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Bio-Engineering of Islet Organoids

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ESOT

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Bio-Engineering of Islet Organoids

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ACADEMIA

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ACADEMIA

6 - 8 APRIL 2022

Rome, Italy

The Academia course provides participants with intensive training on some of the key skills necessary to succeed in research, publishing, and professional interactions. This course is recommended to all transplant professionals who wish to learn how to conduct rigorous research, get published, effectively present their work, and become culturally competent professionals. Topics include: how to conduct a systematic review; statistics; how to prepare and deliver an effective and engaging presentation; communication and unconscious bias. The course combines lectures, workshops, group work, and short presentations by participants.

4 - 5 APRIL 2022

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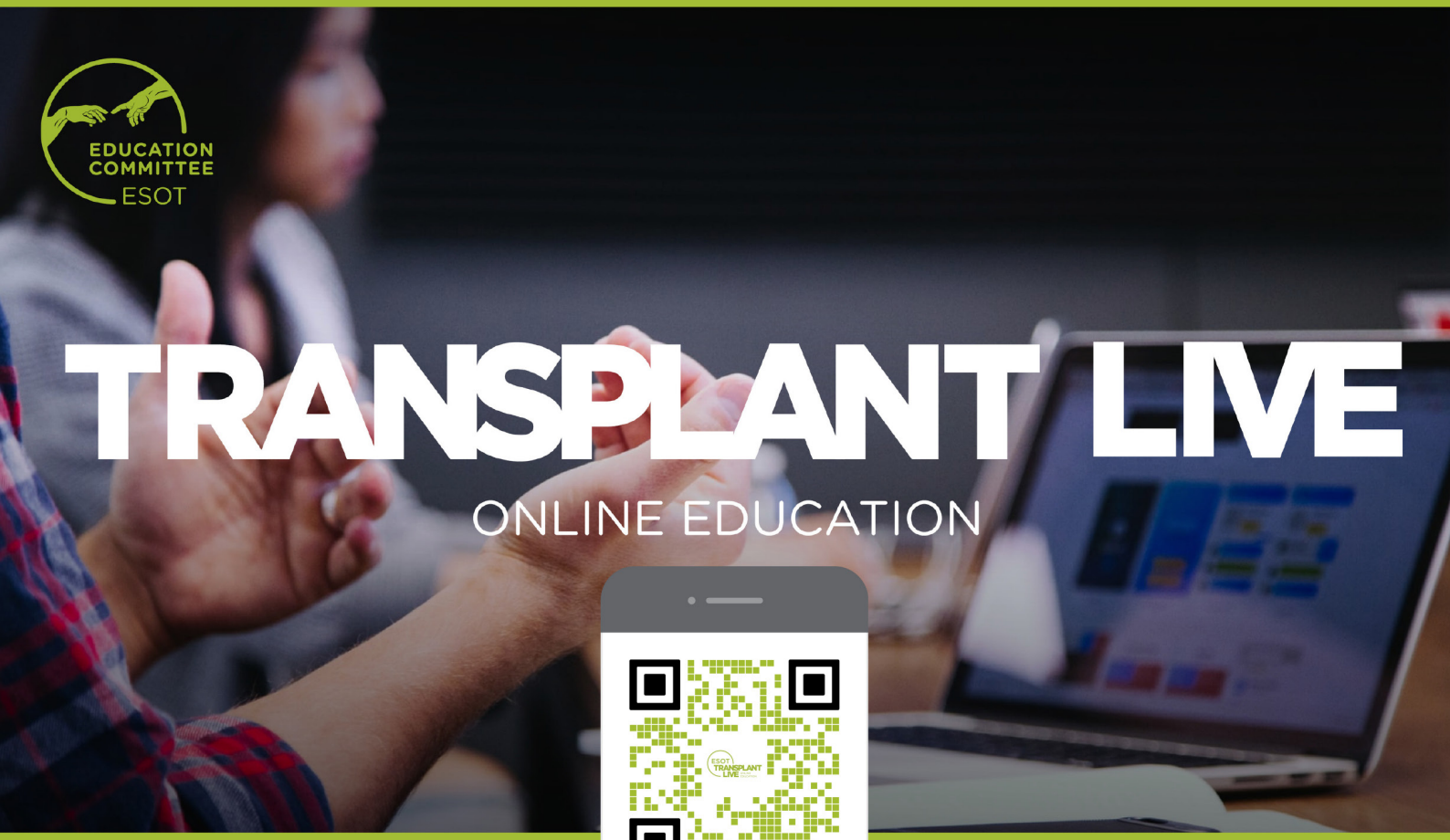

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ESOT | Transplant International

EDITORIAL
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Editorial: Transplant International Goes for GOLD!

Maria Irene Bellini[1], *Nuria Montserrat*[2], *Maarten Naesens*[2], *Thomas Neyens*[3], *Stefan Schneeberger*[2] *and Thierry Berney*[4]*

[1]Social Media Editor, Transplant International, [2]Deputy Editor-in-Chief, Transplant International, [3]Statistical Editor, Transplant International, [4]Editor-in-Chief, Transplant International

Transplant International is starting the New Year with a new publisher. After a rigorous review process, Frontiers Partnerships was considered the best fit four our mission and was selected based on their high quality performance and their enthusiasm for active engagement in our journal (1,2).

One key criterion was our determination to move to a Gold Open Access model. The publication of medical science in open access format has been growing over the past decade, on what is a seemingly irreversible path. Indeed, in 2020, the number of papers published in open access exceeded for the first time those accessible by subscription only (3). We believe that open access publication is part of the dynamic process of open research, which starts with the publication of research in pre-print servers while the manuscript undergoes revisions and improvements, and ends with the granting of full access to source data, for the sake of transparency, reproducibility of experiments, and the fostering of more rigorous science. These considerations are embodied in the FAIR guiding principles for scientific data management - Findability, Accessibility, Interoperability, and Reusability- (4,5) to which Transplant International explicitly adheres.

Open access publishing has become a general request from academic institutions to their scholars, but also, and more compellingly, from most funding agencies who require that all scientific outputs resulting from their grants be made freely available to all (6,7). Open access publishing cannot exist without payment of an author publication fee, which may sometimes generate some frustration, but is covered by an increasing number of funding agencies and institutions.

However, there is much more to open access than the ethics of open science, institutional requests or publication costs. The common goal for all stakeholders in the scientific publication process is to disseminate research and increase its overall quality, acknowledgment, visibility and notoriety. Depending on perspective, a variety of metrics are available. They are useful benchmarking indicators, designed to measure different types of impact. Although often confused, they are not interchangeable. The Impact Factor (IF) is an indicator of where a specific journal is standing in the landscape of scientific titles, but not of a particular article or author. Usage metrics for articles (downloads, views, engagements and captures) or the h-index for authors were designed for this purpose (8). Most of these metrics are driven by citations in the scientific literature and will increase through a higher rate of citations.

Evidence indicates that open access publication confers a citation advantage, at least in selected fields of medical science (9-13). The citation advantage is beneficial to all parties involved, and in particular the authors, but also the academic institutions (14) and the journal (15,16).

Social media have emerged to a leading position among the tools of fast dissemination of scientific output (17,18). They are widely used for this purpose by investigators, but also by social media editors of scientific journals (18). Alternative metric scores (such as Altmetric or PlumX) comprehensively assess usage, captures, mentions, social media posts and citations, but also new categories -such as clinical or policy citations, news articles or mentions, blog posts, comments, reviews or links-that indicate active engagement and repetitive interactions with the public (8). They give an interesting idea of the immediate "social" attention gained by a particular scientific publication, as opposed to the

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h-index which takes years to build for a particular author (8). They measure its impact in the web community, and very often positively influence its future citations (13). There is in fact growing evidence for a link between citations, altmetric scores and open access, at least in certain fields of biomedical research (18-20).

Transplant International is starting this year with a lot of ambitions (1,2), that we believe will be better served by our choice to go for gold. We are confident that our readership, but also the authors submitting their valuable work to Transplant International, will embrace this choice and, as we thank you for your trust, we offer you our best wishes for a successful 2022. Happy New Year !

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 TRIAL WATCH
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Transplant Trial Watch

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Keywords: kidney transplantation, randomised controlled trial, cytomegalovirus, mesenchymal stromal cells, prophylaxis

Randomised Controlled Trial 1

Immunoguided Discontinuation of Prophylaxis for Cytomegalovirus Disease in Kidney Transplant Recipients Treated with Antithymocyte Globulin: A Randomized Clinical Trial
by Paez-Vega, A., et al. Clinical Infectious Diseases 2021 [record in progress].

Randomised Controlled Trial 2

Autologous Bone Marrow-Derived Mesenchymal Stromal Cell Therapy With Early Tacrolimus Withdrawal: The Randomized Prospective, Single-Center, Open-Label TRITON Study
by Reinders, M. E. J., et al. American Journal of Transplantation 2021; 21 (9): 3055–3065.

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

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RANDOMISED CONTROLLED TRIAL 1

Immunoguided Discontinuation of Prophylaxis for Cytomegalovirus Disease in Kidney Transplant Recipients Treated with Antithymocyte Globulin: A Randomized Clinical Trial

by Paez-Vega, A., et al. Clinical Infectious Diseases 2021 [record in progress].

Aims

This study aimed to assess if it is safe and effective to terminate antiviral prophylaxis when cytomegalovirus (CMV)- specific cell-mediated immunity (CMI) is detected following induction treatment and to continue with preemptive therapy (immunoguided prevention), in renal transplant patients.

Interventions

Participants were randomly assigned to either immunoguided prevention or fixed-duration prophylaxis.

Participants
One-fifty CMV-seropositive kidney transplant patients.

Outcomes
Incidence of CMV disease and replication.

Follow-up
Tweleve months

CET Conclusion
This non-inferiority design trial randomized kidney transplant recipients to either fixed-duration CMV prophylaxis, or CMV cell-immunity guided prophylaxis. The authors report both strategies to be equivalent, supporting the idea that prophylaxis can be terminated early in patients with restored cellular immunity to CMV. Immunoguided prophylaxis resulted in less neutropenia. The study design is robust and provides good evidence that CMI-guided prophylaxis is safe and effective in this low-risk population of CMI positive and seropositive patients. Future studies will be needed to evaluate generalizability to other populations, and to establish cost-effectiveness.

Jadad Score
3.

Data Analysis
Per protocol analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov—NCT03123627

Funding Source
Non-industry funded.

Aims
The aim of this post hoc analysis was to investigate the effect of mesenchymal stromal cell (MSC) therapy with early tacrolimus withdrawal in renal transplant patients.

Interventions
Participants in the original trial were randomised to either MSC plus early tacrolimus withdrawal or to standard tacrolimus dose.

RANDOMISED CONTROLLED TRIAL 2

Autologous Bone Marrow-Derived Mesenchymal Stromal Cell Therapy With Early Tacrolimus Withdrawal: The Randomized Prospective, Single-Center, Open-Label TRITON Study

by Reinders, M. E. J., et al. American Journal of Transplantation 2021; 21 (9): 3055–3065.

Participants
Seventy living donor kidney transplant recipients.

Outcomes
The primary outcome was quantitative assessment of interstitial fibrosis. The secondary outcomes were patient death, graft loss, acute rejection, renal function, adverse events, and immunological responses.

Follow-up
Five years.

CET Conclusion
This is a good quality and fair sized randomised controlled trial in renal transplantation. Patients received alemtuzumab induction therapy and then were maintained on prednisolone and tacrolimus. In the study arm, patients also received autologous mesenchymal stem cell (MSC) infusions and then had tacrolimus minimisation and subsequent withdrawal. The intention was to see if fibrosis could be reduced through tacrolimus withdrawal, using MSCs to reduce the risk of rejection in this context. Randomisation was performed by an online system and is likely to be truly random, however the nature of the intervention means that the study was not easily blinded and there is the potential for bias. However, pathologists examining the biopsies were blinded to the allocation and used standardised scoring, which is an important strength of the study. Withdrawals and dropouts are adequately described and the statistical methods are appropriate. The analysis was however not by strict intention-to-treat; one in 12 patients allocated to the study arm had abnormal MSC growth and could not receive that intervention so were excluded from the analysis for example. There were four patients in the control arm who refused to have a follow up biopsy and so were also excluded. These seem small numbers, but in a small trial are significant. The overall fibrosis scores and progression of fibrosis was the same in both arms of the study. Renal function was similar and risk of acute rejection was similarly low between the study arms. There was a significantly higher number of Tregs in the MSC group. A post hoc analysis of 5-years outcomes is presented, but does not indicate any significant differences. The study was too small to identify any significant difference in graft or patient survival. In conclusion, the use of MSC was safe within this study and was not associated with increased risk of rejection when combined with tacrolimus withdrawal.

Jadad Score
3.

Data Analysis
Per protocol analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov—NCT02057965.

Funding Source
Industry funded.

CLINICAL IMPACT SUMMARY

This study from the Netherlands is a good quality randomised controlled trial in renal transplantation and it supports the ongoing investigation of mesenchymal stem cells (MSC) as a potential component of immune suppression regimens.

Renal transplant recipients in the trial received alemtuzumab induction therapy and then were maintained on prednisolone and tacrolimus. In the study arm patients also received two infusions of autologous mesenchymal stem cells (MSCs) and then progressed to tacrolimus minimisation and subsequent withdrawal. The intention was to see if fibrosis could be reduced through tacrolimus withdrawal, using MSCs to safely reduce the risk of rejection in this context. The study was necessarily open-label to the patient and clinicians. However, pathologists examining the biopsies were blinded to the allocation and used standardised scoring, this is an important strength of the study.

Blinded assessment of biopsy scores was similar for both groups and showed similar progression over 24 weeks. There was only one episode of acute rejection in the MSC group on for-cause biopsy and none in the control arm. This was present in a patient on reduced immune suppression due to BK virus infection. There was no graft or patient loss in either arm, but the study was too small to really assess for these outcomes. Protocol biopsies showed a mixture of TCMR and ABMR in three to four patients in each study group during the study period. There were no serious adverse events directly related to the infusion of MSCs and the overall adverse event rate was similar between the study arms.

Whilst there was no statistically significant difference between the groups in terms of most leukocyte cell lines quantified, there was a significant increase in Tregs in the MSC group that persisted up to 52 weeks after transplantation.

The study was too small to identify any significant difference in graft or patient survival, particularly at later timepoints. In conclusion, the use of MSC was safe within this study and was not associated with increased risk of rejection when combined with tacrolimus withdrawal. Whilst there was no apparent difference in fibrosis on biopsy scores, the increase in Tregs is intriguing and there is a potential to see improved GFR at longer follow up in a larger study. This is an exciting potential avenue to improve long-term allograft survival and warrants further exploration in a larger study.

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ORIGINAL RESEARCH
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Bio-Engineering of Pre-Vascularized Islet Organoids for the Treatment of Type 1 Diabetes

Charles-Henri Wassmer[1,2,3†], Fanny Lebreton[1,2,3†], Kevin Bellofatto[1,2,3], Lisa Perez[1,2,3], David Cottet-Dumoulin[2,3†], Axel Andres[2†], Domenico Bosco[2,3], Thierry Berney[1,2†], Véronique Othenin-Girard[4†], Begoña Martinez De Tejada[4†], Marie Cohen[4], Christina Olgasi[5], Antonia Follenzi[5†], Ekaterine Berishvili[1,2,3,6†] and the VANGUARD Consortium[‡]*

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Lack of rapid revascularization and inflammatory attacks at the site of transplantation contribute to impaired islet engraftment and suboptimal metabolic control after clinical islet transplantation. In order to overcome these limitations and enhance engraftment and revascularization, we have generated and transplanted pre-vascularized insulin-secreting organoids composed of rat islet cells, human amniotic epithelial cells (hAECs), and human umbilical vein endothelial cells (HUVECs). Our study demonstrates that pre-vascularized islet organoids exhibit enhanced *in vitro* function compared to native islets, and, most importantly, better engraftment and improved vascularization *in vivo* in a murine model. This is mainly due to cross-talk between hAECs, HUVECs and islet cells, mediated by the upregulation of genes promoting angiogenesis (*vegf-a*) and β cell function (*glp-1r*, *pdx1*). The possibility of adding a selected source of endothelial cells for the neo-vascularization of insulin-scereting grafts may also allow implementation of β cell replacement therapies in more favourable transplantation sites than the liver.

