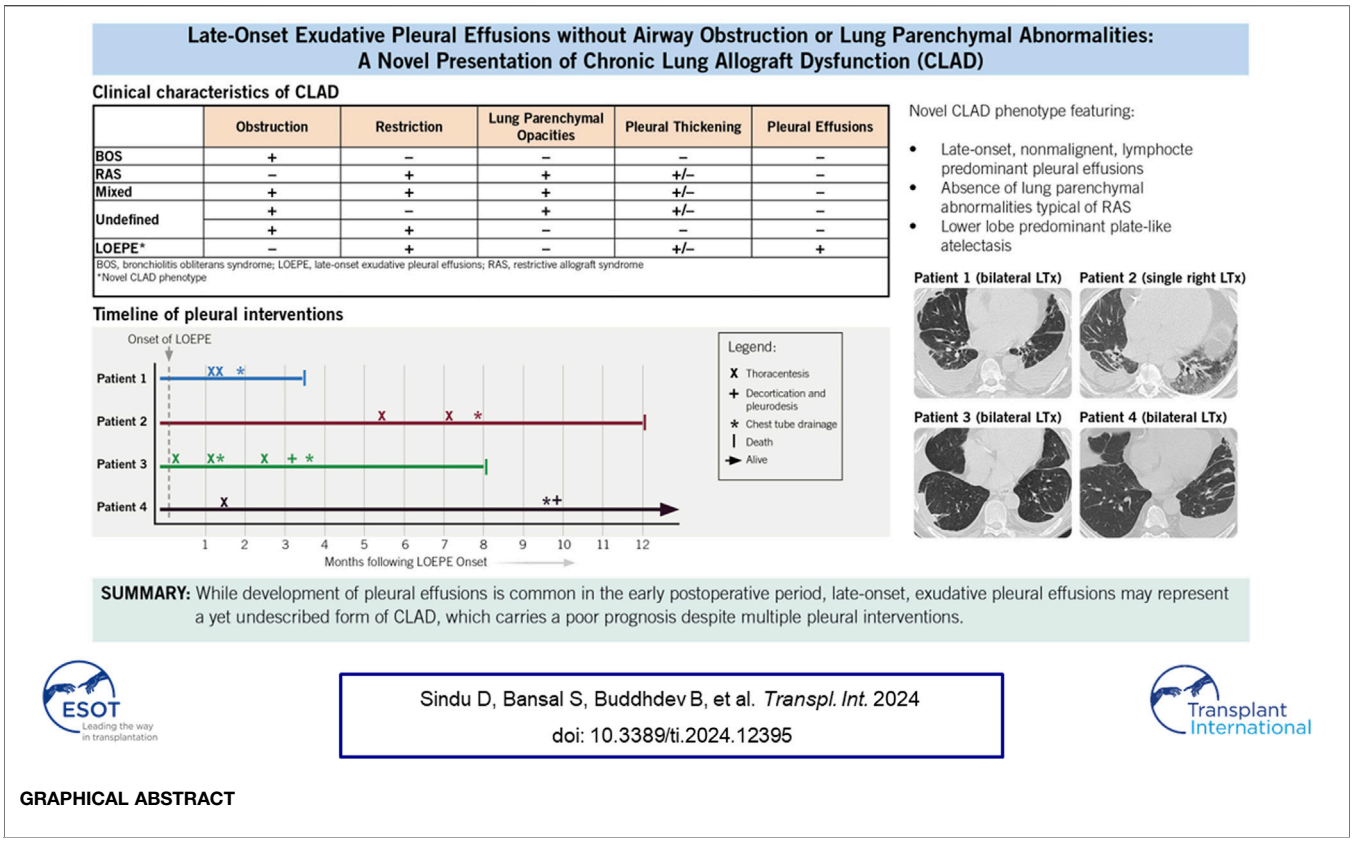
Received: *08 November 2023*
Accepted: *10 January 2024*
Published: *26 January 2024*

Citation:
Sindu D, Bansal S, Buddhdev B, McAnally K, Mohamed H, Walia R, Mohanakumar T and Tokman S (2024) Late-Onset Exudative Pleural Effusions Without Concomitant Airway Obstruction or Lung Parenchymal Abnormalities: A Novel Presentation of Chronic Lung Allograft Dysfunction. Transpl Int 37:12395. doi: 10.3389/ti.2024.12395

INTRODUCTION

Chronic lung allograft dysfunction (CLAD) remains a major challenge after lung transplantation (LT), limiting long-term survival and graft function in lung transplant recipients (LTRs). Bronchiolitis obliterans syndrome (BOS) is the most common phenotype of CLAD, seen in 50%–70% of cases, and is characterized by progressive, irreversible airflow obstruction due to bronchiolar inflammation and fibroproliferation [1]. Restrictive allograft syndrome (RAS), a less prevalent but distinct phenotype, is seen in 10%–30% of LTRs with CLAD and is characterized by restrictive spirometry changes, persistent radiographic opacities, and a markedly worse prognosis

Abbreviations: ACR, acute cellular rejection; BAL, bronchoalveolar lavage; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; DSA, donor-specific antibodies; FEV_1, forced expiratory volume in 1 s; LT, lung transplant; LTR, lung transplant recipient; PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; RAS, restrictive allograft syndrome; TBBx, transbronchial biopsy.



GRAPHICAL ABSTRACT

[2–4]. The 2019 consensus report from the Pulmonary Council of the International Society of Heart and Lung Transplantation also describes mixed and undefined phenotypes to characterize combinations of CLAD presentations [5]. Studies have shown that manifestations of CLAD are heterogeneous, and its diagnosis requires a methodical, guideline-based approach to identify novel phenotypes [2, 5–10]. In addition to clinical phenotypic differences, translational studies indicate that BOS and RAS may also have distinct immunologic profiles [11]. In this case series, we describe four LTRs with a restrictive ventilatory defect characterized by the development of exudative pleural effusions, pleural thickening, and plate-like or rounded atelectasis. We hypothesize that these cases represent a distinct CLAD phenotype with a poor prognosis (**Table 1**).

MATERIALS AND METHODS

This study was approved by our Institutional Review Board (PHX-21-500-198-73-18 dated 07/12/2023) with the need for informed consent waived as data was collected retrospectively by chart review. Analyses of small extracellular vesicles (sEVs) were conducted after approval by our Institutional Review Board (PHXB-16-0027-10-18 dated 7/14/2020) and after obtaining written informed consent from the participant. Small extracellular vesicles were isolated from plasma samples and characterized by Western blot (**Supplementary Methods**). Student's *t* test and paired *t*-test were used when appropriate to compare the relative densities of sEVs isolated from the samples. Statistical analyses were carried out using Prism (GraphPad Software). All patient care was carried out under strict compliance with the International Society of Heart and Lung Transplantation ethics statement.

RESULTS

Patient 1

A 64-year-old man with idiopathic pulmonary fibrosis underwent bilateral LT. His post-LT course was complicated by an episode of CMV viremia, bilateral pleural effusions, and bronchomalacia requiring left main stem bronchus stent placement. He subsequently enjoyed an active lifestyle with stable allograft function and was maintained on standard 3-drug immunosuppression with tacrolimus, mycophenolate mofetil (MMF), and prednisone. Three years after transplant, he developed recurrent squamous cell skin cancer, and MMF was transiently replaced with everolimus in an attempt to slow cancer progression and recurrence. One year later, he had a 15% drop in FEV_1, was treated with an empiric 3-day course of 500 mg methylprednisolone, transitioned off of everolimus and back on MMF, and underwent bronchoscopy with stent placement for severe left bronchomalacia. His lung function returned to baseline and remained stable for the next 9 months but began to

TABLE 1 | Baseline characteristics and clinical features of lung function impairment.

	Patient 1	Patient 2	Patient 3	Patient 4
Baseline clinical characteristics				
Sex	Male	Male	Male	Male
Age at LT, years	64	72	67	63
Indication for LT	IPF	HP	IPF	IPF
Type of LT (single vs. double)	Double	Single, right	Double	Double
PGD-3 at 72 h	No	No	No	No
CMV serostatus (D/R)	D+/R-	D+/R+	D+/R-	D+/R+
Clinical characteristics at CLAD onset[a]				
Age at CLAD onset	69	72.5	69.5	65
Immunosuppressive regimen	Tac, MMF, CS	Tac, MMF, CS	Tac, MMF, CS	Tac, MMF, CS
DSA	negative	negative	negative	negative
Transthoracic echocardiogram				
Systolic function (LVEF)	50%–60%	60%–65%	60%–65%	60%–65%
Diastolic dysfunction	none	Grade 1, mild	Grade 1, mild	Grade 2, moderate
Right heart catheterization				
Mean PAP	26 mm Hg	22 mm Hg	14 mm Hg	18 mm Hg
PCWP	16 mm Hg	15 mm Hg	6 mm Hg	9 mm Hg
Imaging after CLAD onset				
Chest CT	Bilateral pleural effusions, pleural thickening, plate-like and rounded atelectasis	Right-sided pleural effusion, pleural thickening, plate-like and rounded atelectasis	Bilateral pleural effusions, pleural thickening, plate-like and rounded atelectasis	Bilateral pleural effusions, pleural thickening, plate-like and rounded atelectasis
Lung allograft parenchymal changes typical of RAS, consolidations, ground glass opacities, or fibrosis	No	No	No	No
Bronchoscopic findings after CLAD onset				
BAL cultures	No growth	No growth	No growth	No growth
Transbronchial biopsy	A0B0	A0B0	A0B0	A0B0
Pleural effusion: characteristics and management				
Pleural fluid characteristics at CLAD onset[a]	Exudative	Exudative	Exudative	Exudative
White cell count (lymphocytes %)	197/uL (72%)	4083/uL (92%)	102/uL (65%)	2637/uL (87%)
LDH	241 U/L	268 U/L	242 U/L	233 U/L
Protein	2.2 g/dL	3.8 g/dL	4.2 g/dL	4.4 g/dL
Management of pleural effusion	thoracentesis, chest tube drainage	thoracentesis, chest tube drainage	thoracentesis, chest tube drainage, decortication, pleurodesis	thoracentesis, chest tube drainage, decortication, pleurodesis
Outcomes and time measures				
Deceased	Yes	Yes	Yes	No
Cause of death	respiratory failure	respiratory failure	respiratory failure	—
Time from LT to initial FEV_1 decline, months	62	6	30	20
Time from initial FEV_1 decline to death, months	3.5	12	8	—
Time from LT to death, months	65.5	18	38	—