Keywords: regenerative medicine, tissue engineering, β cell replacement therapies, prevascularized iset organoids, human amniotic epithelial cells, HUVECs

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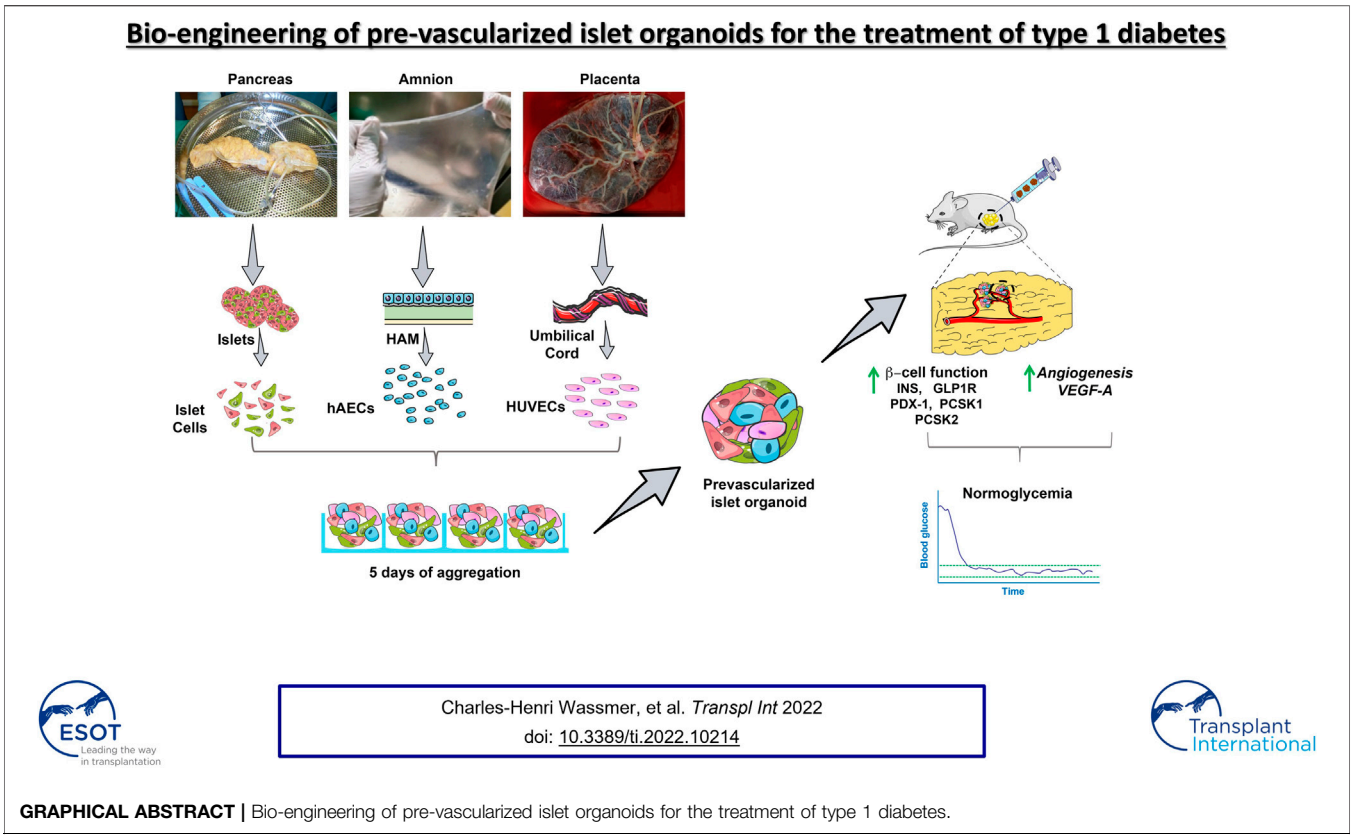
INTRODUCTION

Allogenic transplantation of pancreatic islets is a cell therapy option that holds great promise in the treatment of type 1 diabetes. The development of the Edmonton protocol has drastically increased the success rate of islet transplantation, and has proven to be able to achieve insulin independence in patients with type 1 diabetes (1). Most importantly, pancreatic islet transplantation confers a significant improvement in glycemic control and prevents life-threatening severe hypoglycaemia (2). Despite its efficacy, clinical islet transplantation is facing a number of challenges that limit achievement of steady functional success comparable to whole organ transplantation (3). One of the major challenges is the suboptimal long term graft function caused by the loss of the large portion of intraportally transplanted islets due to the IBMIR reaction, pro-inflammatory microenvironment, low oxygen tension in the liver, impaired vascularization and immunosuppressive drug toxicity (3). Therefore, the search for a suitable alternative transplantation site is a major focus of research in the



GRAPHICAL ABSTRACT | Bio-engineering of pre-vascularized islet organoids for the treatment of type 1 diabetes.

field. Other limiting factors hampering the widespread application of islet transplantation are shortage of donor organs and need for lifelong immunosuppression (4). Xenogenic islets and stem cell-derived beta cells are the two major potentially unlimited sources of insulin-producing tissue (5).

In recent years, substantial progress has been made in generating and characterizing functional stem cell-derived beta cells, which will undoubtedly change the way we will treat type 1 diabetes (6). The first attempts of clinical application of microencapsulated porcine islets or stem cell-derived endocrine tissue incorporated into macrodevices have already taken place (7, 8) and re-enforce the need to identify a site as functional as portal vein infusion but allowing easy graft removal—a site that to date this remains clinically elusive.

Despite the fact that islets represent only 1–2% of pancreatic tissue volume, they receive 10–15% of the total pancreatic blood flow (9). Each islet possesses 1 to 3 pre-arterioles (10), depending on islet size, that rapidly branch out into a multitude of fenestrated capillaries and form an important intra-islet micro-circulation that is five time denser than in the exocrine tissue (11). The cross-talk between endocrine and endothelial cells is vital for proper islet development, configuration and vascularization. Islet cells secrete vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1 in order to recruit endothelial cells (ECs) that are necessary for islet development, survival and function. On the other hand, ECs are involved in cell differentiation, insulin gene expression and cell segregation during embryogenesis (12, 13). In addition, they secrete components of the intra-islet basement membrane that are crucial for proper endocrine function (11).

Islet isolation and culture lead to the disruption of the islet capillary system, with significant loss of ECs due to de-differentiation or necrosis (14). In addition, islets vary in size, ranging from 50 to 400 µm in diameter. In the immediate post-transplantation period, avascular islets are supplied with oxygen and nutrients solely by diffusion until re-establishment of the blood flow, a process that can take about 2 weeks (9). Because of that, larger islets fail to engraft due to insufficient vascularization and subsequent necrosis (15). Significant efforts have been made to develop new strategies to minimize hypoxia-induced β cell death.

Several scientific groups, including our own, have demonstrated that re-aggregation of islet cells in combination with other cell types into homogeneous, round shaped and size-controlled spheroids leads to improvement of function and viability, thanks to heterotypic cell–cell interactions and reproduction of the complex natural morphology of the islet (16–20). In our previous studies, we have shown that incorporation of human amniotic epithelial cells (hAECs) into insulin-secreting organoids protected islet cells from oxidative stress *in vitro*, subsequently improving ß cell viability, function and engraftment (17, 20). Here, we propose an improved approach, in which we engineer pre-vascularized organoids that provide both control over their size and composition, and prompt re-establishment of the cross-talk between ECs and islet

cells, thereby facilitating graft revascularization after transplantation.

MATERIALS AND METHODS

Reagents and Antibodies

All reagents and antibodies used in this study are listed in **Supplementary Tables S1–S3**.

Animals

Animal experiments were performed in accordance with the Geneva veterinary authorities and approved by the Institutional Animal Care and Use Committee of the University of Geneva. Ten-week-old, pregnant female, Lewis rats were purchased from Janvier Laboratory (Le Genest St-Isle, France) and bred in our animal facility at the Geneva University. Fifteen-to 21-week-old male rats were used for pancreatic islet isolation. Six-to 9-week old male B6.129S7-Rag1^{tm1Mom}/J (abbreviated NOD–*Rag1*^{null} bred at Charles River Laboratories, Saint-Germain-Nuelles, France) mice were used as transplantation recipients. All animals were kept under conventional housing conditions with free access to water and food.

Human Tissues

Studies involving human tissues were approved by the Commission Cantonale d'Ethique de la Recherche (CCER; protocol PB_2017-00101), in compliance with the Swiss Human Research Act (810.30).

Placentas were obtained from women undergoing elective caesarean section of uncomplicated, term pregnancies. Informed, written consent was obtained from each donor prior to tissue collection.

Isolation and Culture of Human Umbilical Vein Endothelial Cells and Human Amniotic Epithelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated using a method adapted from a previously published protocol (21). Briefly, the umbilical vein was rinsed, then distended with Collagenase A solution (2 mg/ml) and incubated at 37°C for 12 min. Released cells were then collected by flushing the vein with cold HBSS supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B. Isolated HUVECs were plated in a 75 cm^2 flasks and cultured at 37°C, 21% O_2 and 5% CO_2 in M199 medium supplemented with 20% FBS, 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (1% of a L-Glutamin-Penicillin-Streptomycin stock solution), Fungin 0.1%, 30 µg/ml endothelial cell growth supplement and 100 µg/ml heparin. HUVECs from passage 2 to 7 were used in this study.

hAECs were isolated, cultured and characterized as described previously (10, 14). Freshly isolated hAECs were cultured in DMEM/F-12 medium, supplemented with 10% FBS, 2 mmol/l L-Glutamin, 100 U/ml Penicillin, and 0.1 mg/ml Streptomycin (1% of a L-Glutamin-Penicillin-Streptomycin stock solution, 1 mmol/L sodium pyruvate, 1% MEM NEAA 100X, 0.1% fungin, 0.05 mmol/L 2-mercaptoethanol, 10 ng/ml human recombinant epidermal growth factor (EGF). Only cells at passage 1 were used in this study.

Medium was changed every 48 h. Confluent cells were recovered by mild trypsinization and were cryopreserved for later utilization.

Rat Islet Isolation and Dissociation

Rat islets were isolated by enzymatic digestion (collagenase V) and purified using a discontinuous Ficoll gradient (22–24). Isolated islets were cultured (37°C, 5% CO_2) in DMEM medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, 0.1 mg/ml 1 mmol/L sodium pyruvate and 11 mmol/L glucose for 24 h. Islets were then dispersed into single islet cells (ICs) by incubation in 0.05% trypsin-EDTA (16).

Characterization of Human Umbilical Vein Endothelial Cells and Human Amniotic Epithelial Cells

HUVECs and hAECs were analyzed for expression of previously reported endothelial cell surface markers or specific amniotic epithelial cell surface markers by flow cytometry.

For analysis, cells (2.5×10^5) were stained by incubation for 30 min with primary or isotype control antibody in 100 µl PBS with 0.2% BSA, washed twice with PBS, and analyzed. Antibodies used for HUVECs were: AlexaFluor 657-conjugated anti-CD144 (1:40 dilution), PE-conjugated anti-CD31 and PerCP-Cy 5.5-conjugated anti-CD45 (1:25 dilution). Antibodies used for hAECs were: FITC-conjugated anti-human CD105 (clone 266), BV421-conjugated anti-human CD326 (clone EBA-1), PerCP-Cy5.5 conjugated anti-SSEA4 (clone MC813-70) (1:50 dilution), PE-Cy7 conjugated anti-human CD90 (clone 5E10; 1:100 dilution), PE-conjugated anti-human HLA-E (clone 3D12) and APC-conjugated anti-human HLA-G (clone 87G; 1:20 dilution).

Flow cytometry analysis was performed on a Gallios cytometer using the Kaluza Analysis software.

HUVECs were further characterized by immunostaining. Immunofluorescent assessment was performed on the cells cultured on gelatine-coated glass coverslips. Fixed cells were washed, permeabilized and stained with the following primary antibodies: mouse anti-CD31 (1:50 dilution), rabbit anti-von Willebrand factor (1:100 dilution) and mouse anti-vimentin (1:50 dilution). Cells were then incubated with corresponding Alexa Fluor and FITC-conjugated secondary antibodies. For nuclear counterstaining samples were mounted with aqueous solution containing 4,6 diamidino-2-phenylindole (DAPI).

Functional Assessment of Human Umbilical Vein Endothelial Cells *In Vitro*: Tube Formation Assay

The tube formation assay was performed according to manufacturer's protocols of Corning® Matrigel® Matrix.

Briefly, Matrigel thawed overnight at 4°C was mixed with VEGF (200 ng/ml) and 250 µl of matrix was added to each well osf 24-well plates. After 1 h of incubation at 37°C, cells (8×10^4) were seeded onto the Matrigel and tube formation of HUVECs was observed and photographed using an inverted phase-contrast microscope during 6 h.

Lentiviral Transduction

Lentiviral vector carrying the green fluorescent protein (GFP) under the control of an endothelial specific promoter Vascular endothelial cadherin (VEC/Cdh5) (LV-VEC.GFP) was provided by Prof. A. Follenzi (Università del Piemonte Orientale). HUVECs were transduced with LV-VEC.GFP at passage 3 using a multiplicity of infection (MOI) of 10 (MOI = 10). Transduction efficiency was assessed by fluorescent microscopy and flow cytometry and considered successful when at least 80% of cells showed expression of GFP.

Generation of Pre-Vascularized Islet Organoids

Pre-vascularized islet organoids (PIO) were generated on AggreWell™400 24-well plates by seeding mixture of ICs, HUVECs and hAECs at a ratio of 5:4:1 (800 cells/organoid). Undissociated native islets (NI), ICs spheroids (400 ICs/spheroid), hereafter referred to as pseudo-islets (PI), and IC: HUVEC spheroids (ratio 1:1, 800 cells/spheroid), hereafter referred to as IC + HUVEC served as controls. PIO, PI and IC + HUVEC were cultured for 4 days to allow cell aggregation at 37°C, 21% O_2 and 5% CO_2.

Culture medium for PIO was prepared by mixing equal volumes of complete DMEM, DMEM/F12 and M199 medium, hereafter referred to as organoid medium. IC + HUVEC were cultured in the mixture of complete DMEM and M199 medium at the ratio 1:1. Finally, PI and NI were cultured in complete DMEM medium. Culture medium was changed every other day. Mean diameter of NI, PIO and PI were calculated on the images taken on light microscope using ImageJ software.

In order to observe PIO composition and cell distribution during culture, fluorescent carbocyanine dyes CM-DiL (red) prelabeled hAECs and GFP transduced HUVECs were used. Pictures were taken using an epifluorescent microscope (DMi8 manual microscope).

PIO, PI and NI were collected fixed in formalin and embedded in paraffin. Serial sections of 5 µm were cut and processed for immunofluorescent staining. Slides were stained with the following primary antibodies: guinea pig anti-insulin (1:100), chicken anti-GFP (1:500), and rabbit anti-CK-7 (1:100). The following secondary antibodies were then applied: donkey anti–guinea pig Alexa 555 Fluor-conjugated (1:300), donkey anti–guinea pig FITC-conjugated (1:200), donkey anti-mouse AMCA-conjugated (1:50), goat anti-chicken Alexa Fluor 488 (1:500).

Organoids Sprouting Assay

One hundred PIO were resuspended in a collagen solution, transferred into prewarmed 24-well plates and allowed to gelify for 30 min. Next, 0.1 ml organoid medium supplemented with VEGF-A at the concentration of 200 ng/ml was pipetted on top of each hydrogel containing PIO. The hydrogels were cultured for 24 h at 37°C, 5% CO_2, and 100% humidity. As control, one hundred IC + HUVEC spheroids and PI were cultured in the same way in the hydrogel.

In Vitro Functional Assessment

To assess functional capacity, 300 NI and an equivalent number of PIO and PI, were incubated in duplicates for 1 h at 37°C in Krebs–Ringer solution containing low glucose (2.8 mmol/L) in order to equilibrate the samples. After a change of medium, islets and aggregates were incubated at 37°C for another hour in Krebs–Ringer solution containing low glucose (2.8 mmol/L), followed by 1 h at high glucose (16.7 mmol/L). Supernatants were collected and stored at −20°C. Insulin concentration in supernatants was measured using a rat insulin ELISA kit and normalized to the total insulin content. Results are expressed as the ratio between insulin secreted in high glucose to low glucose, referred to as stimulation index (SI). In addition, total insulin content per IC was measured by dividing the total insulin content by the number of ICs present in the NI, PI and PIO.

Diabetes Induction and Xenogeneic Transplantation

Three days before transplantation mice were subjected to intraperitoneal injection of STZ (180 mg/kg). Non-fasting blood glucose levels were then checked daily using a portable glucometer. Only mice with blood glucose levels over 18 mmol/L for 3 consecutive days were used in this study. Glycemia readings over 28 mmol/L, indicated as "high" on glucometer, were recorded as 30 mmol/L.

A marginal mass of 300 islet equivalents (IEQ) for NI and 1200 PIO, PI and IC + HUVEC were transplanted. Number of organoids was based on the average number of islet cells per IEQ, previously estimated as 1,560 ICs/IEQ (25).

At the day of transplantation, NI and engineered constructs were recovered from culture, packed in PE50 tubing and transplanted into the epididymal fat pad (EFP) of diabetic mice. Non-fasting glucose was assessed daily during the first week and 3 times per week thereafter. Normoglycemia was defined as two consecutive blood glucose levels under 11.1 mmol/L.

Graft Metabolic Function Assessment

Graft capacity to clear glucose *in vivo* was assessed dynamically by intraperitoneal glucose tolerance test (IPGTT) at 30 days after transplantation. Mice were fasted for 6 h and intraperitoneally injected with 2 g of glucose/kg. Blood glucose measurements were taken at 0, 15, 30, 45, 60 and 120 min.