[a]*CLAD, onset is marked by ≥20% decline in FEV_1 for >3 months. The initial FEV_1 decline also coincides with the new onset recurrent pleural effusions.*
Abbreviations: BAL, bronchoalveolar lavage; BOS, bronchiolitis obliterans; CLAD, chronic lung allograft dysfunction; CMV, cytomegalovirus; CS, corticosteroids; D/R, donor/recipient serostatus; DSA, donor- specific antibodies; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; HLA, human leukocyte antigen; HP, hypersensitivity pneumonitis; IPF, idiopathic pulmonary fibrosis; LDH, lactate dehydrogenase; LT, lung transplant; LVEF, left ventricular ejection fraction; MMF, mycophenolate mofetil; PAP, pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; PFT, pulmonary function test; PGD, primary graft dysfunction; RAS, restrictive allograft syndrome; Tac, tacrolimus.

precipitously drop thereafter, with an FEV_1 decline from 3.06 L (87% predicted) to 2.22 L (63%) in 2 months, and then to a nadir of 1.37 L (39% predicted) in another 1.5 months (**Figure 1**). His initial evaluation revealed bilateral costophrenic blunting on chest X-ray, prompting a left-sided thoracentesis with withdrawal of 650 mL of culture-negative, lymphocyte predominant (72%), and exudative pleural fluid. The effusion re-accumulated within a week, and a subsequent high-resolution

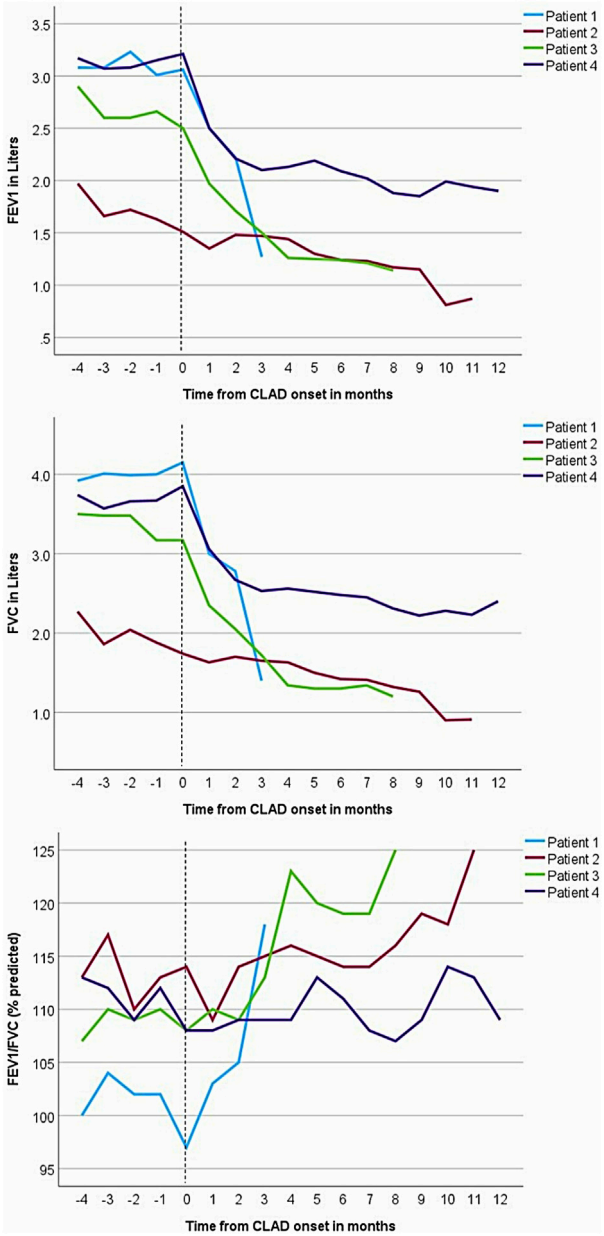


FIGURE 1 | Forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), and FEV_1/FVC trends in the four patients. Spirometry shows a restrictive pattern of decline in lung allograft function. The vertical dashed lines represent the onset of chronic lung allograft dysfunction (CLAD), marked by recurrent pleural effusions.

chest CT showed pleural thickening, bilateral pleural effusions, pericardial effusion, and plate-like and rounded atelectasis (**Figure 2**). Notably, his lung parenchyma appeared normal. Bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsies (TBBx) showed no evidence of infection or acute cellular rejection (ACR); he also never developed donor-specific antibodies (DSAs). He underwent chest tube placement with drainage of pleural fluid and multiple subsequent thoracenteses, which did not improve spirometric flows. A transthoracic echocardiogram showed preserved left ventricular function (55%–60%) and normal diastolic filling. Although a cardiac MRI showed anterior pericardial thickening, functional findings did not meet the criteria for constrictive pericarditis. He died from progressive respiratory failure 64 months after bilateral LT, 3.5 months after his initial drop in FEV_1.

Patient 2

A 72-year-old man with a history of hypersensitivity pneumonitis, coronary artery bypass grafting, and gastroesophageal reflux treated with esophageal fundoplication underwent a single right LT. He was maintained on standard 3-drug immunosuppression. His FEV_1 peaked 1 month after lung transplant [1.97 L (66%)] but began to progressively decline at 6 months, reaching a nadir of 0.87 L (29%) 16 months after LT (**Figure 1**). The decline in spirometric flows was accompanied by a unilateral, right pleural effusion (**Table 1**). A subsequent chest CT revealed right-sided pleural thickening, plate-like and rounded atelectasis, and a notable absence of RAS-like parenchymal changes within the allograft (**Figure 2**). Bronchoscopy with BAL and TBBx showed no evidence of infection or ACR, and he never developed DSAs. He had mild esophageal dysfunction characterized by esophageal stasis and motor incoordination on esophagram but showed normal manometric findings and no evidence of gastroesophageal reflux with a DeMeester score of 1.1. A transthoracic echocardiogram showed normal left ventricular ejection fraction (60%–65%) with mild left ventricular diastolic dysfunction and a raised right ventricular systolic pressure (42 mm Hg). He had a follow-up right heart catheterization (RHC), which revealed a pulmonary arterial pressure (PAP) of 39/11 mm Hg (mean 22 mm Hg) and a pulmonary capillary wedge pressure (PCWP) of 15 mm Hg. Multiple thoracenteses and chest tube drainage did not improve spirometric flows, and analysis revealed culture-negative, exudative, lymphocyte-predominant (92%) pleural fluid. Decortication and pleurodesis were deferred as he was deemed high-risk due to his inability to tolerate single left lung ventilation. He died of respiratory failure 17 months after LT, 12 months after his initial drop in FEV_1.