Lectin Injection

Functional graft vasculature was assessed by infusing DyLight 594-conjugated Lycopersicon Esculentum (Tomato) lectin into the beating left ventricle of mice hearts. Mice were injected with 100 µl of undiluted lectin. Lectin was allowed to circulate for 1 min. Then, the right ventricle was cut to allow blood flow decompression and a volume of 3 ml of PBS was injected into the

left ventricle, followed by 1 ml of 4% PFA. The graft bearing EFPs were collected and fixed overnight in 4% PFA at 4°C. They were then maintained in 30% sucrose at 4°C until used for histology.

Immunohistological Assessment of Recovered Grafts

Grafts were recovered, fixed in formalin and embedded in paraffin. Serial sections of 5 µm were cut and processed for immunofluorescent staining. Tissue samples were permeabilized with 0.5% Triton X-100/PBS for 30 min, followed by 1-h incubation in 0.5% BSA/PBS at room temperature to block unspecific sites. Slides were then incubated with the following primary antibodies: guinea pig anti-insulin (1:100), rabbit anti-CD34 (1:2,000), chicken anti-GFP (1:500), and rabbit anti-VEGF (1:100). The following secondary antibodies were then applied: donkey anti–guinea pig Alexa 555 Fluor-conjugated (1:300), donkey anti–guinea pig FITC-conjugated (1:200), donkey anti-rabbit Alexa 555 Fluor-conjugated (1:300) and goat anti-chicken Alexa Fluor 488 (1:500). Both primary and secondary antibodies were diluted in PBS-0.5% BSA. Finally, slides were mounted with aqueous mounting medium containing DAPI for nuclear staining. Slides were processed on a Zeiss Axioscan.Z1 slide scanner and a Zeiss Axiocam. To analyse vascularization, six pictures per condition were taken and the number of $CD34^+$ cells were counted and normalized by the graft area.

Morphometric analysis was performed using Zen 2.3 Blue Edition software.

Real-Time Quantitative PCR

Graft bearing EFPs recovered at 3 and 30 days after transplantation were processed for PCR analysis. RNA was extracted using the RNeasy minikit and reverse transcribed with a High Capacity cDNA Reverse transcription kit. Gene amplification was performed by RT-PCR using TaqMan Fast Advance Master Mix. Primers used for amplification are listed in **Supplementary Table S4**. *RPLP1* was used as a housekeeping gene to normalize gene expression values. Data were calculated using the comparative cycle threshold Ct method ($2^{-\Delta Ct}$ method) and are expressed in arbitrary units.

Statistical Analysis

Continuous variables are expressed as mean ± SEM. Multiple comparisons were analyzed using one-way ANOVA followed by Dunnett multiple comparisons test while two-way comparisons were analyzed using the Student's t-test. Cumulative number of animals reaching normoglycemia was compared using the log-rank (Mantel-Cox) test. A p value ≤0.05 was considered statistically significant. All statistical analyses were performed with the Prism software 8.0.

RESULTS

Human Umbilical Vein Endothelial Cell Characterization and Transduction

HUVECs reached 80% confluence within 5 days with initial seeding density of 6,000 cells/cm^2. Morphologically, cells displayed typical elliptic shape (**Figure 1A**) and were positive for von Willebrand factor and CD31 (**Figure 1B**). Endothelial origin of the cells was additionally confirmed by flow cytometry. Cells were positive for CD31 and CD144 (97.8% ± 0.7 and 98.1% ± 0.6, respectively) and negative for CD45 (95.8%) (**Figure 1C**).

When cultured on Matrigel, HUVECs formed well-shaped vascular-like structures over a period of 6 h (**Figure 1D**).

To track HUVECs within organoids both *in vitro* and *in vivo*, cells were transduced with LVs carrying green fluorescent protein (GFP) gene under the control of the VEC promotor. HUVEC positivity for GFP was observed during culture and confirmed by flow cytometry 3 days after transduction with 86.6% of GFP+ cells (**Figure 1E** right and left panel, respectively).

Human Amniotic Epithelial Cells Characterization

hAECs used in this study were isolated from six different placentas. Flow cytometry analysis demonstrated strong positivity of hAECs for the embryonic cell surface marker SSEA-4 (88.4 ± 5.0%) and the epithelial cell adhesion molecule (CD326; 95.9 ± 1.3%). HLA-E and HLA-G were expressed in 16.9 ± 4.7% and 48.6 ± 12.3% of the cells, respectively. Finally, expression of CD105 and CD90 by hAECs were 17.6 ± 5.6%, 50.1 ± 7.1, respectively. The results of each hAEC preparation are described in **Supplementary Figure S1**.

Cellular Composition, Endocrine Function and Angiogenic Activity of Pre-Vascularized Islet Organoids

Generation of PIO and PI is described in **Figure 2A**. Aggregation and incorporation of the different cell types occurred within 4 days (**Figures 2B,C**). Mean diameter of NI, PI and PIO was 144.4 ± 6.6, 105.8 ± 1.2 and 134.3 ± 2.3 µm, respectively (**Figure 2D**). NI showed the biggest heterogeneity in size. PI exhibited a significantly smaller mean diameter in comparison with PIO ($p < 0.0001$), due to fewer cellular content. Cellular composition observed by fluorescent microscopy showed that all 3 cell types were present in the PIO (**Figure 2E**). The functional capacity of the constructs was evaluated by glucose-stimulated insulin secretion (GSIS) assay. PI and PIO demonstrated significantly improved insulin secretion in response of glucose stimulation (SI = 7.8 ± 1.5 and 7.7 ± 1.2), compared to NI (SI = 2.0 ± 0.5, $p = 0.013$ and $p = 0.014$, respectively). No significant difference was observed between PI and PIO (**Figure 2F**). In addition, total insulin content/IC was measured and compared between the three groups. PI and PIO demonstrated an increased insulin content/IC (0.01 ± 0.003 and 0.008 ± 0.002 pmol/L, respectively) in comparison with NI (0.002 ± 0.0004 pmol/L). These dramatic enhancement of static GSIS secretion in our constructs compared to unmodified native islets indicate that better oxygen and nutrient access, and improved transport of glucose and insulin, enhanced survival and function of PI and PIO. Our findings are consistent with previous reports on better *in vitro* performance of smaller pseudoislets (26, 27).

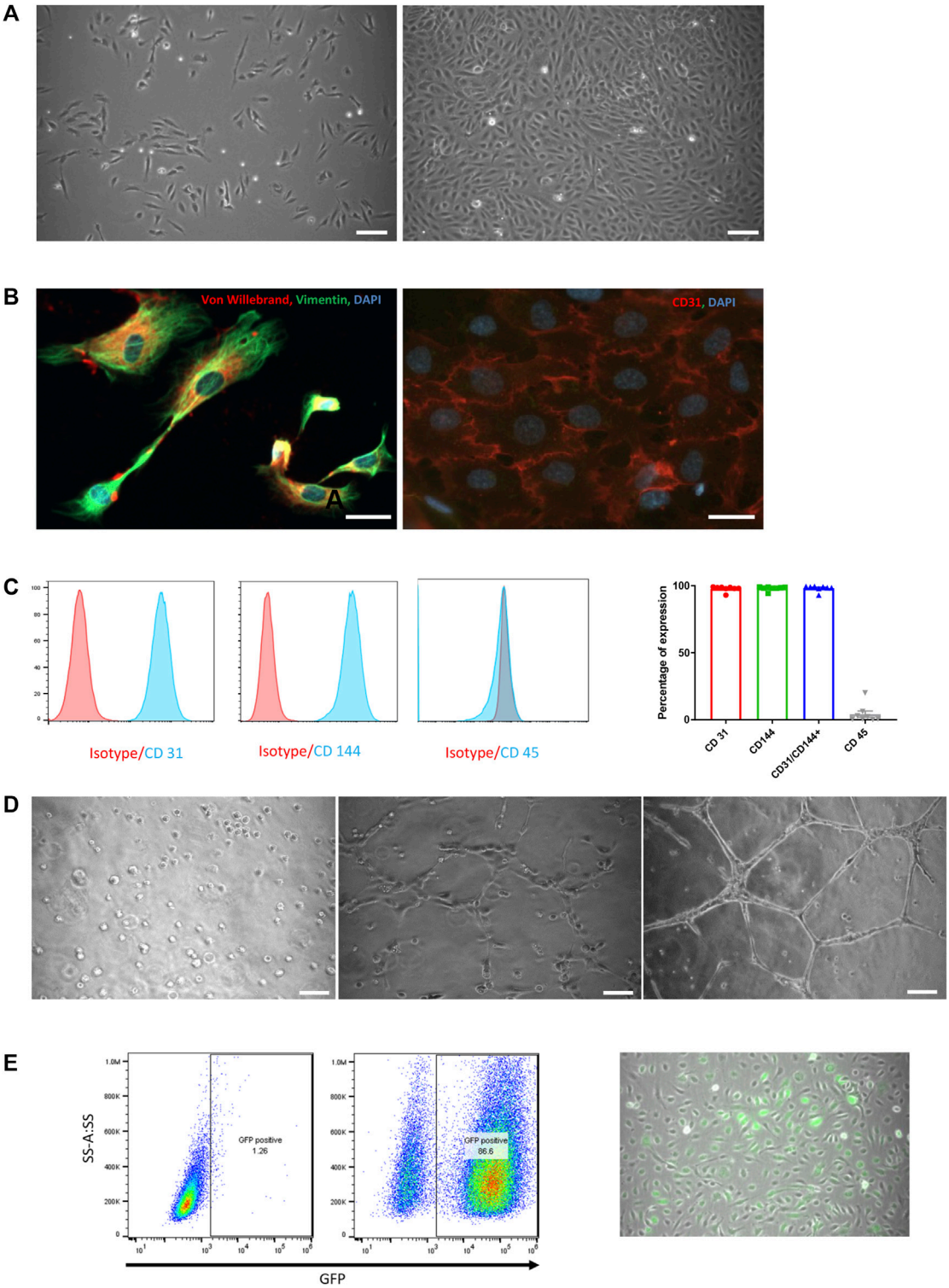


FIGURE 1 | HUVEC characterization and *in vitro* functional assessment. **(A)** Phase-contrast microscopic pictures of HUVEC in culture at day 1 and day 5. Scale bar = 50 µm. **(B)** Immunofluorescence staining of cultured HUVEC with von Willebrand (red) and Vimentin (green, left panel) and CD31 (red, **right panel**). Nuclei are labelled with DAPI (blue). Scale bar = 25 µm. **(C)** FACS analysis on HUVEC for CD31, CD144 and CD45 with their respective isotypes **(left panels)** and expressed as the percentage of positivity of expression on 8 consecutive preparations (mean ± SEM, **right panel**). **(D)** Phase-contrast microscopic pictures of tube formation assessment on Matrigel at 0 h, 2 and 6 h. Scale bar = 50 µm. **(E)** Assessment of GFP transduction success by flow cytometry analysis **(left panel)** and by phase-contrast microscopic images (right panel). GFP-positive cells are spontaneously green, scale bar = 50 µm.

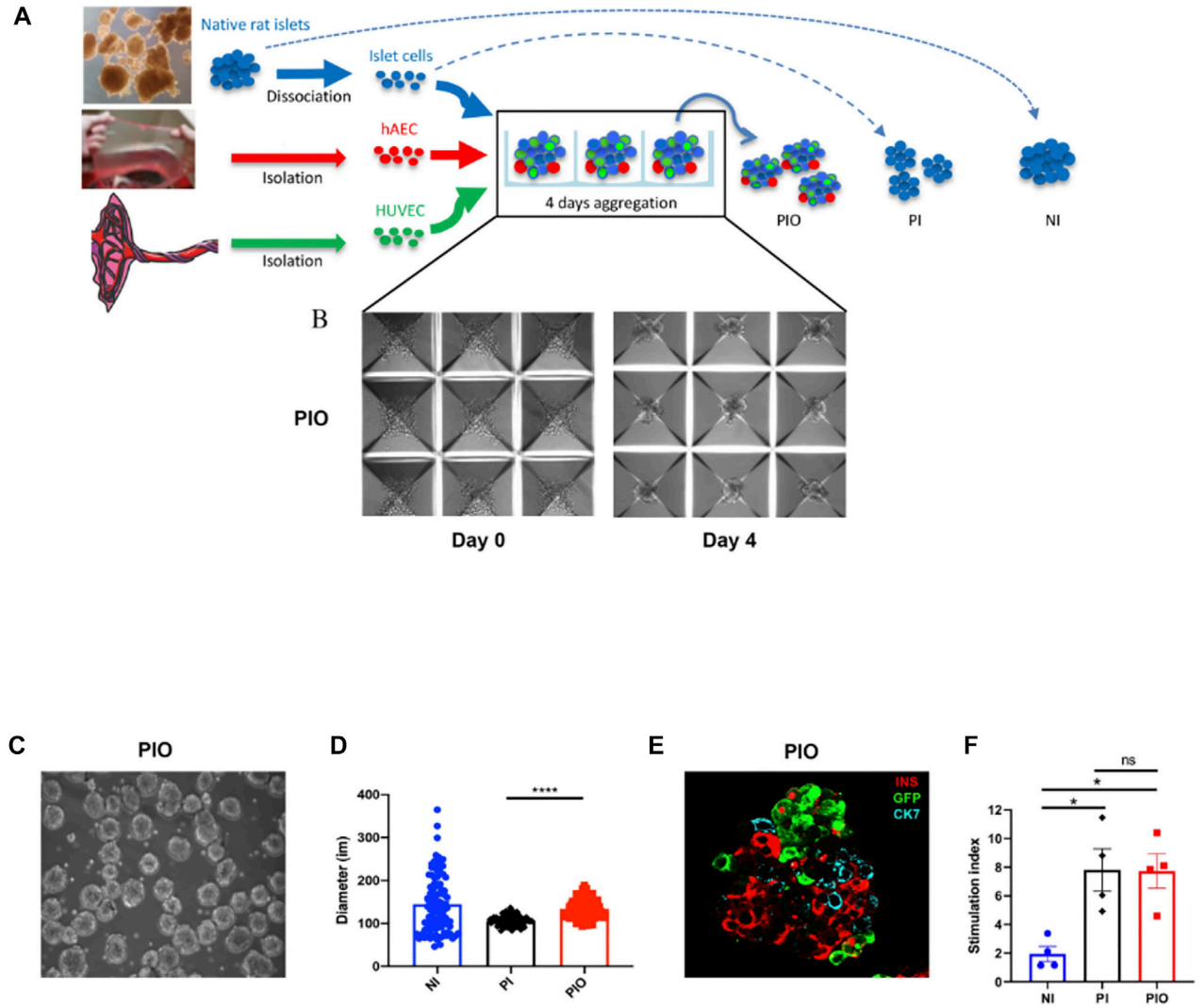


FIGURE 2 | Organoids generation. **(A)** Schematic representation of PI and PIO generation in culture. **(B)** Light microscope pictures of the PIO cultured in AggreWell™400 24-well plates at day 0 and day 4. Scale bar = 100 µm. **(C)** Light microscope pictures of the PIO after collection from the wells. **(D)** Average diameter of each condition calculated at 4 days of culture (n = 100/condition). **(E)** Representative immunofluorescence stainings of PIO. Islet cells are stained for insulin (red), HUVECs for GFP (green) and hAECs for CK7 (blue). Scale bar = 25 µm. **(F)** *In vitro* function assessed by GSIS and represented by the stimulation index (n = 4). All data are expressed as mean ± SEM. *p < 0.01, ***p < 0.001, one-way ANOVA with Dunnett's multiple comparison test.

To investigate the angiogenic potential of the PIO, collagen-based sprouting assays were performed. Our results demonstrated that PIO showed more extensive sprouting in surrounding matrix compared to IC + HUVEC (**Supplementary Figure S2**). In contrast, no sprouting was observed from PI (data not shown). Furthermore, immunofluorescence revealed GFP positive cells confirming their endothelial nature.