Patient 3

A 67-year-old man with idiopathic pulmonary fibrosis underwent bilateral LT and was maintained on standard 3-drug immunosuppression. His posttransplant gastroesophageal evaluation revealed reflux with an elevated DeMeester score of 45.1 and poor peristalsis on manometry. He was offered fundoplication, but the patient elected medical management with aspiration precautions. He was hospitalized at an outside institution with worsening dyspnea and new bilateral pleural effusions 30 months after LT. His transthoracic echocardiogram revealed normal left ventricular systolic function and mild diastolic dysfunction. Bronchoscopy with BAL and TBBx showed no evidence of infection or ACR. Aspiration was thought to contribute to his respiratory decline, and he was

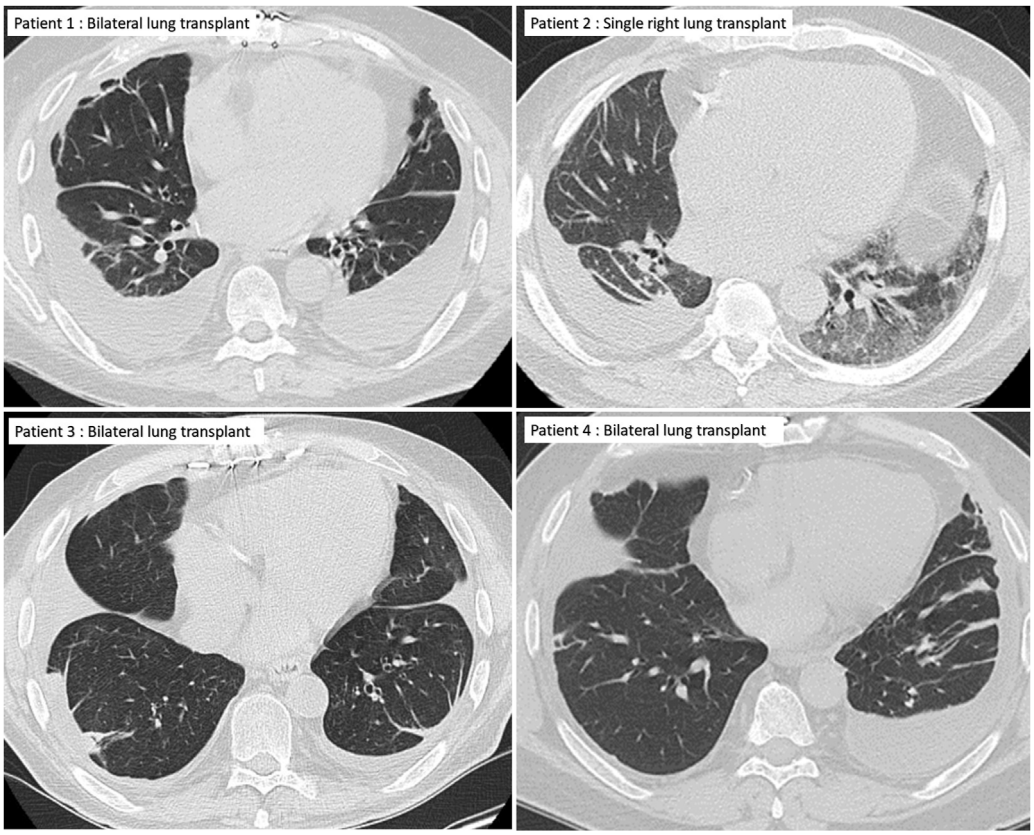


FIGURE 2 | Chest CT of the four patients showing pleural effusions and plate-like atelectasis but no lung parenchymal changes typical of restrictive allograft syndrome.

transitioned from an oral diet to a trial of tube feeding but continued to have a decline in spirometric flows (**Figure 1**) and recurrent pleural effusions (**Figure 2**) with a lack of appropriate lung parenchymal expansion after thoracentesis or chest tube placement. Pleural fluid analysis revealed a lymphocyte-predominant (65%), exudative effusion, and his chest CT showed pleural thickening, plate-like and rounded atelectasis within the allograft, and an absence of RAS-like lung parenchymal changes. He subsequently underwent a partial right decortication and bilateral pleurodesis, but his spirometric flows continued to decline, with his FEV_1 reaching a nadir of 1.14 L (35% predicted) 7 months after his initial decline. He died 1 month later from respiratory failure, 38 months after LT and 8 months after his initial drop in FEV_1.

Patient 4

A 63-year-old man with idiopathic pulmonary fibrosis underwent bilateral LT and was maintained on standard 3-drug immunosuppression. He had an uneventful posttransplant course until he developed a gradual spirometric decline 20 months after transplant (**Figure 1**). His chest CT initially showed bilateral, loculated pleural effusions, and subsequent imaging revealed pleural thickening and plate-like and rounded atelectasis but no RAS-like lung parenchymal changes (**Figure 2**). Bronchoscopy with BAL and TBBx showed no evidence of infection or ACR, and he never developed DSAs. A transthoracic echocardiogram showed normal left ventricular systolic function but moderate left ventricular diastolic dysfunction. He also underwent RHC, which showed no evidence of pulmonary arterial hypertension [PAP: 28/9 mm Hg (mean 18 mm Hg); PCWP: 9 mm Hg]. He was on chronic diuretic therapy and had multiple thoracentesis procedures, which did not improve spirometric flows, and analysis revealed culture-negative, exudative, lymphocyte-predominant (87%) pleural fluid. He then underwent video-assisted thoracoscopic surgery with decortication and doxycycline pleurodesis 9 months after his initial spirometric decline. A pleural biopsy (**Supplementary Figure S1**) showed pleural fibrosis with organizing hemothorax, and he received two doses of intrapleural tissue plasminogen activator to break up loculations. His spirometric flows initially stabilized postoperatively, but he complained of intractable chest pain and dyspnea on exertion. In addition, sEVs isolated from the patient's plasma contained elevated levels of NFkB, 20S proteasome, lipocalin, TNFα, and TGFβ compared to control samples from stable LTRs (**Figure 3**; **Supplementary Figure S2**),

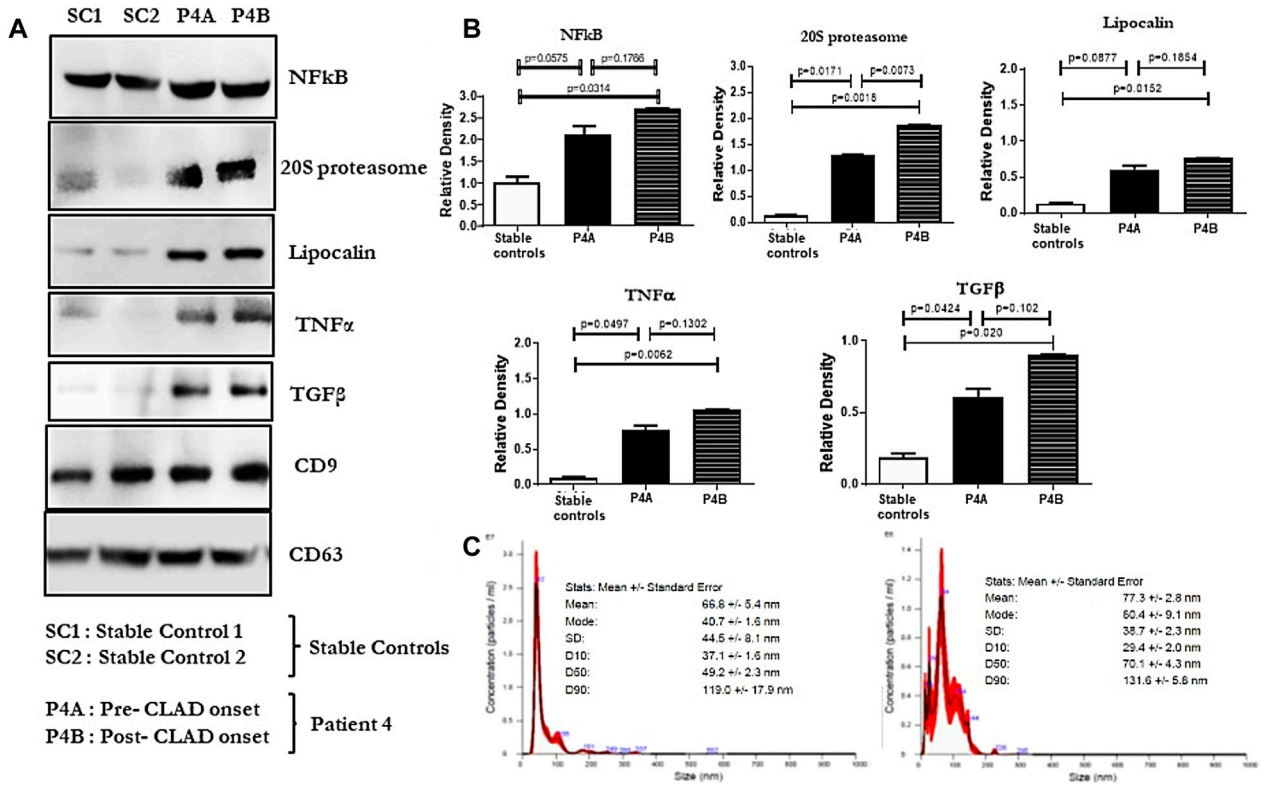


FIGURE 3 | (A): Western blot of small extracellular vesicle (sEV) proteins NFkB, 20S proteasome, lipocalin, TNFα, and TGFβ from plasma samples of lung transplant recipients. SC1 and SC2 denote sEV proteins from 2 stable controls; P4A and P4B denote sEV proteins in the plasma of patient 4, 9 months before and 1 year after the onset of CLAD, respectively. CD9 and CD63 were used as controls to normalize the blots for NFkB, 20S proteasome, lipocalin, TNFα, and TGFβ. **(B)**: Densitometry and statistical analysis of western blots for stable controls and pre and post CLAD-onset samples of patient 4. Relative density graphs are represented as bar plots. **(C)**: Nanosight images depicting the size of the sEVs isolated from both samples of patient 4.

and the concentrations of these inflammatory immunologic markers increased before the onset of CLAD. He remains alive 33.5 months after LT; however, his FEV_1 is at a nadir of 1.9 L, a 39.7% decline from baseline.