Pre-Vascularized Islet Organoids Improve Glycaemic Control in Immunodeficient Diabetic Mice

To assess whether incorporation of hAECs and HUVECs into the islet organoids could promote engraftment and function *in vivo*, diabetic NOD–*Rag1*^{null} mice were transplanted with a marginal mass of PIO (n = 14), NI (n = 13) and PI (n = 9). Mice transplanted with PIO demonstrated significant improvement of glycaemic control compared to both controls. Average blood glucose levels were significantly lower in the PIO group compared to NI and PI (**Figure 3A**). Normoglycemia was reached in 78.6% of animals (11/14) in the PIO group, in comparison with 55.6% (5/9) and 46.2% (6/13) for the PI and NI groups, respectively (**Figure 3B**). Median time to achieve normoglycemia was 6 days in the PIO group, 21 days in the PI group and >30 days in the NI group. To investigate secretory function of the graft, IPGTT was performed at 30 days post-transplantation. Mice transplanted with PIO and non-diabetic controls (NDC) showed lower blood glucose levels

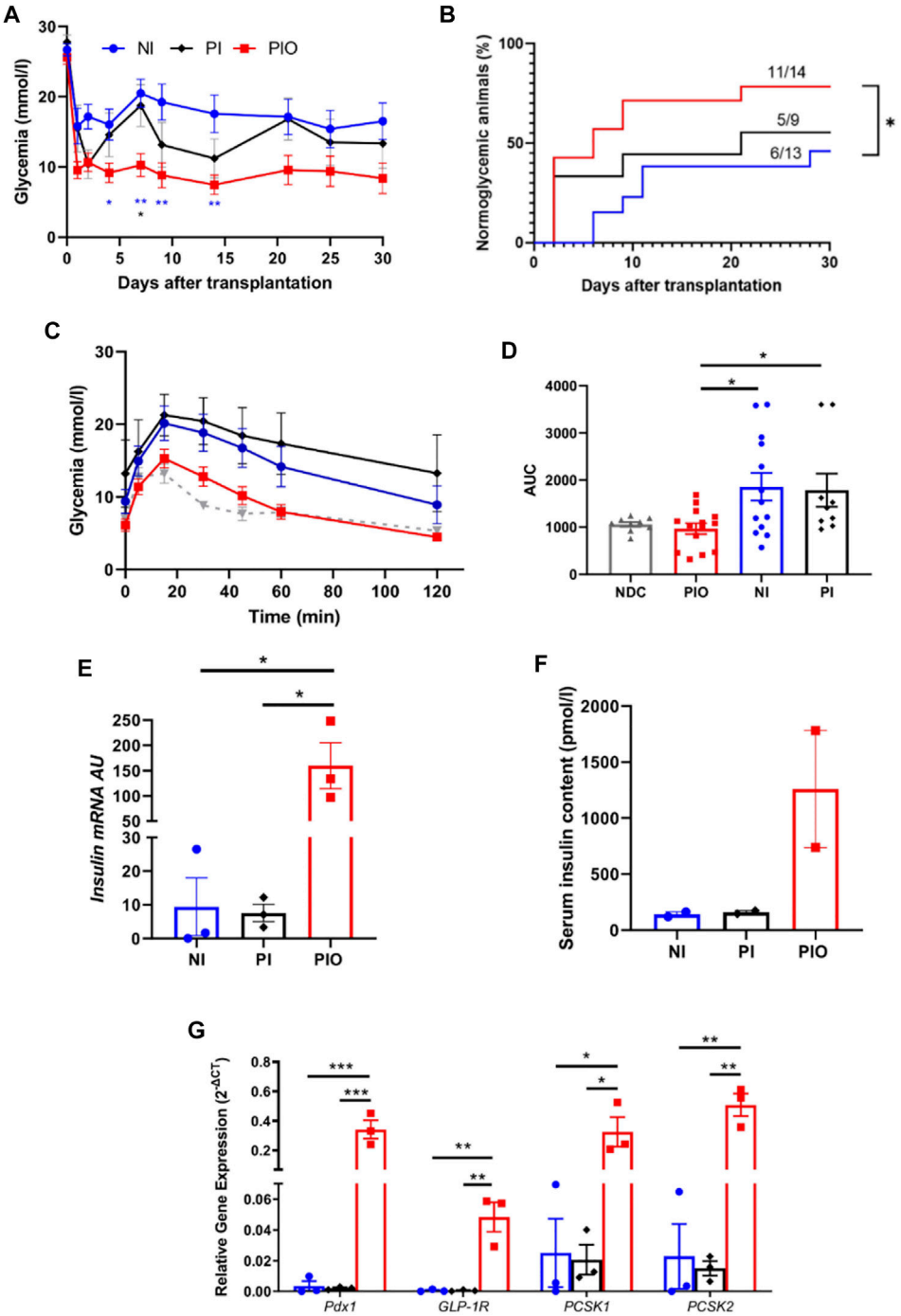


FIGURE 3 | *In vivo* function of organoids in immunodeficient, diabetic mice. **(A)** Glycemia level measured over 30 days in NOD-*Rag1*^{null} mice transplanted with 300 NI (n = 13, blue circle) and their equivalent number of PI (n = 9, black diamond) and PIO (n = 14, red square). Mean glucose level was compared at 4, 7, 9, 14, 21 and 30 days by a one-way ANOVA with Dunnett's multiple comparison test. All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01. **(B)** Cumulative number of mice reaching normoglycemia over 30 days. Comparison made using the log-rank (Mantel-Cox) test, *p < 0.05. **(C–D)** Glycemia level of each group during the intraperitoneal glucose tolerance test performed at 30 days post-transplantation **(C)** and their corresponding AUC values **(D)**. Grey triangles represent the non-diabetic control (NDC) group (n = 9). **(E)** Insulin mRNA expressed by NI, PI and PIO at 30 days post-transplantation; insulin mRNA was analyzed by qPCR, arbitrary units (AU) after normalization to housekeeping genes. Data shown are mean ± SEM, *p < 0.05, one-way ANOVA with Dunnett's multiple comparison test, n = 3. **(F)** Insulin concentration measured by ELISA in mice serum at 30 days post-transplantation. All data are expressed as mean ± SEM, one-way ANOVA with Dunnett's multiple comparison test, n = 2. **(G)** *pdx1, glp-1r, pcsk* and *pcsk2* expressed in PIO (red columns), PI (black columns) and NI (blue columns) at 30 days after transplantation, data presented as arbitrary units (AU) after normalization to housekeeping genes. Data shown are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and comparisons were made by a one-way ANOVA with Dunnett's multiple comparison test, n = 3.

when compared to animals transplanted with PI and NI (**Figure 3C**). This is illustrated by the increasing area under the curve (AUC) of the different groups, with PIO (966.8 ± 113.7), PI (1783 ± 351.1, $p = 0.05$ vs. PIO) and NI (1856 ± 294.5, $p = 0.014$ vs. PIO; **Figure 3D**).

We further investigated whether the improved glycemic control in the PIO group was associated with insulin production from the transplanted β cells. Remarkable upregulation of rat insulin mRNA levels in the graft was found in the PIO group in comparison to controls (PIO vs. PI, $p = 0.013$, PIO vs. NI, $p = 0.013$; **Figure 3E**). These results were supported by insulin measurements in the serum taken from the same mice (**Figure 3F**). Although a statistical significance wasn't achieved, a ten-fold increase in insulin levels was detected in the PIO group (1,259 ± 521 pmol/L), in comparison to both controls (NI: 140.6 ± 22.1 pmol/L, PI: 159.8 ± 14.4 pmol/L, p = ns).

Glp-1r, *pdx1* are known to be critical for promoting insulin secretion (28–31). Therefore, we investigated whether these genes were involved in the improved secretory outcomes of PIO. Gene expression analyses revealed upregulation of genes involved in β-cell function (*pdx1*, *pcsk1*, *pcsk2* and *glp-1r*) in PIO at 30 days post-transplantation, compared to controls (*pdx1*: PIO vs. PI, $p = 0.0009$, PIO vs. native islet, $p = 0.0009$; *glp-1r*: PIO vs. PI, $p = 0.002$, PIO vs. native islet, $p = 0.002$; *pcsk1*: PIO vs. PI, $p = 0.02$, PIO vs native islet $p = 0.021$ and *pcsk2*: PIO vs. PI, $p = 0.0005$, PIO vs. native islet, $p = 0.0006$; **Figure 3G**). Interestingly, at an earlier time points (3 days), a similar increase in gene expression was observed in PI and PIO in comparison with NI group, although without reaching statistical differences (**Supplementary Figure S3**). These data indicate that incorporation of accessory cells into the organoids supports long term secretory function of β cells.

Transplantation of Pre-Vascularized Islet Organoids Accelerates Graft Revascularization

To evaluate engraftment and revascularization, graft-bearing EFPs were removed at 30 days post-transplantation and processed for histology. Immunohistochemical staining for CD34, a marker for endothelial cells, showed that vessel density was significantly higher in the PIO samples (22.6 ± 3.5 CD34 + cells/cm^2) than in the NI samples (7.6 ± 0.9, $p = 0.002$; **Figures 4A,B**). Furthermore, in the PIO group, vessels were observed not only around graft, but mainly within β-cell positive area.

To investigate whether the blood vessels formed within the engrafted tissue constructs become functional and contribute to graft perfusion, we used intravascular injection of fluorescently labeled Lectin. Histological assessment of the Lectin-perfused grafts demonstrated the presence of functional Lectin positive vascular network within the PIO, in contrast only few vessels were present within NI (**Figure 4C**).

Next, we examined the mechanisms by which supportive cells (HUVECs and hAECs) contributed to rapid neovascularization of the graft. To this end, we investigated whether these cells might induce the production of angiogenic factors, such as *vegf-a* (**Figure 4D**). We observed, that rat *vegf-a* mRNA expression was significantly higher in PIO group (0.365 ± 0.033 AU) compared to NI (0.038 ± 0.005 AU; $p = 0.0006$) group. This finding was further confirmed by immunohistochemical staining for *vegf-a* of recovered samples, demonstrating higher fluorescent intensity in the PIO compared to NI (**Figure 4E**). These data indicate that incorporation of HUVEC and hAEC into PIO contribute to graft revascularization.

Human Amniotic Epithelial Cells Incorporation Into Organoids Improves Function and HUVEC-Derived Revascularization

Finally, we evaluated whether incorporation of hAECs into the organoids was essential for the engraftment and vascularization of the PIO. To this end, we added an additional group of mice transplanted with spheroids composed of IC: HUVEC (1:1 ratio) to the three existing groups.

Figure 5 summarizes the results obtained with this group. Blood glucose control was significantly lower in the IC + HUVEC group in comparison to the PIO group (**Figure 5A**). The IPGTT performed at 30 days post-transplantation demonstrated a poor glucose clearance in the IC + HUVEC group (**Figure 5B**). Response to increased blood glucose levels was significantly lower than for the PIO group as demonstrated by the AUC (2044 ± 578.1 vs. 966.8 ± 113.7, $p = 0.008$, respectively; **Figure 5C**).

After demonstrating that incorporation of supportive cells into the PIO improved graft revascularization, we investigated the degree to which these cells were contributing to new vessel development in the graft. To easily identify donor-derived new vessels, GFP-transduced HUVECs were incorporated into the PIO. Graft-bearing EFPs were recovered at 30 days post-transplantation and processed for immunohistological analysis. Interestingly, GFP positive cells were found inside the graft in the PIO group, while none was found in the IC + HUVEC group (**Figure 5D**). Both human and mouse vessels were positively stained by anti-CD34 confirming the establishment of anastomoses between donor derived HUVECs and mouse blood vessels. Furthermore, GFP/CD34 double positive endothelial cells were found at the graft periphery, inside capillaries containing erythrocytes, indicating that HUVECs were able to migrate and merge with a murine vascular system, forming functionally perfused blood vessels, as shown in **Figure 5E**. These data indicate that hAECs support HUVECs inside the organoids and thus contribute to accelerated revascularization.

DISCUSSION

Impaired and delayed revascularization of the graft is a major issue in islet transplantation and represents a main limitation to the search for extrahepatic sites for islet transplantation. Common vascularization strategies focus either on the combination of accessory cells with islets (32) or

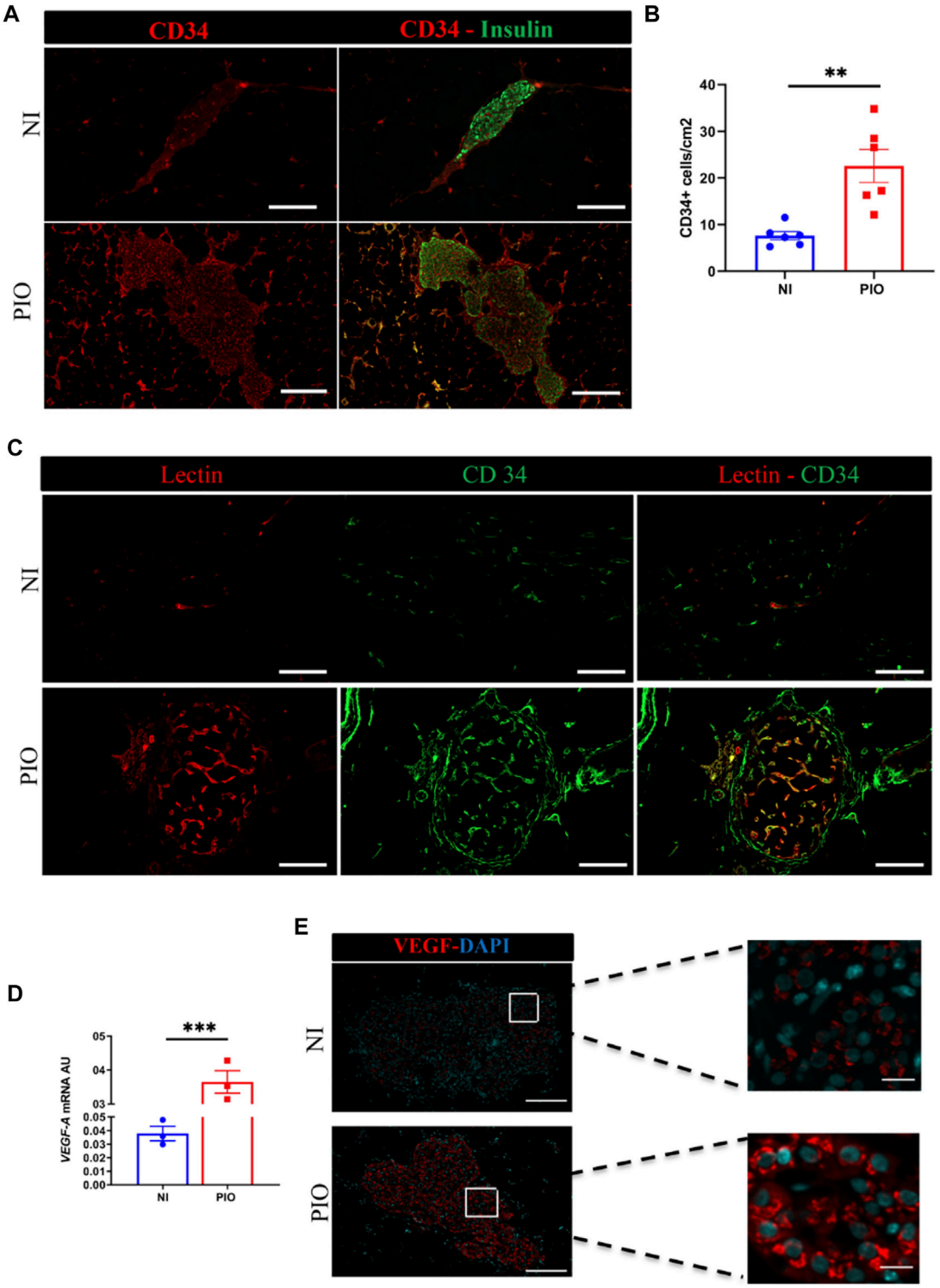


FIGURE 4 | *In vivo* revascularization assessment by immunohistological analysis. **(A)** The blood vessels of the graft detected at day 30 post-transplantation using CD34 (red) and insulin (green) immunostaining. Grafts Scale bar = 50 µm. **(B)** Quantitative analysis of revascularization was achieved by calculating the number of CD34 positive cells in the insulin positive area and the result was divided by the graft surface area. This was realized in two graft regions per mouse and in 3 mice per group. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, comparisons were made by a 2-tail unpaired Student *t* test. **(C)** Assessment of vessel functional capacity by mice injection of 100 µl of lectin. Capillaries are labelled in red and endothelial CD34+ cells in green. Scale bar = 50 µm. **(D)** *vegf-a* mRNA expression analyzed by qPCR at 30-days post-transplantation in PIO and NI groups; data presented as arbitrary units (AU) after normalization to housekeeping genes. Data shown are expressed as mean ± SEM. ***$p < 0.0006$, 2-tail unpaired Student t test, $n = 3$. **(E)** Recovered grafts stained for VEGF-A at day 30 after transplantation. Scale bars = 100 µm.

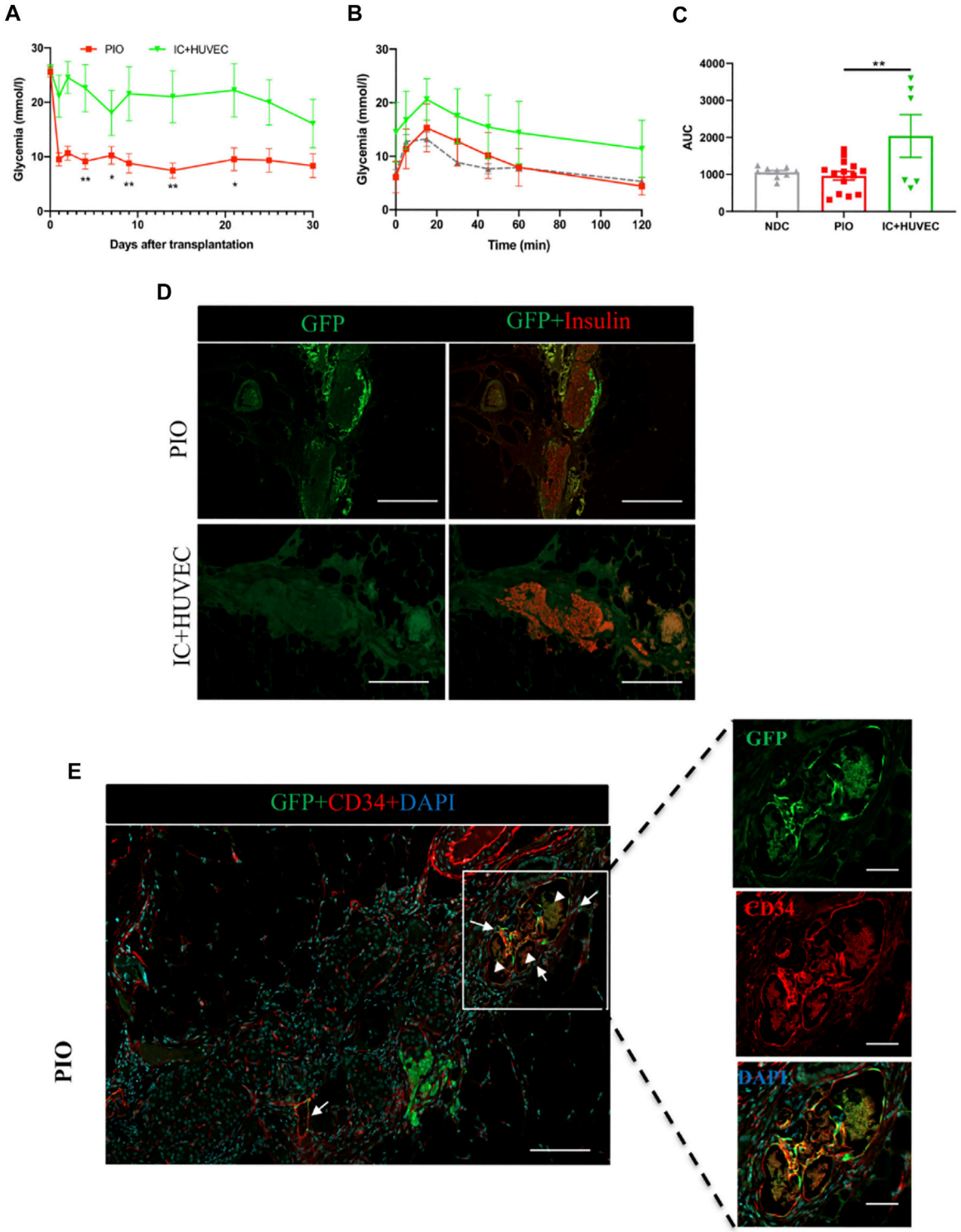


FIGURE 5 | *In vivo* function of IC + HUVEC spheroids, in immunodeficient, diabetic mice. **(A)** Mean glucose levels measured in NOD-*Rag1*^{null} mice transplanted with PIO (n = 14, red squares) and IC + HUVEC (n = 6, green inverted triangles). Mean glucose level was compared at 4, 7, 9, 14, 21 and 30 days post-transplantation by a 2-tail unpaired Student t test. All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01. **(B,C)** Intraperitoneal glucose tolerance test performed at 30 days post-transplantation and their corresponding AUC. Grey triangle represents the non-diabetic control (NDC) group (n = 9). Comparisons were made by a one-way ANOVA with Dunnett's multiple comparison test. All data are expressed as mean ± SEM. *p < 0.05, **p < 0.01. **(D)** Graft-bearing EFP recovered at 30 days post-transplantation and stained for GFP (green) and insulin (red). Scale bar = 100 µm. **(E)** Immunohistological staining for GFP (green), CD34 (red) and DAPI (blue). The yellow color represents the GFP-HUVECs with positive staining of anti-CD34. Arrows indicate chimeric blood vessels. Arrowheads indicate red blood cells. Scale bar for top panel = 100 µm and for the 3 bottom panels, 20 µm.

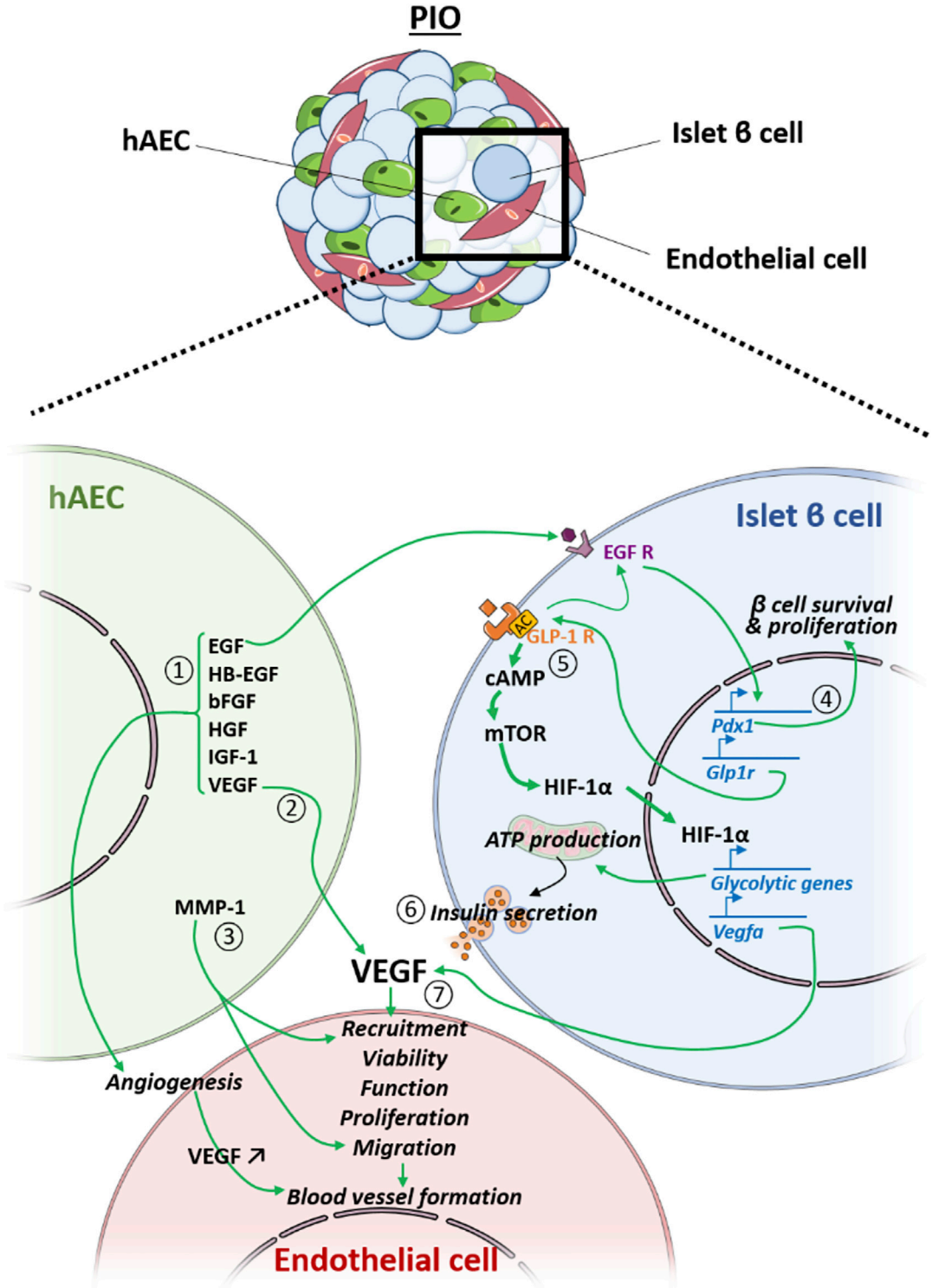


FIGURE 6 | Crosstalk between the hAEC, the endothelial cell (EC) and the islet β cell (IC) within the PIO. hAEC enhances revascularization of the PIO in a direct manner by secreting 1) angiogenic factors and 2) *vegf* that improve EC viability, function, proliferation and blood vessel formation, and 3) by producing ECM-degrading proteases (MMP-1) that facilitate EC migration and sprouting. Additionally, hAECs secrete EGF that 4) upregulates IC *pdx1* expression, leading to higher IC survival and proliferation, as well as 5) *glp1-r* expression, leading to an up-regulation of glycolytic genes and *vegf-a* through the mTOR/HIF-1a pathway, resulting in 6) an improved insulin secretion and 7) a better revascularization of the PIO.

incorporation of endothelial cells into islet-like constructs generated from embryonic stem cell-derived ß cells (30) or ß cell lines (31), and are mainly based on *in vitro* testing. In this study, we successfully generated functional pre-vascularized islet organoids using multiple cell types. The major finding of this study is that incorporation of hAECs and HUVECs into insulin-producing organoids hastens the rate of graft revascularization, and subsequently results in better engraftment of the β-cell mass.

HUVECs are the most commonly used, robust source of human endothelial cells in regenerative medicine and tissue engineering (33). However, limited proliferative potential of these cells hinders their clinical application. hAECs isolated from the amniotic membrane of discarded placenta is considered a non-controversial stem cell source (34). These cells demonstrated profound anti-fibrotic, anti-inflammatory, non-tumorigenic and low antigenic properties (35, 36). Furthermore, hAECs possess pluripotent stem cells characteristics, can be isolated in large quantities and are thus considered as an evolving therapeutic tool for the development of various clinical applications (35). Previously, we have shown that the generation of insulin-secreting organoids from primary IC in combination with hAECs improved islet engraftment and vascularization primarily by stimulating *VEGF-A* production from the graft *via* HIF1- α signaling pathway (17, 20). Therefore, in this study, we evaluated whether hAECs could accelerate the angiogenic potential of mature endothelial cells (HUVECs). Our results show that chimeric, prevascularized insulin secreting organoids are capable of establishing new vascular networks *in vitro* and *in vivo* when co-cultured with hAECs and HUVECs. The enhancement of the angiogenic potential of HUVECs by hAECs can be explained by three possible mechanisms: 1) *via* the secretion of ECM-degrading proteases facilitating EC migration and sprouting (37), 2) by up-regulating VEGF expression in endothelial and islet cells (38), and 3) by the reduction or suppression of inflammatory responses (39, 40). Our *in vivo* experiments have demonstrated the superiority of pre-vascularized islet organoids for insulin secretion and revascularization.

Another important finding is the existence of a cross-talk between the islet, endothelial and amniotic epithelial cells associated within one organoid (summarized in **Figure 6**), and that this communication can be successfully employed for improving outcomes of islet transplantation. In terms of revascularization, we observe that both blood vessel density and number of functional vessels were significantly higher in the grafts explanted from mice transplanted with PIO in comparison to control groups. *VEGF-A* is a proangiogenic factor that recruits endothelial cells and circulating endothelial progenitors (11). Our results demonstrated significant upregulation of *VEGF-A* gene expression in the grafts explanted from mice transplanted with pre-vascularized organoids. Immunohistochemical analysis of the explanted grafts confirmed that the major producers of *VEGF-A* were islet cells. This finding was in agreement with our previous studies, demonstrating that hAECs markedly increase production of *VEGF-A* in islet cells *via* paracrine signalling (17). In addition, hAECs themselves are known to secrete *VEGF-A* (41), which on the other hand could also enhance performance of HUVECs within the organoids. To verify this hypothesis, we used GFP-HUVECs and tracked transplanted cells inside the graft. We found GFP-HUVECs both inside and in the vicinity of the graft. At the same time, GFP-HUVECs were also detected to be integrated into the peri-islet functional blood vessels containing red blood cells. This indicates that the donor derived endothelial cells anastomosed with the murine vascular system and formed functionally perfused blood vessels. Interestingly, the same was not observed in mice transplanted with IC + HUVECs, in which no GFP-HUVECs were found in the recovered grafts. In addition, almost no blood circulation was observed inside the graft area. This indicates that hAECs contribute to the process of endothelial cell remodelling and stabilization finally leading to mature vessel formation. Our findings are in agreement with previously reported data, demonstrating that hAECs enhance EC viability, function, proliferation, migration and blood vessel formation *in vitro* and *in vivo* (41). Furthermore, amniotic cells secrete additional factors that are critical for angiogenesis, such as EGF, HB-EGF, bFGF, HGF, IGF-1 (42). Taken together, these data suggest that hAECs promote revascularization both directly by secreting angiogenic factors and indirectly by stimulating *VEGF-A* secretion by islet cells.

Accelerated revascularization can also provide important survival cues to the islet cells. Another important challenge to islet transplantation is to achieve stable, long-term insulin independence, preferably with single donor islet transplantation. In this study, improved revascularization was accompanied by prompt return of severely diabetic mice to a normoglycaemic state after transplantation of minimal mass of prevascularized islet organoids. Mice transplanted with PIO showed significantly improved insulin secretion and better glucose clearance compared to mice transplanted with PI, NI and IC + HUVECs. Investigations of underlying mechanisms showed that superior function of β-cells in PIOs was mediated by the GLP-1R signalling pathway. GLP-1R has been found to regulate homeostasis of β-cell mass by inducing β-cell proliferation and protecting against apoptosis. On the other hand, activation of the GLP-1R leads to the activation of multiple downstream pathways, including EGF receptor signalling (43), which in turn stimulates proliferation of β cells (44). EGF has been shown to enhance glucose-dependent insulin secretion and upregulate PDX1 expression (20). Although the precise mechanisms underlying this pattern of increased gene expression in the PIOs are not fully understood, we speculate that growth factor expression profile of hAECs, mainly EGF, could stimulate upregulation of the expression of genes involved in β-cell function (GLP-1R, PDX-1).

CONCLUSION

In this study, we demonstrate a novel approach to generate pre-vascularized islet organoids by combining primary ICs with two

additional supportive cell types, HUVECs and hAECs, and address some of the challenges of clinical islet transplantation such as donor supply scarcity, impaired islet engraftment and revascularization. Furthermore, our data demonstrate that hAECs not only promote cell viability and engraftment, but most importantly, play a primordial supporting role in the development of HUVEC-derived neo-vessels within the transplanted tissue.

However, to generate large numbers of uniform, size-controlled and functional prevascularized islet organoids, a scalable platform technology is a prerequisite to ensure standardization and reproducibility for new and innovative beta cell replacement strategies.

Addressing this challenge, recently, we showed that several spheroid generating methods are suitable to assemble uniform, size-controlled and functional islet-like clusters (45). The compared techniques included native islets as controls (IEQs), a self-aggregation technique, the hanging drop technique, the agarose 3D microwell technique and the Sphericalplate SP5D. We demonstrated that up to 9000 islet organoids can be easily generated per plate.

Moreover, the SP5D can be automatized, and robotic-mediated spheroid generation can further reduce variability and therefore improve standardization and reproducibility.

Taken together, these findings could be a basis for the design of novel extra-hepatic, extra-vascular islet transplantation sites.

CAPSULE SENTENCE SUMMARY

The pre-vascularized islet organoids were generated from dissociated islet cells, human amniotic epithelial cells (hAECs), and human umbilical vein endothelial cells (HUVECs). Our study demonstrates that pre-vascularized islet organoids exhibit enhanced *in vitro* function and most importantly, improved engraftment and accelerated vascularization *in vivo* in a murine model.

VANGUARD CONSORTIUM

The School of Medicine of the Università del Piemonte Orientale "Amedeo Avogadro": Chiara Borsotti, Simone Merlin. IRCCS Ospedale San Raffaele: Lorenzo Piemonti, Antonio Citro, Silvia Pellegrini. Ludwig-Maximilians-Universität München: Jochen Seissler, Lelia Wolf-van Buerck, Mohsen Honarpisheh. Lyon Claude Bernard University: Olivier Thaunat. Erasmus University Medical Center Rotterdam: Emma Massey, Antonia Cronin, Eline Bunnik, Dide de Jongh. European Society for Organ Transplantation: Luca Segantini, Giovanna Rossi. Kugelmeiers AG: Patrick Kugelmeier, Petra Wolint. Accelopment Switzerland Ltd. : Marco Cavallaro, Julia Götz, Jeanette Müller.

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 Commission Cantonale d'Ethique de la Recherche (CCER), in compliance with the Swiss Human Research Act (810.30). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Geneva.

AUTHOR CONTRIBUTIONS

C-HW: Performing experiments, data analysis and interpretation, manuscript writing. FL, DC-D, and KB: Performing experiments. MC, TB, CO, AF, and DB: Manuscript editing. LP: Technical support. AF, CO, VO-G and BT: Provision of study material. EB: Conception and design, supervision of project, financial support, administrative support, manuscript writing, final approval of manuscript. All other authors edited and approved the manuscript.