DISCUSSION

Chronic lung allograft dysfunction is the most common cause of death among long-term survivors of LT. In 2019, Verleden et al [5] published a consensus statement defining and standardizing the nomenclature and clinical phenotypes of CLAD to facilitate collaboration between centers investigating its pathogenesis, prevention, and treatment. They defined CLAD as a substantial and persistent decline (≥20%) in FEV_1 from baseline, with a predominantly obstructive, predominantly restrictive, or mixed obstructive and restrictive ventilatory pattern that is not explained by other conditions, including pleural effusion. A second consensus statement in the same year by Glanville et al [2] specifically focused on RAS and defined its diagnostic criteria as a ≥20% decline in FEV_1 from baseline and persistent opacities on chest imaging. The absence of RAS-like radiographic opacities [4, 8] and the presence of pleural effusions among our patients excludes them from the currently accepted definitions of RAS, thereby highlighting their unique physiology and phenotype.

Development of recurrent pleural effusion is a well-known LT complication [12, 13], but a yet undescribed manifestation of CLAD. In a large German study of 1223 LTRs, Joean et al [14] identified 113 (9.2%) patients with clinically significant pleural effusions requiring thoracentesis. They observed a bimodal distribution of pleural effusion onset with 67 (59%) patients developing pleural effusion within the first 6 months after LT at a median of 63 days [interquartile range (IQR) 39–96 days] followed by a second peak in 46 patients (41%) who developed pleural effusion at a median of 838 days (IQR 287–1,197 days). The odds of developing a malignant effusion or a cardiogenic effusion were significantly higher in the late-onset group [OR 3.55; CI (1.11–11.32) and OR 5.96; 95% CI (1.95–18.17), respectively], and the late-onset group had lower overall survival than a matched control group [HR 2.43, 95% CI (1.27, 4.62), $p < 0.05$]. However, the survival difference did not retain statistical significance after excluding malignant pleural effusions. In contrast, the LTRs in our series showed a markedly reduced survival rate and poor prognosis after the development of late-onset,

exudative, nonmalignant pleural effusions, despite the absence of a concurrent illness to drive morbidity and mortality. This supports our hypothesis that the pleural effusions seen in our cohort are different from previously described late-onset pleural effusions [12] and instead represent a phenotypically unique manifestation of CLAD.

Consequent to persistent and treatment-refractory pleural effusions, the LTRs in our case series developed a progressive restrictive ventilatory defect with spirometric declines mirroring those of RAS (>20% decline in FEV_1, concurrent FVC decline, FEV_1/FVC >0.7 in all four patients along with >10% TLC decline in one patient). However, an important phenotypic distinction remains between our patients and patients with RAS—our patients never developed pulmonary opacities typical of RAS. The radiographic patterns of RAS on chest CT typically include parenchymal abnormalities followed by progressive pleuro-pulmonary fibrosis [2, 4, 8, 15, 16]. Dettmer et al [15] developed a CT-score for inflammation based on the presence of central and peripheral consolidations, central and peripheral ground-glass opacities, and pleural abnormalities. Patients with restrictive CLAD had an inflammation score >2 (mean 3.43 vs. 0.60 for patients without restrictive CLAD, $p < 0.001$) and had significantly shorter survival than patients with a score ≤2. In contrast, LTRs in our study had an inflammation score ≤2 due to the absence of lung parenchymal abnormalities, but still developed life-limiting and rapidly progressive respiratory failure. Furthermore, while pleural thickening and fibrosis are well-described abnormalities among patients with RAS, the presence of pleural effusions is unique to this cohort and supports our hypothesis that this may be a novel presentation of CLAD.

Our group has previously demonstrated that elevated levels of immunologic markers can be found in circulating sEVs before the onset of CLAD [17], and the exosomal contents between patients with BOS and those with RAS can vary [11]. Veraar et al [18] also showed that lipocalin-2 was elevated in patients with RAS, and increased serum concentrations predicted worse CLAD-free survival in stable patients. Furthermore, Sacreas et al [19] suggested that the fibrotic process of RAS mirrors that of idiopathic pulmonary fibrosis and may be driven by mesothelial-to-mesenchymal transition, characterized by differentiation of pleural mesothelial cells into myofibroblasts after stimulation by TGFβ. All of these findings align with our preliminary data, which showed elevated levels of NFkB, 20S proteasomes, lipocalin, and TGFβ in circulating sEVs isolated from patient 4 before and after the onset of CLAD. Lastly, Iasella et al [20] demonstrated enhanced type-1 immunity among patients with CLAD, characterized by an increase in the concentration of BAL and airway epithelial inflammatory markers including TNFα. We also detected elevated levels of TNFα in patient 4, but within circulating sEVs rather than the airways. These findings suggest that the immunologic and inflammatory milieu identified in our patient may mirror that of other patients with CLAD, despite the difference in their clinical presentation.

Our study is descriptive in nature and has a small number of patients, thereby limiting our ability to draw definitive conclusions. In addition, TLC was not measured uniformly in all patients, and the death of 3 of 4 patients precluded molecular analysis. However, despite these limitations, our observations remain novel and important, as recognition of specific CLAD phenotypes and a better understanding of CLAD subpopulations are essential for developing novel diagnostic and therapeutic strategies [7, 19, 21, 22]. Currently, patients who do not fit into one of the four main categories of CLAD outlined in the 2019 consensus statement (BOS, RAS, mixed, or undefined) remain unclassified, as is the case with the patients in our cohort, who have a restrictive ventilatory defect, exudative pleural effusions, and no evidence of RAS-like opacities. As Levy et al [7] highlighted in 2020, a classification system that permits a sizable portion of patients to be unclassified will be problematic in future clinical decision-making. Further research is vital to unravel the underlying mechanisms of CLAD [23], refine diagnostic criteria, and develop tailored therapeutic strategies. This is especially important in our patient cohort as multiple interventions including pleural fluid drainage, decortication, and pleurodesis proved morbid and ineffective, despite having a potential therapeutic target within the pleural space. Larger studies are needed to confirm our findings and guide therapeutic interventions in this unusual subset of LTRs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at our institution (PHX-21-500-198-73-18 dated 07/12/2023). The Ethics Committee/Institutional Review Board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because of the retrospective study design. However, the study of immune mechanisms using molecular analysis was approved by the Institutional Review Board (PHXB-16-0027-10-18 dated 7/14/2020), and the patients who provided blood samples provided written informed consent to participate in the study. The studies were conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

DS, SB, BB, KM, HM, RW, TM, and ST made substantial contributions to the conception of the work, design of the analysis, drafting of the manuscript, or revising it critically for important intellectual content, and final approval of the version to be published, in accordance with the ICMJE guidelines. DS, SB, and ST worked on the conception and design of the work, the acquisition of the data, data analysis,

the interpretation of the data for the work, and the drafting of the manuscript. All authors are accountable for all aspects of the work and agree to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. 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.

ACKNOWLEDGMENTS

The authors thank Kristine Nally for editorial assistance and Marco Marchionni for graphic design. We thank Jesse Canez for technical and material assistance for molecular analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12395/full#supplementary-material

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BRIEF RESEARCH REPORT
published: 23 January 2024
doi: 10.3389/ti.2024.12019

Predicting Tacrolimus Concentrations in the Skin of Adult Kidney Transplant Recipients: A Feasibility Study

Felicity Sartain[1], Andrea K. Viecelli[2], Margaret Veitch[3], Michael E. Franklin[4], Brian W. Dymock[5], James W. Wells[3,6] and Scott B. Campbell[2,7]*

[1]Department of Medicine, Cairns Hospital, Cairns, QLD, Australia, [2]Department of Kidney and Transplant Services, Princess Alexandra Hospital, Brisbane, QLD, Australia, [3]Frazer Institute, Faculty of Medicine, The University of Queensland, Brisbane, QLD, Australia, [4]Department of Clinical Pharmacology, Princess Alexandra Hospital, Brisbane, QLD, Australia, [5]Queensland Emory Drug Discovery Initiative, UniQuest, The University of Queensland, Brisbane, QLD, Australia, [6]Dermatology Research Centre, Frazer Institute, Faculty of Medicine, The University of Queensland, Brisbane, QLD, Australia, [7]Faculty of Medicine, The University of Queensland, Brisbane, QLD, Australia

Solid organ transplant recipients are at an increased risk of developing skin cancers due to chronic immunosuppression, particularly with calcineurin inhibitors. Tacrolimus is the most prescribed calcineurin inhibitor in this patient cohort, and understanding tacrolimus concentrations in the skin will facilitate the development of anti-cancer preventive and therapeutic strategies. Here, we show that in mice, tacrolimus blood levels peaked rapidly ~1 h post last oral dose while skin levels rose more slowly and remained high for at least 6 h. Subsequently, tacrolimus skin and blood concentrations were assessed in 15 kidney transplant recipients. The mean age was 61 years, the average time post-transplant was 7 years (range 0–21 years) and 87% were male. The average skin sampling time post tacrolimus dosing was 6 h 32 min. Skin tacrolimus concentrations ranged from 7.1 ng/g to 71.2 ng/g and correlated with blood concentrations (r = 0.6). Mouse and human mean skin concentrations were in a similar range. Our data suggests that tacrolimus measurements in the blood may be used to approximate tacrolimus concentrations in the skin of kidney transplant recipients, and further exploited for the delivery of anti-cancer therapies designed to antagonize the immunosuppressive effects of tacrolimus in the skin.