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CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10214/full#supplementary-material

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GLOSSARY

AMCA Aminomethylcoumarin Acetate
AUC area under the curve
bFGF basic fibroblast growth factor
BSA Bovine Serum Albumine
CCER Commission Cantonale d'Ethique de la Recherche
CK-7 Cytokeratin 7
DAPI 4′,6-diamidino-2-phénylindole
DMEM Dulbecco's Modified Eagle Medium
EC endothelial cell
EFP epididymal fat pad
EGF epidermal growth factor
FBS fetal bovine serum
GFP green fluorescent protein
GLP-1R Glucoagon-like peptide 1 receptor
hAEC human amniotic epithelial cell
HB-EGF heparin binding epithelial growth factor
HBSS Hanks' balanced salt solution
HGF Hepatocyte growth factor
HIF1-a Hypoxia-inducible factor 1-alpha
HUVEC human umbilical vein endothelial cell
IC islet cell
IEQ islet equivalent
IGF-1 insulin-like growth factor-1
IPGTT intraperitoneal glucose tolerance test
LV lentiviral
MEM-NEAA Minimum essential medium non-essential amino acids
MOI multiplicity of infection
NI native islet
NDC non-diabetic control
PBS Dubbelco's Phosphate buffer saline
PCSK1 Proprotein Convertase Subtilisin/Kexin Type 1
PCSK2 Proprotein Convertase Subtilisin/Kexin Type 2
PDX-1 pancreatic and duodenal homeobox 1
PFA Paraformaldehyde
PI pseudo-islet
PIO prevascularized islet organoid
RPLP1 ribosomal protein lateral stalk subunit P1
RT-PCR reverse transcriptase polymerase chain reaction
SI stimulation index
STZ streptozotocin
VEC vascular endothelial cadherin
VEGF-A Vascular endothelial growth factor A

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ORIGINAL RESEARCH
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An Inventory of Deceased Donor Family Care and Contact Between Donor Families and Recipients in 15 European Countries

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Families of organ donors play an important role in the deceased organ donation process. The aim of this study was to gain insight into donor family care by creating an inventory of practice in various European countries. A questionnaire about donor family care and contact between donor families and recipients was developed. Representatives of the organ donor professionals of 15 European countries responded (94%). The donor coordinator plays a key role in care for the donor family. All countries provide information about the donation results to the families, although diminished due to privacy laws. Anonymous written contact between donor families and recipients is possible in almost all countries and direct contact in only a few. Remembrance ceremonies exist in most countries. Half of the respondents thought the aftercare could improve. This first inventory shows that differences exist between countries, depending on the organisation of the donation process, the law and the different role of the professionals. Direct contact between donor families and recipients is rarely supported by the donation organisation. To date there has been limited research about the experience of donor family aftercare and we would urge all donation organisations to consider this as a priority area.

Keywords: organ donation, donor family care, contact donor and recipient, remembrance ceremonies, family after care

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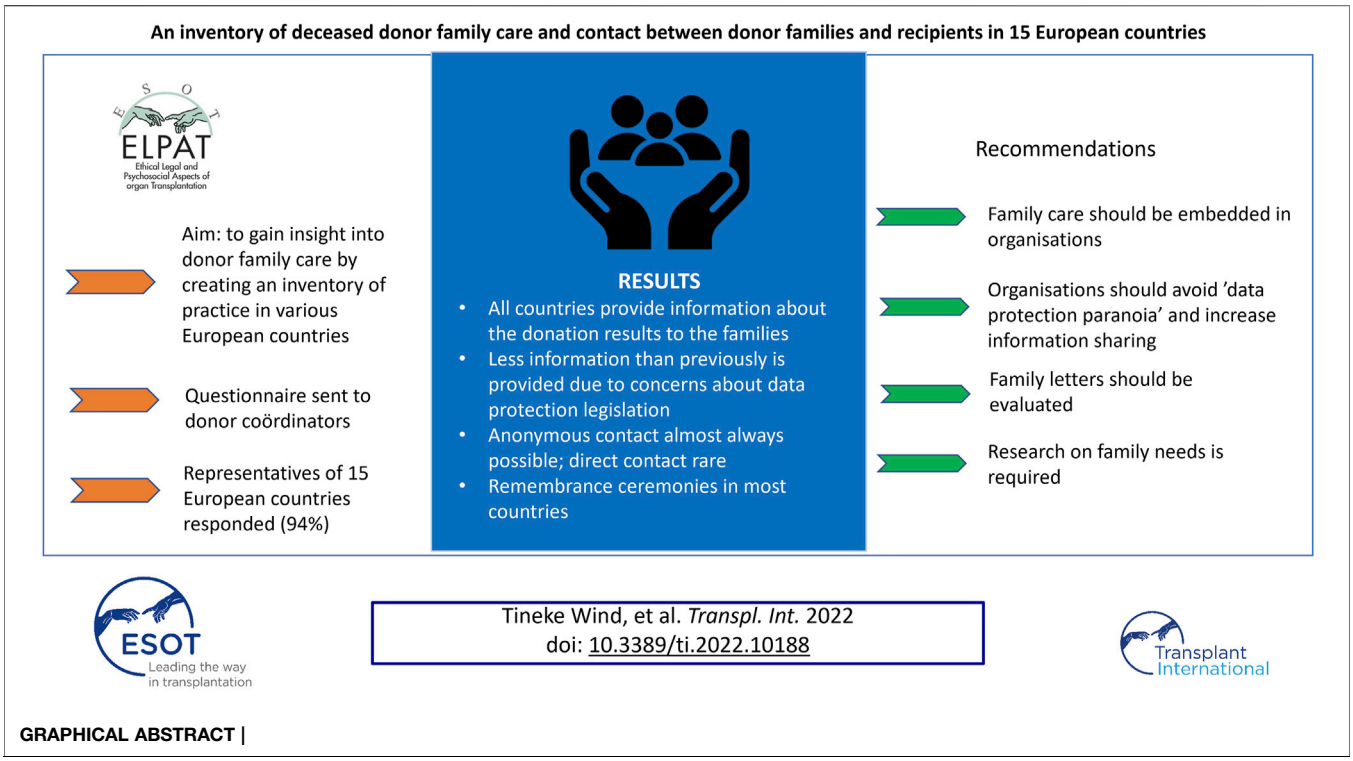
INTRODUCTION

Organ transplantation is a well-accepted medical treatment for organ failure. The main source of donor organs is patients dying in the intensive care unit (ICU), after declaration of brain death (donation after brain death, DBD), or after withdrawal of life sustaining treatment (donation after circulatory death, DCD). In the ICU, "patient-centred care" is the predominant model, which means that individual's specific health needs and desired health outcomes are the driving force behind all health care decisions and quality measurements (1). Patient-centred care includes family-centred care, as most ICU patients cannot make decisions or communicate for themselves due to the severity of the illness and sedation (2). Families in this context are not limited to blood relations or a singular unit but are composed of various individuals who are close to the deceased.



GRAPHICAL ABSTRACT |

If the decision is taken that ICU treatment is futile, the end-of life care process will start. Organ donation is an integral part of end-of life care for many patients (3). The family of a potential organ donor plays an important role in the deceased organ donation process. Depending on the consent system of the country in question, information regarding the wish of the patient or consent may be needed from the family (4, 5) and in most countries the agreement or support of the family is sought before donation proceeds. Communication with the family is therefore important to gain insight in the decision or attitude of the patient regarding organ donation (6, 7).

Organ donation can be overwhelming and stressful for families for many reasons, including the duration of the process and grief at the death of a loved one (8). However, donation can also lead to longer term positive outcomes, particularly as the knowledge that the donation helped other people can lessen the burden of bereavement (9). This highlights the importance of communication with, and care of, the donor family during and after the donation process (9, 10).

The aim of this study is to gain insight into donor family care by creating an inventory of practice in various European countries. We focus on two aspects: the formal communication with the family during and after the donation process and the possibility of contact between donor families and recipients.

METHODS

The Deceased Donation Working Group of ELPAT (the Ethical, Legal, and Psychological Aspects of Transplantation section of the European Society for Organ Transplantation) developed a questionnaire. The items of the questionnaire were based on our professional experiences with the donation process. The questionnaire contained two parts: communication and care for donor families during and after the donation and contact between donor families and recipients.

The first part of the questionnaire contained 16 questions, of which 13 were multiple-choice questions with the ability to provide additional comments, and three open questions. Subjects covered included: guidance of the family during the donation process, information and care provided to families after donation and the provision of remembrance ceremonies for the family.

The second part contained eight questions, of which six were multiple-choice and two open questions. Subjects covered included: contact, what kind of contact, possibility and experience of meetings between donor families and recipients.

Pilot testing of the questionnaire was done with five organ donor coordinators, and using their comments, minor modifications were made to the questionnaire. For each country, one representative was approached by mail or telephone during early 2019; the study was explained and consent was obtained to participate. Each representative was chosen because of their anticipated in-depth knowledge of their country's donation process, and their ability to obtain information from a diverse group to reduce heterogeneity and subjectivity in the replies.

If there were, according to the representative likely to be regional differences in the country, additional representatives were approached as required.

TABLE 1 | Countries and first contact by donor coordinator with family, remembrance ceremonies and meeting transplant recipient.

Country	DC contact with family in relation to donation consent	Remembrance ceremonies organised	Meeting donor family and recipient
Belgium	Before/after/no contact*	Yes	Yes
Croatia	After	No	No
Denmark	No contact	Yes	No
France	Before	Yes	No
Finland	Before	No	No
Germany	After	Yes	No
Hungary	No contact	Yes	No
Iceland	Before	Yes	No
Netherlands	After	Yes	Yes
Norway	No contact	Yes	No
Slovenia	Before/after	No	No
Spain	Before/after	Yes	No
Sweden	No contact	No	Yes
Switzerland	Before/after	Yes	No
United Kingdom	Before	Yes	Yes

Regional differences.

RESULTS

From the 16 approached countries, 15 responded (94%). In three countries (Belgium, France and Netherlands), more questionnaires were returned from different regions, to explore regional differences. Respondents were all experts on the donation process in their country, although their specific job titles varied; for example, organ donor coordinators, transplant coordinators or specialist nurse for organ donation. As these names differ for the person who fulfills the similar task of coordinating the donation process, we use for clarity in this article one term for all the above job titles: donor coordinator (DC).

DC Contact With Family in Relation to Donation Consent

The organisation of the donation and the roles of the professionals involved in the donation process differ by country. Communication with and support of the donor family in the ICU was, in most countries, organised in cooperation between the DC, the intensivist and the ICU nurse. The DC coordinates the donation process on the ICU, has contact with the family and coordinates the organ retrieval procedure.

Table 1 outlines the timing of DC contact with the family in relation to consent for donation. In five countries there is no direct contact between the DC and the family (Denmark, Hungary, Sweden, Norway, and some regions of Belgium) and the DC coordinates the donation process at distance from an office, in close contact with the intensivist. In three countries (Croatia, Germany and Netherlands) only after consent is given for donation can there be contact between the DC and the family. In eight countries, contact between the family and the DC is possible before there is consent for donation. For example, in the U.K. the DC (called the specialist nurse—organ donation) plays an important and active role in the request for donation. Support and guidance of the family on the ICU during the donation process is provided by the intensivist and ICU nurse in all countries, while in 11 countries also the DC is involved in family guidance throughout the donor procedure.

Care for Donor Families After Donation

All countries provide information to the donor family after the donation procedure. This information is given by letter, by telephone and in some countries face to face at the family home or in the hospital. The information is provided by the DC; only in the four countries where there is no contact between the DC and the family, the intensivist provides the information.

Respondents from most countries say the way in which information is provided depends on family wishes. In three countries, a national organisation provides the information (Hungary, Slovenia, United Kingdom). In some countries, the donor family is invited to the hospital a few months after the donation procedure, to evaluate and discuss the donation process.

The kind of information that is provided after the donation procedure varies; information about which organs/tissues are transplanted, information about the recipients gender, age or health. In two countries (Hungary and Finland), only a standard "thank you letter" is sent without any information about the recipients. During the last years, the information provided has reduced in many countries (n = 7), e.g., now the information about age, gender, time on the waiting list, and the health of the recipient, is limited. Some countries now only provide the information that the organ is transplanted or not. A reason suggested by participants for less information sharing was data protection legislation. Some stated that this is a pity, because less meaningful letters are sent to the family. Another factor suggested was social media, due to the fear that donor families will search for recipients' information. This was mentioned for two countries.

The representatives from six countries expressed overall satisfaction with the care provided for donor families (Netherlands, Belgium, United Kingdom, France, Finland Iceland). The main concern from the other country representatives who were not satisfied was that there was no or not enough aftercare for donor families. Structural organised care for families after donation was missing, were some comments, like: "There is no organised care, donor family care should be implemented." "More national follow up of donor families is needed." A lack of structured organised after care was a frequent observation, highlighting there was too much variation per hospital or region. Comments: "There should be more follow up with donor families after they leave the hospital, to learn more about their grieving process and implement the lessons learned." Information was lacking concerning the effects, positive or negative, of the donation to this grieving process. In some countries the opinion was that donor after care is too variable per region or hospital and should be implemented on a national level, to guarantee donor after care for all donor families. Comments: "smaller hospitals are less experienced; there is a wish to give more support to all families, not only to those who ask for help or information."

We were interested if there was a difference in care for donor families on the ICU and families of regular ICU patients who die in the ICU. From the respondent countries, almost half are of the opinion that the care differs. The general opinion was that more attention is given to donor families during the donation process, especially if there is a DC present to coordinate the donor procedure, or a trained professional to give information and support to the family and who can help them in the beginning of the grieving process. Donor families also receive more care after the donation (aftercare) than families of deceased ICU patients, was the opinion of seven countries. "Donor families are invites to special ceremonies," "donor families receive 'thank you letters.'" "Yes, the care is different, donor families are invited to the donor hospital 6 weeks after the donation, to talk about their experience." Families from regular ICU patients who die don't have these special ceremonies in general. However, some state the care is equal, also families of non-donors are invited to the hospital for after care.

Remembrance Ceremonies for Donor Families

In 11 countries remembrance ceremonies are organised for donor families, only in four countries (Croatia, Finland, Slovenia, Sweden) was this not the case (**Table 1**). The ceremonies are organised at different levels: at a national level, a regional level, or at a hospital level. Examples of these different ceremonies are: a donor family day, a transplant honours day, a donor memorial day, a remembrance walk with donor families, a national donor monument, a hospital donor monument, a donor tribute evening. Sometimes smaller ceremonies are organised at a hospital level, like a farewell ceremony organised by a priest. There are hospitals that have a monument in their hospital in honour of their donors. In one country (the United Kingdom) a posthumous national award consisting of a special certificate and pin is offered to all the donor families, to pay respect to the donor through the donor family. During the annual ceremonies in some countries, recipients are also present as well as representatives from transplant centres to give support to families.

Who is responsible for organising the ceremonies differs per country: private organisations, a recipient organisation, local organisations from donor hospitals, or the event can be organised by the transplant foundation, or regional teams. All countries who provide ceremonies, state that they were satisfied with these ceremonies. Comments on the ceremonies: "very helpful for donor families," "families are happy with the attention," "well visited meetings," "important to share experiences and emotions with other families." According to some countries improvement could be the presence of a professional during the meetings, like a DC, to answer questions and give specific information.

Contact Between the Donor Family and Recipient(s)

In all countries but one (Croatia), written anonymous contact between recipient and donor family is possible, through an intermediate, the DC. In one country (Switzerland) a website was developed, where recipients and donor families can post their thoughts, thanks, experiences, and histories. Here was also a guide/template for a "thank you letter" to be used for transplant patients to a donor family. Initiative for contact is taken more by recipients than by donor families.

Four countries responded that a formalised process exists for donor family and recipient(s) to meet face-to-face (**Table 1**). However, the circumstances and conditions differ. For example, in the Netherlands, this is only possible through an organisation where donor families and recipients can report themselves, without involvement of professionals. Because anonymity is regulated by law, professionals cannot be involved. The organisation matches the donor family and recipient and arrange the meeting. In Belgium during donor day, a meeting is possible. In Sweden meetings happen, but without health care personnel. In the United Kingdom, the meetings are held in a mutual convenient place, well prepared with the support of the DC. Because the satisfaction of the donor family is not routinely measured, most countries state that family experience are not known. Two countries (the United Kingdom and Belgium) are positive about the meetings. Comments of other countries suggests that there is a lot of discussion about meetings between donor families and recipients. Comments included:

> "Is this a good thing"?
>
> "Should there be a role for the health care professionals during these meetings?" "Expectations should be well managed"
>
> "Is the motivation of the donor family and the recipient the same?"
>
> "Meetings don't feel right, not intent to cooperate as a professional."

"What if there is some pressure felt from the donor family to the recipient"?

"Meetings can only be possible if the donor family and recipient find each other through social media."

A few general comments were made, and suggestions to improve the care for donor families. For example, offering the family a conversation with a psychologist, if they are in need for support or to be more active in approaching recipients to send a kind of thank you letter to the donor family.

Letters to Donor Families

To be able to compare if the information provided to the donor families from the DC changed over the years, we asked participants to send examples of two letters: a recent letter and one from approximately 10 years ago. Letters were received from five countries. In four countries, the letters were changed, and in all of these cases less information was given about the recipients. Information about time on the waiting list, age (only an age period) or health information about the recipient was no longer given in the latest letters. The recent letters were simpler and more straight forward, consisting only of information concerning whether the transplantation of the organ was successful, with no more detailed information about the health process of the recipient. The reason for this change was reported as being stricter privacy legislation in the different countries.

DISCUSSION

This study is the first survey about the care of donor families, during and after the donation process and contact between the donor families and the recipients in 15 European countries. It shows that there is a variability between countries, and in some countries small differences between centres or regions. For example, the role of the DC and the moment this professional is participating in the donation process. Depending on the way donation is organised there are also differences in the communication with and care of the donor family. Communication during the donation process with the family is mostly led by the DC; only in a small number of countries is there no direct contact between the DC and the donor family. Generally, DC contact with the donor family starts after consent for donation, though a minority of DCs have contact before consent. There is some evidence that early contact with the family and involvement of the DC in the request for donation can have a positive effect on the perceived support for the family and the consent rate for donation (11).

The amount of information provided to the family about the outcome of the donation and transplantations depends on the legislation and its interpretation, in a specific country. In several countries this information has become more limited in recent years due to data protection legislation. This means that less information about the recipients can be given, mainly restricted to age range, transplant outcomes and sometimes gender of the recipients. It is possible that concerns about privacy and strict interpretations of data protection legislation may not be justified; if a recipient consents to the processing of more data in order to facilitate higher levels of communication with donor families, this would be permitted by the General Data Protection Regulation. However, different countries may have stricter national laws in place, or transplant professionals may be being given legal advice that takes a very strict interpretation of data minimisation where that is not necessary.

Face-to-face meetings between donor family and recipients only take place in a few countries, but there is concern about the impact these meetings can have on both donor families and recipients. There are some studies about the contact between the donor families and transplant recipients (12, 13). Outcomes of the studies differ, but benefits are seen from contact; letters from recipients to donor families are appreciated by donor families and contribute to positive feelings. Expressing gratitude to the family of the person who made the donation possible can be important. However, there are also several reasons for not having contact: protection from emotional stress, not to be reminded of this stressful period and the loss of the loved one, and the wish to leave a difficult period behind. Families who met with recipients, reported that it eased their pain and gave some positive meaning to their loss (14). On the other hand, negative feelings, such as disappointment in the person who received the organ, can also occur. In most countries surveyed, anonymity must be assured and the law is perceived as preventing healthcare professionals from facilitating direct contact between donor families and recipients.

The aftercare given to donor families can be different from the care given to non-donor families on the ICU. This could be influenced by providing more and longer intensive contact with the donor family, more extensive conversations and explanations about the donor procedure by the intensive care staff and coordinator of the donation procedure. In the literature, families who consented to donation felt more supported than families of non-donors (15). Increased satisfaction of families of ICU patients is seen in previous studies, when a family support coordinator is brought in, an extra person who cares for the needs of the family (16). The positive effect of such a person is also seen in the consent rate for donation (17). In this study, almost half of the respondents still thought there was need for improvement of the aftercare for donor families. Too little is known about the impact of the donation procedure on the grieving process. Studies that focus on the impact of organ donation on the grieving process, also show that the act of donation can assist families in their grief (18).

Remembrance ceremonies exist for donor families in almost all countries, and the impression of the DC is that the ceremonies benefit the donor family, but satisfaction with the ceremonies is not routinely measured. There is attention to the needs of donor families, but a lack of specific information about these needs is also mentioned. No specific studies are performed to measure the impact of ceremonies on the donor family.

Data collection was completed before the COVID pandemic, so it had no impact on the results. However, the pandemic may have affected the provision of donor family support, such as public remembrance ceremonies, which are temporarily reduced.

Reflections and Recommendations

Given the results of our research, we can make several recommendations. Donor family aftercare is an essential part of the donor process and should be delivered in a structured way and embedded into the organisational process. In order to establish best practice for a country, research on family views is needed. What services do donor families need? This research can be done using groups, such as donor family advisory group, or interviews with donor families. Research should also be conducted to evaluate letters from a donor family perspective; what information benefits the donor family? Are meetings between recipients and donor families beneficial for both? Those working with donor families and recipients should reflect on this inventory, and comparative practices, and consider whether they are meeting the needs of donor families. Furthermore, organisations can tend toward risk aversion in data protection at the expense of helping families; this tendency should be avoided. Research evaluating the impact of COVID on donor family care should also be performed.

Conclusion

This first inventory of 15 European countries about the care provided to donor families during and after donation and contact between donor families and recipients, shows, as expected, that there are differences between the countries. These differences depend on the organisation of donation, the law (and its interpretation) and the different roles of the professionals involved in donation. Donor family aftercare is provided in all countries and some countries provide remembrance ceremonies. In most countries, direct contact between donor families and recipients is not supported by the donation organisation. To date there has been limited research about the experiences and satisfaction of donor family aftercare and we would urge all donation organisations to consider this as a priority area.

Strengths and Limitations of the Study

This is the first multi-country study to compare the way care to donor family and aftercare is provided to donor families and contact between donor families and recipients, which provides valuable insights. Since this is a first inventory, it provides only an initial overview of the different aspects. More research is necessary to explore in depth in how communication and aftercare is given, and the experiences and satisfaction of the donor families, with including possible suggestions for improvement.

Participating Countries

Belgium, Croatia, Denmark, Finland France, Germany, Hungary, Iceland, the Netherlands, Norway, Slovenia, Spain, Sweden, Switzerland, United Kingdom.

CAPSULE SUMMARY STATEMENT

Families of organ donors play an important role in the deceased organ donation process. Little is known about the care for donor families. Although there are differences between the countries, families are provided with information about the transplant results and remembrance ceremonies are organised. Aftercare for donor families could improve and be more structural organised. Meetings between donor families and recipients exist. With this inventory of 15 European countries we gain insight in the daily practise, which is important, to learn from other countries and to know where future research should focus on, like the experience and needs of donor families.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

The research idea was developed by all authors as part of the ELPAT group, TW and NJ developed the questionnaire, all authors contributed to the final questionnaire, TW, distributed and analysed the questionnaire surveyed TW wrote the final version. NJ, AF, and DS contributed to finalize the article or finalised the article.

CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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MiR-21 in Lung Transplant Recipients With Chronic Lung Allograft Dysfunction

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Background: Micro-RNA-21 (miR-21) is a post-translational regulator involved in epithelial-to-mesenchymal transition (EMT). Since EMT is thought to contribute to chronic lung allograft dysfunction (CLAD), we aimed to characterize miR-21 expression and distinct EMT markers in CLAD.

Methods: Expression of miR-21, vimentin, Notch intracellular domain (NICD) and SMAD 2/3 was investigated in explanted CLAD lungs of patients who underwent retransplantation. Circulating miR-21 was determined in collected serum samples of CLAD and matched stable recipients.

Results: The frequency of miR-21 expression was higher in restrictive allograft syndrome (RAS) than in bronchiolitis obliterans syndrome (BOS) specimens (86 vs 30%, $p = 0.01$); Vimentin, NICD and p-SMAD 2/3 were positive in 17 (100%), 12 (71%), and 7 (42%) BOS patients and in 7 (100%), 4 (57%) and 4 (57%) RAS cases, respectively. All four markers were negative in control tissue from donor lungs. RAS patients showed a significant increase in serum concentration of miR-21 over time as compared to stable recipients ($p = 0.040$).

Conclusion: To the best of our knowledge this is the first study highlighting the role miR-21 in CLAD. Further studies are necessary to investigate the involvement of miR-21 in the pathogenesis of CLAD and its potential as a therapeutic target.

Keywords: mir-21, chronic lung allograft dysfunction, bronchiolitis obliterans syndrome, restrictive allograft syndrome, lung transplantation

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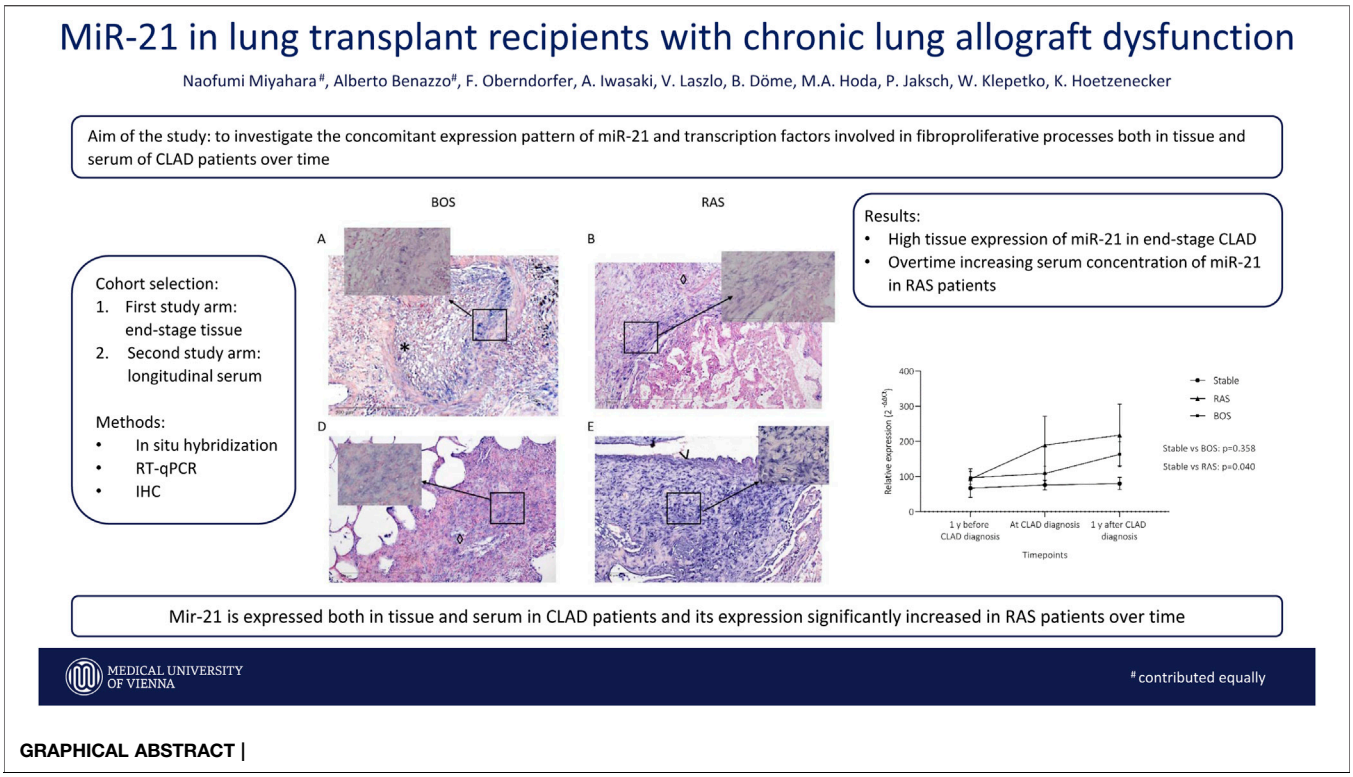
Abbreviations: CLAD, chronic lung allograft dysfunction; LTx, lung transplantation; BOS, bronchiolitis obliterans syndrome; RAS, restrictive allograft syndrome; EMT, Epithelial mesenchymal transition; TGF-β1, transforming growth factor-β1; miR-21, micro RNA-21; FFPE, formalin-fixed paraffin-embedded; COPD, chronic obstructive pulmonary disease; AMR, Antibody-mediated rejection; ACR, acute cellular rejection; LB, lymphocytic bronchiolitis; ISHLT, International society of heart and lung transplantation; ISH, *in situ* hybridization; IHC, immunohistochemistry; NICD, notch intracellular domain; SMAD, Sma and Mad-related protein.



GRAPHICAL ABSTRACT |

INTRODUCTION

Chronic lung allograft dysfunction (CLAD) represents the main cause of long-term morbidity and mortality after lung transplantation (LTx). CLAD can manifest either as bronchiolitis obliterans syndrome (BOS) or restrictive allograft syndrome (RAS), mixed phenotype or as an undefined entity. CLAD affects up to 50% of lung transplant recipients within 5 years (1). Although significant efforts have been made to unravel the pathophysiology of CLAD, the main causative factors as well as therapeutic targets are still elusive. After an initial epithelial and endothelial injury, a series of immune and inflammatory stimuli trigger the activation of different pro-fibrogenic processes (2). These include activation of specific signaling pathways, activation of resident mesenchymal stromal cells and macrophages, proliferation of myofibroblasts, deposition of collagen by fibroblasts as well as epithelial-to-mesenchymal transition (EMT) (3, 4). Activation of transforming growth factor-β1 (TGF-β1), tyrosine kinase, Notch, and integrin signaling pathways leads to the deposition of extracellular matrix and to the phenotypic transition of epithelial cells into mesenchymal cells. MicroRNA-21 (miR-21) is a post-translational regulator of several signaling pathways involved in EMT. Moreover, it is a central regulator of the TGF-β1/SMAD (5) and is highly expressed during the development of lung fibrosis (6).

This study aimed to investigate the concomitant expression pattern of miR-21 and transcription factors involved in fibroproliferative processes both in tissue and serum of CLAD patients over time. The rationale of this study was to explore the role of miR-21 as predictive biomarker and potential therapeutic target against fibroproliferative derangements observed in CLAD development.

MATERIALS AND METHODS

Cohort Selection and Tissue Samples

This work was based on two study arms. The first study arm included tissue specimens from explanted CLAD allografts at time of retransplantations. The second study arm used prospectively collected serum samples from lung transplant recipients in follow-up at our institution. This study was approved by the Institutional Review Board of the Medical University of Vienna, Austria (EK-No.2106/2017).

First Study Arm

All patients who received retransplantation at the Medical University of Vienna, for CLAD between 2009 and 2017 were included in this single-center study (17 BOS and seven RAS patients). Mixed and undefined phenotypes were excluded. Moreover, patients who changed their CLAD phenotype between onset and retransplantation were not included in the study. Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens from explanted lung allografts were used for quantitative real-time PCR and histopathological analysis.

Specimens for IHC and ISH were obtained from the most affected of the five explanted lobes. Resected donor lung parenchyma obtained from size-reduced transplantations was used as control tissue ($n = 4$, female $n = 3$, median age = 42 ± 10). Control tissue was histologically analyzed and, if free from any parenchymal diseases, was used as healthy control.

Second Study Arm

A case-control cohort was identified nested within a longitudinal cohort of lung transplant recipients at the Medical University of Vienna, who consented for storage of serum. At our institution a total of 710 patients consented for prospective storage of biological samples including serum, plasma and BAL for scientific purposes from 2009 to 2014. Among them, 213 recipients developed CLAD during follow-up. Controls were chosen among 497 CLAD-free recipients by matching according to gender, age, underlying diagnosis, type of transplantation and type of induction therapy. Thirty patients in each group were initially identified, however only for 25 CLAD and 26 non-CLAD recipients, serum samples were available for all three time points defined by the study protocol. MiR-21 concentration was measured in serum samples of 51 lung recipients (13 BOS, 12 RAS, 26 stable patients) at the three defined timepoints. The timepoints for CLAD group were: 1 year before CLAD diagnosis or matched, at the time of CLAD diagnosis and 1 year after CLAD diagnosis. The first time point of serum sampling in the control arm was matched to the time point in the CLAD arm. This approach was chosen to address possible differences in miR21 expression related to the time passed since LTx. Antibody-mediated rejection (AMR) was defined according to the last ISHLT recommendations (7). Higher grade acute cellular rejection (ACR) and lymphocytic bronchiolitis (LB) were defined as ≥ A2 and B2, respectively (8). Cumulative A and B scores are the sum of all A and B scores divided by the number of biopsies performed in the follow-up per patient. BOS and RAS were diagnosed according to the most recent ISHLT classification (9) determined by two transplant physicians. At time of sampling, no patient had signs or diagnosis of ACR, AMR, infection systemic inflammation. Patients with an established CLAD diagnosis received azithromycin (250 mg three times a week) until retransplantation. Seven patients underwent extracorporeal photopheresis (ECP), due to further deterioration. In all patients, ECP was not started before 1 year after CLAD diagnosis (the third timepoint for serum collection).

Quantitative Real-Time PCR (RT-qPCR) of miR-21

For quantification of tissue miR-21, RNA was extracted by 5 × 10 µm tissue sections from FFPE blocks using miRCURY™ RNA Isolation Kit—FFPE samples (Exiqon, Vedbaek, Denmark), according to the manufacturer's instructions. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, Massachusetts, United States) and individual miRNA-specific RT primers (Exiqon, Vedbaek, Denmark). Micro-RNA levels were quantified in duplicates from 4 µl cDNA, with SYBR Green PCR Master Mix and specific primers of the miRCURY LNA™ miRNA PCR assay, using the following settings on an Applied Biosystems™ 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, United States): 2 min, 50°C; 10 s, 95°C; 40 cycles of 10 s, 95°C; 1 min, 60°C.