OPEN ACCESS

Correspondence
James W. Wells,
j.wells3@uq.edu.au

Received: *08 September 2023*
Accepted: *11 January 2024*
Published: *23 January 2024*

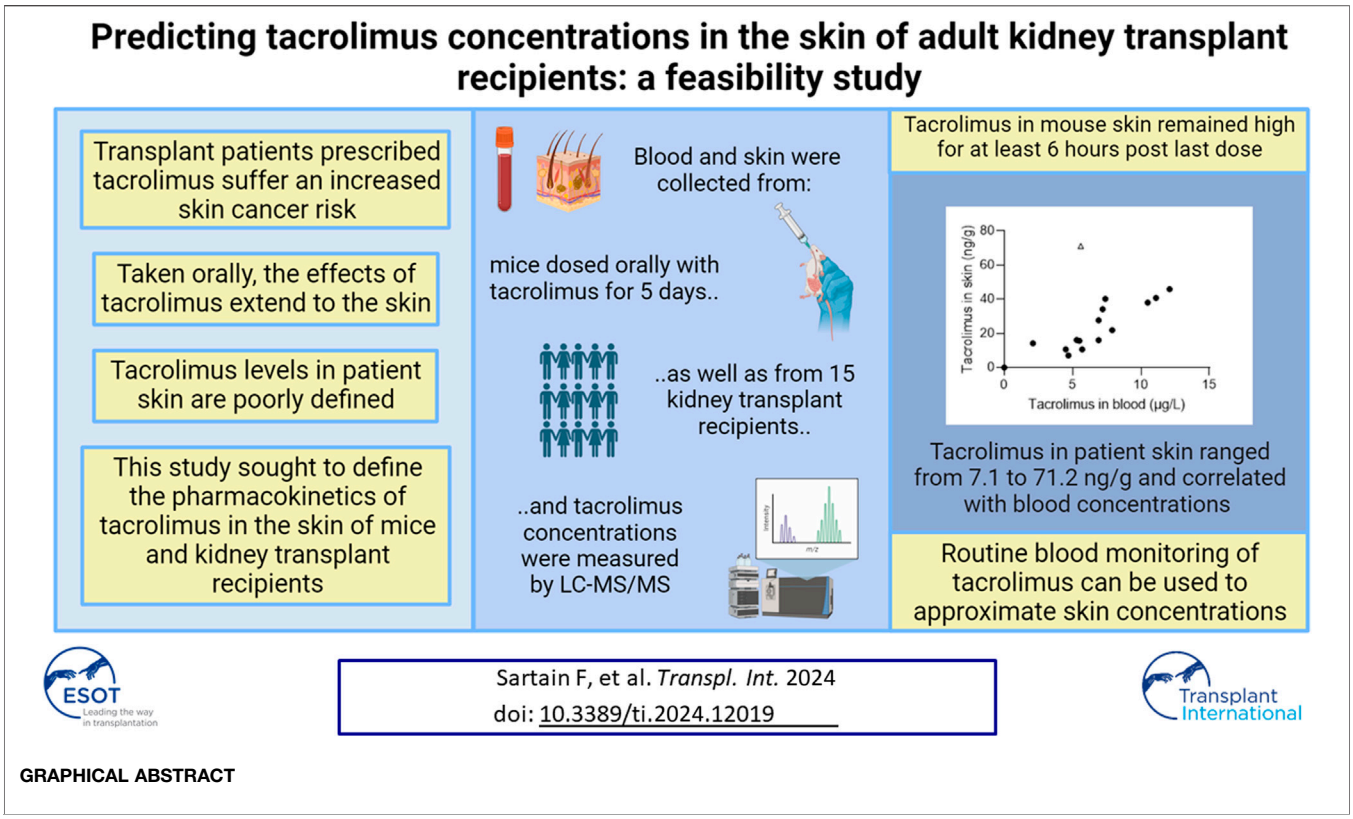
Citation:
Sartain F, Viecelli AK, Veitch M, Franklin ME, Dymock BW, Wells JW and Campbell SB (2024) Predicting Tacrolimus Concentrations in the Skin of Adult Kidney Transplant Recipients: A Feasibility Study. Transpl Int 37:12019. doi: 10.3389/ti.2024.12019

Keywords: kidney transplantation, organ transplant, skin cancer, tacrolimus, drug concentration, skin, calcineurin inhibitors

INTRODUCTION

Solid organ transplant recipients are at an increased risk of developing malignancies as a consequence of their immunosuppression, with calcineurin inhibitors thought to be particularly responsible [1–3]. Skin cancer is the most common cancer type in kidney transplant recipients [4, 5]. The pathogenesis of skin cancer in transplant recipients involves predisposing risk factors and this is amplified by the carcinogenic effect of immunosuppressive medications. For instance, calcineurin

Abbreviations: DNA, deoxyribonucleic acid; FKBP12, 12-kDa FK506-binding protein; mTOR, mammalian target of rapamycin.



GRAPHICAL ABSTRACT

inhibitors impair the capacity of the immune system to repair or destroy ultraviolet damaged cells [6]. Current pharmacologic therapies to prevent occurrence of skin cancers include retinoid therapy, nicotinamide and the modulation of immunosuppression by converting from calcineurin inhibitors to mammalian target of rapamycin (mTOR) pathway inhibitors [5, 7–9].

Tacrolimus, a calcineurin inhibitor, is commonly used to prevent rejection in solid organ transplants and acts by preventing the transcription of key pro-inflammatory cytokines within T cells which are necessary to drive an effective immune response [10, 11]. Tacrolimus also inhibits the presentation of exogenous antigens through the inhibition of antigen processing pathways and significantly inhibits helper T cell differentiation and cytokine secretion by CD4 memory T cells [3]. Nucleotide excision repair is inhibited by calcineurin inhibitors whereas calcineurin overexpression enhances cellular nucleotide excision repair [12]. Given that tacrolimus inhibits the capacity for ultraviolet-induced DNA repair, it is hypothesized that the inhibition of tacrolimus effects on cells in the skin may improve the ability of Sun damaged cutaneous cells to repair and thus may reduce the development of skin cancer. Importantly, this could happen without impeding the important systemic effects that tacrolimus has on the prevention of transplant rejection.

Recently, we have described the development of a novel and competitive tacrolimus inhibitor, Q-2361 [13]. Q-2361 is a reversible antagonist of the tacrolimus-FKBP12 binding interaction. The tacrolimus-FKBP12 complex binds to calcineurin forming a ternary complex thereby inhibiting calcineurin. A 400-1000-fold concentration of Q-2361 over tacrolimus facilitates human T cell function in the presence of tacrolimus. Transplant patients are known to have normal numbers of T cells in their skin despite many years of immunosuppression [14], and the local application of Q-2361 to squamous skin cancers growing in tacrolimus-suppressed mice has been shown to lead to T cell-mediated tumor rejection [13]. Thus, to progress the topical application of this compound towards clinical studies in patients, it is important to understand the concentration range of tacrolimus in patient skin compared to mouse skin and whether tacrolimus patient skin concentrations can be approximated from routine blood measurements of tacrolimus. Therefore, we aimed to test the hypotheses that it is possible to measure tacrolimus concentrations in the skin of adult kidney transplant recipients and that skin measurements correlate closely with blood measurements.

PATIENTS AND METHODS

Mice

All animal procedures were approved by the University of Queensland Animal Ethics Committee; Approval Number UQDI/512/17. C57BL/6J mice were purchased from the Animal Resources Facility (Perth, Australia). All mice used were 12-week females and were housed under

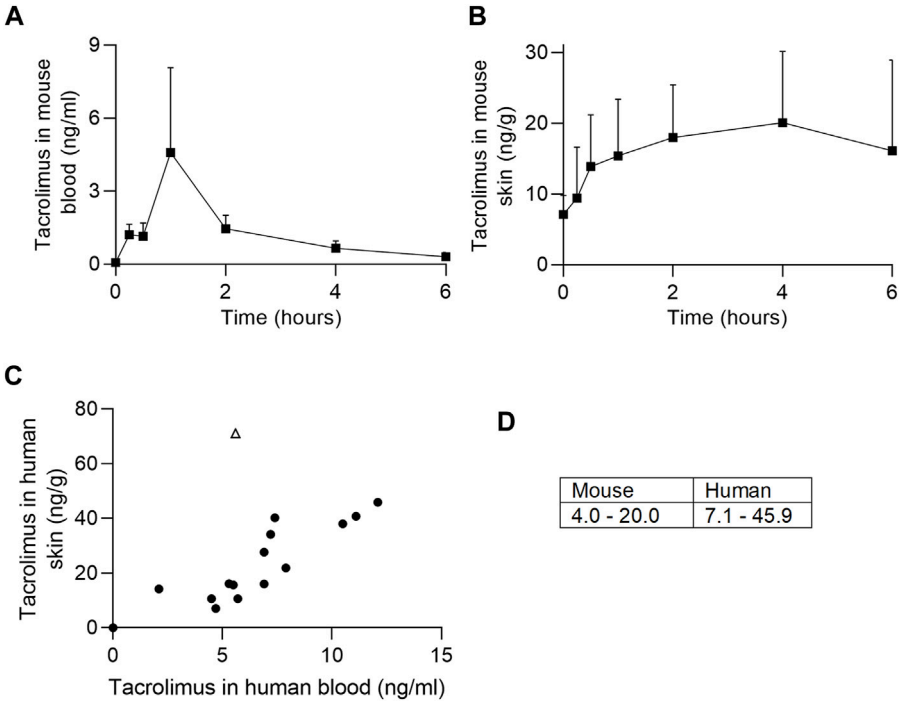


FIGURE 1 | Tacrolimus assessments in blood and skin. **(A, B)** Tacrolimus levels in mouse blood and skin (respectively) following 4 days of twice-per-day oral dosing. $n = 4$ mice/timepoint, error bars represent standard error of the mean. **(C)** Comparison of tacrolimus levels in patient blood and skin. The outlier (pictured as an open triangle) was from a skin sample taken above an upper lip (tacrolimus skin concentration 71.2 ng/g). Correlations are shown with ($r = 0.6$, $p < 0.05$) and without ($r = 0.88$, $p < 0.0001$) outlier. **(D)** Mouse and human mean tacrolimus concentration data ranges in the skin (ng/g), excluding one human outlier with tacrolimus skin concentration of 71.2 ng/g.

specific pathogen-free conditions at the Translational Research Institute Biological Research Facility (Brisbane, Australia).

Oral Dosing With Tacrolimus in Mice

Mice were dosed orally with tacrolimus (MedChemExpress, Monmouth Junction, NJ, USA; 1 mg/kg) twice per day with a 7-hour interval for 4 days via oral gavage. On Day 5 the mice were orally dosed, and then cardiac bleeds and skin harvests were performed at the indicated timepoints. 110 µL of blood was transferred to a cryovial containing 10 µL 0.5M EDTA, shaken, snap-frozen on dry ice, and stored at −20°C. Approximately 2 cm^2 of back skin was harvested, weighed, snap-frozen on dry ice, and stored at −20°C. The quantification of tacrolimus in blood was performed as previously described [15].

Patient Study Setting and Design

Ethics approval was gained from the Metro South Human Research Ethics Committee (Approval number: HREC/2019/QMS/50547). Written consent from each adult kidney transplant recipient for involvement in this project was obtained prior to the removal of their presumed skin cancer. Eligible patients were adult kidney transplant recipients (≥18 years of age) who were on once or twice daily tacrolimus dosage and were planned to undergo a skin excision. Patients were excluded if they had a bleeding disorder or if they were on any anticoagulation other than aspirin. Included patients needed to have a planned surgical procedure which was likely to result in excess skin being available for sampling.

Skin Sampling

Suspicious skin lesions were excised by the surgical team. Two 2–3 mm punch biopsies were immediately taken from the ends of the excised skin sample by the study investigators and placed on ice for transport to the laboratory (10 min). Once in the laboratory the skin biopsies were weighed, snap-frozen on dry ice, and stored at −20°C. The patients also had a blood tacrolimus level collected the same day of the excision, which was sent directly to the hospital clinical pharmacology department for assessment.

Skin Tacrolimus Quantification

The quantification of tacrolimus in skin was performed as described [15]. Briefly, samples were placed in a tissue grinding tube (Precellys® Lysing Kit; MK28-R; Bertin Technologies, Montigny-le-Bretonneux, France) containing 1 mL of Titrisol buffer (Merck KGaA, Darmstadt, Germany) and 60 µL of internal standard (ascomycin; Fujisawa Pharmaceutical Company, Osaka, Japan). Samples were homogenized using a Precellys® 24 Homogenizer (Bertin Technologies) at 6,500 rpm, 30 s for 5 cycles, with the tubes

TABLE 1 | Baseline characteristics of study participants.

#	Sex	Age	Kidney disease	Year	Medication	Type and duration of dialysis	Weight (kg)
1	M	55	Reflux Nephropathy	2007	Tac, Aza, Pred	HD – 4 months	65.2
2	M	51	IgAN	2015	Tac, Lef, Pred	Pre-emptive	85
3	M	54	Renovascular Disease	2008	Tac, Myco, Pred	PD – uncertain duration	59
4	F	58	GN	2008, 2014	Tac, Myco, Pred	PD – 2 years btw transplants	96
5	M	59	Glomerulonephritis	2010	Tac, Myco, Pred	HD – uncertain duration	101.75
6	M	48	Lupus Nephritis	1998, 2005	Tac, Myco, Pred	HD – 13 years	83
7	M	65	PCKD	2019	Tac, Myco, Pred	HD – 1.5 years	95
8	M	56	IgAN/HSP	2010	Tac, Myco, Pred	Unknown	91
9	M	69	ADPKD	2005	Tac, Myco, Pred	HD – 1 year	71
10	M	60	Uncertain	2014	Tac, Myco, Pred	PD – 2.5 years	105
11	M	68	Nephritis	2012	Tac, Myco, Pred	PD – 2.5 years	102
12	F	69	ADPKD	2019	Tac, Myco, Pred	PD – 2.5 years	56.35
13	M	65	Post lung transplant, ATN	2017	Tac, Myco, Pred	HD – 5 years	95
14	M	72	Glomerulonephritis	2014	Tac, Myco, Pred	HD – 1.5 years	119.2
15	M	60	Caroli's Disease	2013	Tac, Myco, Pred	HD – <1 year	94

#, patient number; M, Male; F, Female; IgAN, IgA Nephropathy; PCKD, Polycystic Kidney Disease; ADPKD, Autosomal polycystic kidney disease; GN, Glomerulonephritis unspecified; ATN, Acute Tubular Necrosis; Tac, Tacrolimus; Aza, Azathioprine; Myco, Mycophenolate; Lef, Leflunomide; Pred, Prednisone; HD, Haemodialysis; PD, Peritoneal Dialysis; HSP, Henoch Schoenlein Purpura; Year, year of transplant.

TABLE 2 | Tacrolimus measurements in blood and skin.

#	Total daily dose of Tac	Time since last dose (Hrs:Mins)	Time between samples (mins)	Tac in blood (ng/mL)	Skin Biopsy site, Tac conc (ng/g)	Mean Tac skin conc (ng/g)
1	2 mg BD (4 mg daily)	6:53	67[a]	11.1	Right lateral thigh, 40.8	40.8
2	6 mg BD (12 mg daily)	6:50	10[b]	5.6	Left upper lip, 71.2	71.2
3	1 mg BD (2 mg daily)	7:30	15[b]	5.5	Left forearm, 15.7	15.7
4	0.5 mg BD (1 mg daily)	9:35	7[b]	2.1	Left hand, 14.2	14.2
5	4 mg BD (8 mg daily)	5:35	10[a]	6.9	Neck, 27.0 Left shoulder, 28.4	27.7 (±0.9)
6	4.5 mg BD (9 mg daily)	4:40	5[a]	4.5	Right shoulder, 8.6 Right calf, 12.8	10.7 (±2.9)
7	1 mg mane/2 mg nocte (3 mg daily)	7:05	15[a]	4.7	Back, 7.1	7.1
8	1 mg mane/0.5 mg nocte (1.5 mg daily)	7:20	20[a]	10.5	Left ear, 38.0	38.0
9	1 mg BD (2 mg daily)	Unknown	5[a]	5.3	Right calf, 16.2	16.2
10	1 mg BD (2 mg daily)	8:10	15[b]	7.4	Left side of nose, 40.2	40.2
11	0.5 mg mane, 1 mg nocte (1.5 mg daily)	5:30	20[a]	7.9	Right forearm #1, 28.3 Right forearm #2, 17.6 Left lower calf, 23.4 Left shoulder, 18.5	21.9 (±4.9)
12	5 mg BD (10 mg daily)	4:45	30[a]	12.1	Left dorsum of hand, 38.9 Forehead, 52.9	45.9 (±9.8)
13	2 mg mane, 1.5 mg nocte (3.5 mg daily)	7:30	35[b]	6.9	Right knee, 16.1	16.1
14	0.5 mg mane, 1 mg nocte (1.5 mg daily)	6:45	8[b]	5.7	Right knee, 10.7	10.7
15	1.5 mg mane, 1 mg nocte (2.5 mg daily)	10:00	29[a]	7.2	Left cheek, 38.7 Left intra orbital, 36.3 Right forearm, 27.7	34.2 (±5.7)

#, patient number.

[a]Blood sample taken before skin sample.

[b]Skin sample taken before blood sample; Tac, Tacrolimus. Numbers in brackets in right hand column represent standard deviation.

placed on ice for at least 1 min between each cycle to prevent drug degradation. 5 mL tert-butyl methyl ether was added to each sample, and the liquid-liquid extraction process was performed manually by inverting the tubes for at least 5 min. Tubes were then centrifuged at room temperature at 1,800 g for 3 min to separate all the layers, and the tert-butyl methyl ether fraction containing partitioned tacrolimus was collected and evaporated using a Sample Concentrator (Techne Dri-Block, DB-3D, Cambridge, England) at 35°C and the residue subsequently reconstituted with 200 µL 50% methanol by vortexing

thoroughly. Reconstituted samples were transferred into UPLC max recovery sample vials (Waters Corporation, Milford, MA) and analyzed using LC-MS/MS (Alliance HT LC system interfaced to a Quattro Micro tandem mass spectrometer; Waters Corporation) in the hospital clinical pharmacology department. Excess skin retrieved post abdominoplasty from a patient not on tacrolimus was used as a control.

Statistical Analysis

Statistical and Pearson's correlation coefficient analysis were carried out using GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA). There was no power calculation attached to the number of participants. Sample collection was concluded following visual evidence of a correlation between blood and skin tacrolimus levels.

RESULTS

Pharmacokinetics of Tacrolimus in Mouse Skin

To understand how tacrolimus concentrations in the blood and skin change at defined time points post oral dosing, we administered tacrolimus orally to mice twice daily over 5 days. At defined time points post last dose blood and skin were harvested and tacrolimus concentrations assessed by liquid chromatography with tandem mass spectrometry. As shown in **Figure 1**, tacrolimus blood levels peaked rapidly approximately 1 h post last oral dose (**Figure 1A**) while skin levels rose more slowly and remained high for at least 6 h (**Figure 1B**). The data suggests that skin tacrolimus levels do not rise and fall as quickly as they do in blood, and remained high for at least 6 h post last dose.

Pharmacokinetics of Tacrolimus in Kidney Transplant Recipient Skin

Thirty-one patients were approached and consented. Of the thirty-one patients consented only fifteen patients proceeded to have skin lesions excised and thus were included in the study. Of the fifteen patients consented two (13%) were female and thirteen (87%) were male. The mean age was 61 years (range 48–72 years) and the average time since first transplant was 7 years (0–21 years). The majority of the patients (87%) were on the immunosuppressive regimen of tacrolimus, mycophenolate and prednisone. The average time post oral tacrolimus dosing was 6 h 32 min. Four of the patients had been treated for previous rejection. Two patients had had a second kidney transplant. Baseline characteristics of the patients who were included in the study are provided in **Table 1**.

Twelve patients had an identifiable cutaneous malignancy on histopathology. Of the 23 samples obtained, 5 were classified as basal cell carcinomas, 4 samples were squamous cell carcinomas, 9 were intraepidermal carcinomas and 3 were solar keratoses. Two samples did not contain any pre-malignant or malignant tissue. These samples were taken from a variety of different anatomical locations as listed in **Table 2**.

The timing of skin excision was documented for all the patients involved in the study. The mean time between the skin sample excision and tacrolimus blood collection was 18.5 min (range 5–67 min). Five patients had multiple excisions taken and the tacrolimus cutaneous concentration was measured independently in all the specimens. Excess cutaneous tissue post abdominoplasty from a patient not taking tacrolimus was sent to the laboratory as a control. The tacrolimus concentration was 0 ng/g in this sample. Tacrolimus was detectable in the skin in all patients on oral tacrolimus.

The skin concentration of tacrolimus was calculated in twenty-three samples from fifteen patients (**Table 2**). Skin tacrolimus concentrations ranged from 7.1 ng/g to 71.2 ng/g. There was one clear outlier in the data: patient number two had a tacrolimus blood concentration of 5.6 μg/L and the concentration obtained from the skin sample was 71.2 ng/g. In patients in whom multiple skin samples were taken for tacrolimus skin concentration measurement, the mean tacrolimus concentration was used for the correlation calculations. The blood concentration of tacrolimus correlated with the concentration of tacrolimus detected in the skin samples (**Figure 1C**) with a Pearson's correlation coefficient of 0.6 (with the outlier included; open triangle) or 0.88 (with the outlier excluded). The mean concentration ranges in mouse versus human skin were similar (**Figure 1D**) indicating that mouse is a suitable model for drug testing increasing the relevance and translatability of mouse data.

DISCUSSION

To our knowledge this is the first study to demonstrate that tacrolimus can be measured in the skin of persons taking oral tacrolimus. In this small study it was demonstrated that the skin concentration of tacrolimus correlated with the blood concentration. Notably, several patients had multiple skin excisions taken from different sites and largely all the samples from each individual demonstrated a comparable skin tacrolimus concentration.

In this study, the tacrolimus blood level was collected in the early afternoon which is not the usual time that a trough level would be collected. This blood measurement was specifically collected to compare to the measured skin concentration of tacrolimus and determine whether there was any correlation. Higher tacrolimus concentrations were generally seen in the samples taken from patients' faces (i.e. patients 8, 10, 12, 14, 15). However, it is not clear from this small data set whether or not areas more prone to Sun exposure exhibit higher skin concentrations of tacrolimus. We also assume there was a variable amount of fat in each sample depending on the anatomical location and this may also affect the pharmacokinetic distribution of tacrolimus.

Previous studies have measured tacrolimus concentration in the skin after topical application of tacrolimus [16]. A study comparing the delivery systems for topical tacrolimus measured tacrolimus concentration in human skin by liquid chromatography tandem-mass spectrometry. They also

detected inflammatory markers such as IL-6 and IL-8. The researchers found that extensive barrier disruption resulted in the enhanced penetration of topically applied tacrolimus and uptake by immune cells in the skin. Other studies have measured the blood concentration of tacrolimus post skin application [17]. One study, using 0.1% tacrolimus ointment, found that systemic exposure tended to increase proportionally as the size of the treated body surface area increased, however the highest blood level was only 3% of the usual tacrolimus level measured in the blood of liver transplant patients receiving tacrolimus orally.

Given the significant issue of skin cancer post-transplant, effective therapies are urgently required to address this problem. Encouragingly new therapies and techniques are being actively studied to reduce the incidence of post-transplant skin cancer. A recent randomized, double-blind, placebo-controlled, single-arm trial explored whether topical sirolimus would reduce the incidence of skin cancer in solid organ transplant recipients with a history of skin cancer [18]. Participants had topical sirolimus applied to one forearm and hand for 12 weeks. At 12 weeks, the number of keratotic lesions had reduced in each patient by 31 +/- 5% and at 24 months there was a 3-fold decrease in intraepithelial carcinomas, however no difference in squamous cell carcinoma numbers were observed.

Our previous studies in mice show that a simple solution-based Q-2361 topical formulation achieved high (>30 μg/g) and sustained residence in skin with negligible drug levels in the blood [13]. In the current study, it was determined that the range of tacrolimus skin concentrations varied from 7.1 ng/g to 71.2 ng/g in patients receiving a total daily tacrolimus dose of between 1- and 12 mg of tacrolimus daily. It is entirely feasible, therefore, that appropriately formulated and topically applied Q-2361 could result in levels of Q-2361 over tacrolimus needed to locally rescue T cell function in patient skin. Furthermore, as a result of the correlation between blood and skin tacrolimus levels, the level of tacrolimus in patient skin can be approximated from routine blood analysis, without subjecting patients to additional skin biopsies, assisting with accurate dosing estimates for Q-2361 in future clinical trials.

Our study had several limitations. As the skin excisions were from different anatomical locations (depending on where the concern for malignancy was) the location and thickness of tissue was not standardized. It can also be assumed that different parts of skin have different degrees of Sun damage compared to others and we are uncertain of how that could affect tacrolimus concentrations. Finally, there was no power calculation attached to the chosen number of participants. Rather, recruitment ceased once there were enough numbers to see visual evidence of correlation between skin and blood levels.

Potential future research directions include sampling a larger cohort of patients and including the collection of information on race, skin type, and Sun exposure history. Collectively, this would allow for a deeper understanding of the variability in skin tacrolimus concentrations between different anatomical locations and among patients, and permit comparative analysis of skin tone, tacrolimus concentration, Sun exposure, and skin cancer prevalence.

There is an urgent unmet clinical need for new therapies to help address the issue of cutaneous malignancy in transplant recipients. This study importantly demonstrates that in mice and patients taking oral tacrolimus the drug concentrations can be measured in the skin and these levels appear to correlate between species and with blood tacrolimus concentrations in humans. Thus, this provides a rationale for the development of topical therapies such as Q-2361 which antagonize tacrolimus locally in the skin, aiming to reduce the development of skin cancer and potentially even treat established malignancy. Q-2361 is efficacious in mouse models of squamous cell cancer [13]. Clinical trials are now required to explore the efficacy of Q-2361 and whether individualization of the quantity and frequency of the treatment is required given the variability of tacrolimus skin concentrations between 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.

ETHICS STATEMENT

The studies involving humans were approved by Metro South Human Research Ethics Committee (Approval number: HREC/2019/QMS/50547). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by University of Queensland Animal Ethics Committee; Approval Number UQDI/512/17. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology: SC, JW, and AV. Funding acquisition: SC, JW, and AV. Experimentation, investigation and sample analysis: MV, AV, and MF. Writing–data analysis, original draft, review and editing: FS, MV, AV, MF, BD, JW, and SC. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

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LETTER TO THE EDITOR
published: 19 January 2024
doi: 10.3389/ti.2024.11985

A Novel Case of CMV Resistance to Valganciclovir and Maribavir in a Renal Transplant Patient

Helen Pearce[1], *Emma K. Montgomery*[2], *Neil Sheerin*[2,3]* *and Helena Ellam*[4]

[1]Newcastle Hospitals Trust, Newcastle upon Tyne, United Kingdom, [2]Renal Services, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom, [3]Immunity and Inflammation Theme, Faculty of Medical Sciences, Translational and Clinical Research Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, [4]Department of Virology, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom

Keywords: CMV infection, resistance, renal, transplant, maribavir

Dear Editors,

Cytomegalovirus (CMV) is one of the most common viruses causing infectious complications after kidney transplantation [1, 2]. Anti-viral prophylaxis and pre-emptive therapy are the mainstays of CMV prevention. Maribavir is an oral benzimidazole riboside drug thought to have potent, selective multimodal anti-CMV activity, thus conferring protection against CMV strains resistant to traditional anti-viral drugs [3]. Whilst valganciclovir remains the first line oral anti-viral treatment in the management of CMV, maribavir offers a promising oral alternative to previous second line nephrotoxic drugs, foscarnet and cidofovir [4]. Approved by the U.S. Food and Drug Administration in November 2021, maribavir has been recommended by National Institute for Health and Care Excellence (NICE) for patients with resistance to at least one other first-line medication used in the management of CMV [4, 5]. Despite maribavir's novel mode of action, we report a case of resistance to both ganciclovir and maribavir in a patient following kidney transplantation.

A 70-year-old male with end-stage kidney disease, underwent kidney transplantation on 27/11/22. The patient was high risk for CMV disease (donor CMV seropositive/recipient CMV seronegative). Histocompatibility report issued day 44 post-transplant and showed donor/recipient serologic equivalents of HLA-B, -C, -DRB1, -DQB1, and–DPB1 antigens were matched, aside from HLA-A (donor HLA type A*02/A*24, recipient HLA type A*68). Post transplantation, the patient was initiated on CMV prophylaxis in the form of oral valganciclovir, 450 mg three times weekly for 100 days, alongside his maintenance immunosuppressant regime (tacrolimus (Adoport) 5 mg BD, mycophenolate mofetil (MMF) 500 mg BD and prednisolone 5 mg OD).

In the weeks following transplantation, the patient reported no symptoms suggestive of CMV disease. However, day 53 post-transplant, a CMV viraemia was detected from a whole blood CMV assay (viral load 72,800 IU/mL, eGFR 28, lymphocyte count 0.73 10^9/L, tacrolimus level 13.9 ug/L) whilst the patient remained on prophylactic valganciclovir. Subsequently, the patient's valganciclovir was increased to treatment dose (450 mg OD), MMF was suspended, tacrolimus dose decreased to 3 mg AM, 4 mg PM and prednisolone dose increased to 10 mg OD. Weekly CMV monitoring was instituted thereafter. Blood tests day 100 post-transplant showed the patients eGFR was stable at 34, the patient's lymphocyte count was 1.14 10^9/L and tacrolimus level 8.8 ug/L. Since CMV viral titres remained high despite being on treatment-dose valganciclovir, a sample was sent for genotypic resistance testing on day 103 post-transplant (UL54 and UL97 regions sequenced by Sanger sequencing). Results received on day 109 post-transplant identified the presence of UL97 C603W mutation (a common mutation which confers resistance to ganciclovir) and valganciclovir was subsequently stopped. The patient remained asymptomatic, however, on day 116 post-transplant blood tests showed the patient's alanine transaminase levels were elevated at 160 IU/L, eGFR 28, lymphocyte count 0.99 10^9/L and tacrolimus level 12.8 ug/L. In the context of

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***Correspondence**
Neil Sheerin,
neil.sheerin@newcastle.ac.uk

Received: 29 August 2023
Accepted: 10 January 2024
Published: 19 January 2024

Citation:
Pearce H, Montgomery EK, Sheerin N and Ellam H (2024) A Novel Case of CMV Resistance to Valganciclovir and Maribavir in a Renal Transplant Patient. Transpl Int 37:11985. doi: 10.3389/ti.2024.11985

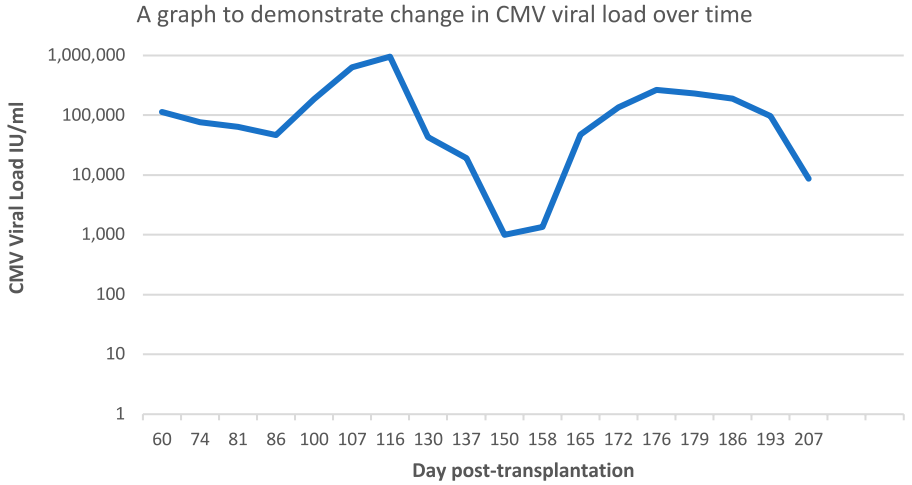

FIGURE 1 | A graph to demonstrate change in CMV viral load, in-keeping with CMV resistance emergence.

increasing viral titres, maribavir therapy (400 mg BD) was initiated. It should be noted that maribavir strongly antagonises the action of valganciclovir, an effect thought to be precipitated by interference in the phosphorylation process, therefore these two medications must not be used in conjunction with one another [6]. Further amendments were made to the patient's immunosuppressant regime, specifically a reduction in tacrolimus dose to 3 mg BD, day 116 post-transplant. In the initial phase following initiation of maribavir, the patient's CMV viral load steadily decreased. The patient's tacrolimus dose was further reduced to 2 mg BD in response to increasing trough levels, due to the known interaction between tacrolimus and maribavir. Blood tests day 144 post-transplant showed the patient's eGFR remained at 28, alanine transaminase levels decreased to 88 IU/L, lymphocyte count increased to 2.23 10^9/L and tacrolimus level 10 ug/L.

Day 165 post-transplant there was a significant rise in the patient's CMV viral load, despite full treatment compliance (**Figure 1**), and a sample was sent for repeat genotypic resistance testing (UL54, UL97, UL56 and UL89 regions sequenced by Sanger sequencing). This identified the development of two new mutations in addition to the previously identified C603W mutation in the UL97 region: T409M mutation in the UL97 region (which is known to confer resistance to maribavir) and T503I mutation in the UL54 region (conferring resistance to ganciclovir and cidofovir). No drug resistance-associated mutations were identified in the UL56/UL89 regions. These results were confirmed by genotypic resistance testing at a second laboratory. Maribavir therapy was subsequently stopped on day 179 post-transplant. It should be noted that T409M is a common mutation, well described in the literature, and confers high level resistance to maribavir. Interestingly it often develops after an initial suppression of a patient's CMV viral load, as seen in this case [7]. In the week following termination of maribavir, the patient's CMV viral load decreased, which could be indicative of self-cleared infection. Further anti-viral treatment was not given and day 207 post-transplant, the following changes were made to the patient's immunosuppression regime: tacrolimus (Adoport) 2 mg BD, mycophenolate mofetil 500 mg remained suspended, prednisolone reduced to 10 mg/5 mg alternate days. The patient's kidney transplant function remains satisfactory and liver function remains stable following termination of maribavir (Day 207 post-transplant: eGFR 38, alanine transaminase 48 IU/L and lymphocyte count 2.34 10^9/L and tacrolimus level 5.6 ug/L).

In summary, to our knowledge, this is the first reported case of resistance to both valganciclovir and maribavir outside of clinical trials in the UK. Although resistance to maribavir is described in the SOLSTICE clinical trial [3, 8], our case highlights the need for clinicians to be vigilant when initiating treatment with maribavir. It should also be acknowledged that foscarnet may be a preferred option over maribavir when treating refractory CMV diseases with high viral loads [9]. A low threshold for CMV resistance testing is recommended if a patient's CMV viral load increases whilst on treatment. Maribavir is known to increase exposure to tacrolimus [10] and our case highlights the importance of monitoring concomitant immunosuppressant blood concentration at initiation, co-administration, and discontinuation of maribavir.

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

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

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

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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Transplant International

Official journal of the European Society for Organ Transplantation

Editorial Office

Avenue du Tribunal Fédéral 34
CH – 1005 Lausanne
Switzerland

Tel +41 (0)21 510 17 40
Fax +41 (0)21 510 17 01

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