Serum RNA, including miRNAs, was extracted from 200 µl patient serum, by using the miRNeasy Serum/Plasma Advanced kit (QIAGEN, Germany) according to the manufacturer's instructions. cDNA was synthesized from 2.5 µl of serum-RNA by using individual miRNA-specific RT primers contained in the 5× miRCURY RT reaction buffer and 10× miRCURY RT enzyme mix (QIAGEN, Germany), by using the following thermal cycler conditions: 60 min, 42°C; 5 min, 95°C. Circulating miRNA levels were quantified in duplicate from 4 µl cDNA, with SYBR Green PCR Master Mix and specific primers of the miRCURY LNA™ miRNA PCR assay, using the amplification condition explained above.

RT-qPCR data were analyzed via the comparative threshold cycle (Ct) method [6]. The concentration of circulating miR-21 was expressed as $2^{-\Delta\Delta Ct}$ and compared with to control samples.

In Situ Hybridization (ISH)

ISH was performed according to the manufacturer's protocol (QIAGEN, Hilden, Germany) (10), with some modifications. A double-DIG labeled miRCURY LNA™ microRNA detection probe with the sequences 5′-TCAACATCAGTC TGATAAGCTA-3′ and a U6 probe (positive control) was used, while a scrambled probe served as negative control. U6 small nuclear RNA (snRNA) is a noncoding RNA transcript used in pre-mRNA splicing expressed in all cells. Therefore, ISH for U6 revealed an intense signal in cell nuclei. Tissue sections (6 µm thick) were deparaffinized in descending ethanol solutions (99, 96, 70%) and digested with Proteinase-K (15 µg/ml) for 30 min at 37° using an Abbott hybridizer System. Then, LNATM probes were denatured and diluted in QIAGEN ISH buffer. Hybridization with 40 nM MiR21 probe, 1 nM U6 probe and 40 nM scramble probe was performed at 50°C for 60 min followed by stringent washes in 5 × SSC, 1 × SSC and 0.2 × SSC at 50°C and blocking. The digoxigenins were recognized by a specific anti-DIG antibody conjugated with Alkaline phosphatase (AP). Samples were stained with freshly prepared NBT/BCIP substrate reagent containing 0.2 mM Levamisole (2 h at 30°C) and slides incubated with KTBT buffer. Nuclear Fast Red was used as counter stain. Sections were analyzed microscopically.

MiR-21 intensity in fibroblast cytoplasm and extracellular matrix was retained for scoring purposes with a minimum cut-off at 10% of cells. Cases were classified as: 0 = negative or faint expression; 1 +: low expression (<25%); 2 +, moderate expression (25–50%); 3 +, strong expression (>50%). Cases with a score of three were regarded as positive in Kaplan-Meier curves.

Immunohistochemistry (IHC)

All stainings were performed on sections of 2–3 µm thickness. IHC was conducted according to a standard protocol using the

TABLE 1 | Patient demographics.

Characteristics		Study arm—tissue			Study arm—Serum			
		BOS[a] *n* = 17	RAS[b] *n* = 7	*p*-value	BOS (*n* = 13)	RAS (*n* = 12)	Controls (*n* = 26)	*p*-value
Female		12 (70)	5 (71)	0.967	7 (53%)	3 (25%)	14 (53%)	0.21
Age, year (mean ± SD[c])		27.3 ± 11.7	26.3 ± 11.8	0.907	46 ± 14	52 ± 10	46 ± 13	0.41
Underlying diagnosis	COPD[d]	1 (5.9%)	1 (14.3%)	0.207	5 (39%)	11 (92%)	13 (50%)	0.332
	Fibrosis[e]	7 (41.2%)	0		3 (23%)	0	2 (8%)	
	iPAH[f]	3 (17.6%)	1 (14.3%)		2 (15%)	0	1 (4%)	
	CF[g]	5 (29.4%)	4 (57.1%)		3 (23%)	1 (8%)	5 (19%)	
	Others	1 (5.9%)	1 (14.3%9		0	0	5 (19%)	
CMV[h] risk	D+/R−	3 (17.5%)	3 (42.9%)	0.409	7 (54%)	5 (42%)	4 (15%)	0.087
	D+/R+	9 (52.9%)	2 (28.5%)		2 (15%)	3 (25%)	9 (46%)	
	D−/R+	2 (14.8%)	2 (28.5%)		3 (23%)	3 (25%)	8 (30%)	
	D−/R−	2 (14.8%)	0		1 (8%)	1 (8%)	5 (19%)	
Primary transplant type	Single	4 (23%)	0 (0%)	0.159	2 (15%)	1 (8%)	2 (8%)	0.753
	Double	13 (76%)	7 (100%)		11 (85%)	11 (92%)	24 (92%)	
Intraoperative VA ECMO[i]		11 (64.7%)	3 (42.9%)	0.324	5 (39%)	3 (25%)	13 (50%)	0.530
Prolonged postoperative VA ECMO		6 (35.3%)	1 (14.3%)	0.303	1 (8%)	1 (8%)	4 (15%)	0.712
Induction therapy	No induction	12 (70.6%)	1 (14.3%)	0.041	10 (77%)	10 (84%)	11 (42%)	0.333
	rATG[j]	3 (17.6%)	4 (57.1%)		3 (23%)	1 (8%)	11 (42%)	
	Alemtuzumab	2 (11.8%)	2 (28.6%)		0	1 (8%)	4 (16%)	
Higher grade ACR[k] (A ≥ 2)		4 (23.5%)	1 (14.1%)	0.612	2 (15%)	1 (8%)	6 (23%)	0.588
Time to higher grade ACR from time of LTx[l], months (mean ± SD)		12 ± 14	0.1	0.500	26 ± 18	14	6.5 ± 4	0.064
Higher grade LB[m] (B ≥ 2)		7 (41.2%)	3 (42.3%)	0.939	6 (46%)	2 (17%)	4 (15%)	0.114
Time to higher grade LB from time of LTx[l], months (mean ± SD)		12 ± 15	0.7 ± 1.2	0.250	21 ± 17	9 ± 12	23 ± 28	0.370
Clinical AMR[n]		2 (11.8%)	1 (14.3%)	0.865	0	0	0	—
Time to higher grade AMR from time of LTx[l], months (mean ± SD)		94 ± 63	18	0.667	—	—	—	—
Cumulative A score		0.23 ± 0.20	0.14 ± 0.25	0.114	0.33 ± 0.24	0.24 ± 0.025	0.19 ± 0.22	0.489
Cumulative B score		0.60 ± 0.42	0.54 ± 0.62	0.534	0.85 ± 0.45	0.62 ± 0.25	0.51 ± 0.28	0.094
Azythromycin therapy for CLAD[o]		10 (58.8%)	5 (71.4%)	0.562	9 (69%)	12 (100%)	0	0.036
Extracorporeal photopheresis for CLAD		8 (50%)	4 (57.1%)	0.752	6 (46%)	1 (8%)	0	0.035
Time to CLAD from time of LTx[l], months (mean ± SD)		34.4 ± 34.3	52.3 ± 54.1	0.494	59.1 ± 37.5	38.4 ± 29.3	—	0.21
Time to Re-LTx from time of LTx[l], months (mean ± SD)		70.4 ± 58.8	68 ± 51.7	0.852	—	—	—	—

[a]*Bronchiolitis obliterans syndrome.*
[b]*Restrictive allograft syndrome.*
[c]*Standard deviation.*
[d]*Chronic obstructive pulmonary disease.*
[e]*Idiopathic pulmonary fibrosis.*
[f]*idiopathic pulmonary arterial hypertension.*
[g]*Cystic fibrosis.*
[h]*Cytomegalovirus.*
[i]*Veno-Arterial extracorporeal membrane oxygenation.*
[j]*Rabbit anti-thymocyte globulin.*
[k]*Acute cellular rejection.*
[l]*Transplantation.*
[m]*Lymphocytic bronchiolitis.*
[n]*Antibody-mediated rejection.*
[o]*Chronic lung allograft dysfunction.*

following antibodies: Vimentin (Clone V9, Biocare medical) at dilutions of 1:300, Notch intracellular domain (NICD) (Clone A-8, Santa Cruz Biotechnology) at dilutions of 1:50, p-Sma and Mad-related protein (SMAD) 2/3 (Clone C-8, Santa Cruz Biotechnology) at dilutions of 1:100, β-catenin (Clone 14, BD Transduction laboratories) at dilutions of 1:100 and E-cadherin (Clone NCH-38, DAKO) at dilutions of 1:2. Staining was either performed with a BenchMark Ultra or a BenchMark XT (Ventana, Tucson, AZ). Negative and positive controls demonstrated appropriate immunolabeling for each staining.

The proportion of epithelial cells or fibroblasts and extracellular matrix that were positive for each marker was classified as: 0 = negative or faint expression; 1 +: low expression (<25%); 2 +, moderate expression (25–50%); 3 +, strong expression (>50%). IHC and ISH were reviewed and scored by two independent researchers (A.B., F.O.), and cases with at least 1 + were regarded as positive.

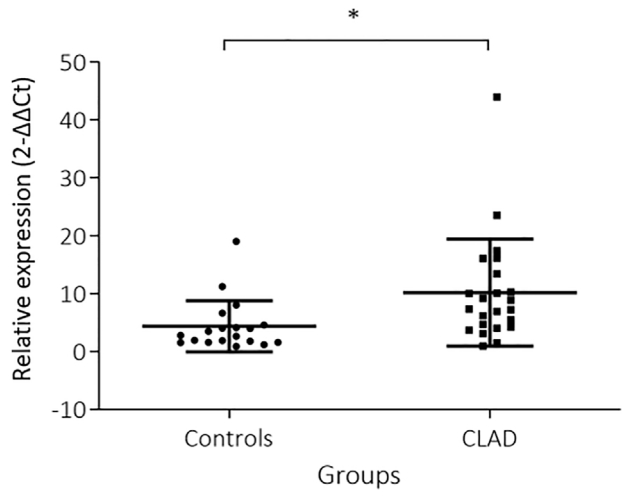


FIGURE 1 | Relative expression of miR-21 in CLAD allografts. Expression of miR-21 in explanted lung allografts and normal lung tissue is shown. MiR-21 is strongly expressed in CLAD tissue whereas it is low in control tissue (p = 0.002). MiR-21 expression is presented as $2^{-\Delta\Delta Ct}$ values (Mean + Standard error).

Statistical Analyses

Categorical variables are reported as percentage, continuous variables as mean (min-max). A X^2 test, Fisher exact test or one-way ANOVA test was used to test differences. Correlations were quantified with Pearson's correlation coefficient. The values of serum miR-21 ($2^{-\Delta\Delta Ct}$) for CLAD patients and stable patients were compared by 2-way repeated measure ANOVA test to evaluate significant trends over time. A p-value of 0.05 or less was considered statistically significant. All the statistical analyses were conducted with IBM SPSS Statistics version 25 (IBM, Chicago, IL) and graphics were designed with GraphPad Prism 8.

RESULTS

Patient Demographics

Clinical characteristics of the 24 patients included in the histological analysis are shown in **Table 1**. Seventeen (70%) patients were female, age at transplantation was 26 ± 11 years and at CLAD diagnosis 33 ± 12 years. The most frequent underlying diagnosis was cystic fibrosis (9, 38%), followed by interstitial lung disease (7, 29%) and idiopathic pulmonary arterial hypertension (4, 16%). Thirteen (54%) patients did not receive any induction therapy, 7 (29%)

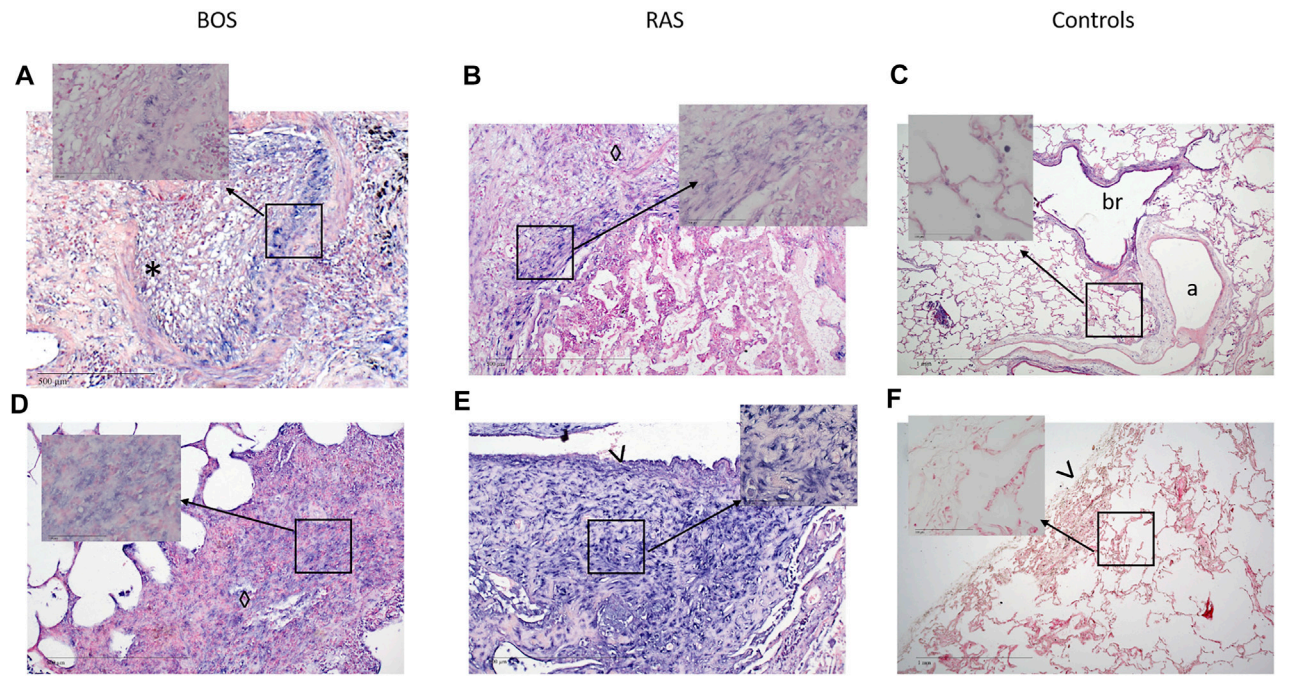


FIGURE 2 | Representative images of miR-21 *in situ* hybridization in BOS and RAS lesions and in healthy lung tissue. Images of a completely obliterated bronchiole **(A)** and interstitial **(D)** staining of miR-21 (blue) in a BOS sample (original magnification **(A,D)** = ×10, insert original magnification **(A,D)** = ×40). Interstitial **(B)** and subpleural **(E)** fibrosis with architectural distortion in a RAS sample, showing diffuse positive miR-21 staining (original magnification **(B,E)** = ×10, insert original magnification **(B,E)** = ×40). Images of healthy lung parenchyma **(C)** and pleura **(F)** of control lungs (original magnification **(C,F)** = ×4, insert original magnification **(C,F)** = ×40). miR-21 = microRNA-21, ISH = *in situ* hybridization, *, obliterated bronchiole; ◊, interstitial fibrosis; >, pleura; a, artery; br, bronchiole. Nuclear Fast Red was used as counter stain.

TABLE 2 | Expression of EMT markers and miR-21 in BOS and RAS.

		Grading							
		Intra/Peribronchial				Interstitium			
		0	1+	2+	3+	0	1+	2+	3+
BOS[a]	Vimentin	0 (0)	4 (23.5)	9 (53)	4 (23.5)	8 (47)	4 (23)	3 (18)	1 (6)
	NICD[b]	5 (29)	9 (53)	0 (0)	3 (18)	8 (47)	5 (29)	3 (18)	0 (0)
	SMAD[c]	10 (59)	7 (41)	0 (0)	0 (0)	10 (59)	5 (29)	1 (6)	0 (0)
	β-catenin	17 (100)	0 (0)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	0 (0)
	E-cadherin	17 (100)	0 (0)	0 (0)	0 (0)	17 (100)	0 (0)	0 (0)	0 (0)
	miR-21	12 (70)	3 (18)	2 (12)	0 (0)	9 (53)	4 (23)	1 (6)	2 (12)
RAS[d]	Vimentin	1 (14)	5 (72)	1 (14)	0	0 (0)	2 (28)	3 (42)	2 (28)
	NICD	2 (29)	4 (57)	1 (14)	0 (0)	3 (42)	2 (28)	0	2 (28)
	SMAD	2 (29)	4 (57)	1 (14)	0 (0)	3 (42)	3 (42)	0 (0)	1 (14)
	β-catenin	7 (100)	0 (0)	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	0 (0)
	E-cadherin	7 (100)	0 (0)	0 (0)	0 (0)	7 (100)	0 (0)	0 (0)	0 (0)
	miR-21	0 (0)	2 (28.5)	2 (28.5)	3 (43)	1 (14)	0 (0)	2 (28)	4 (57)

[a]*Bronchiolitis obliterans syndrome.*
[b]*Notch intracellular domain.*
[c]*Sma and Mad-related protein.*
[d]*Restrictive allograft syndrome.*

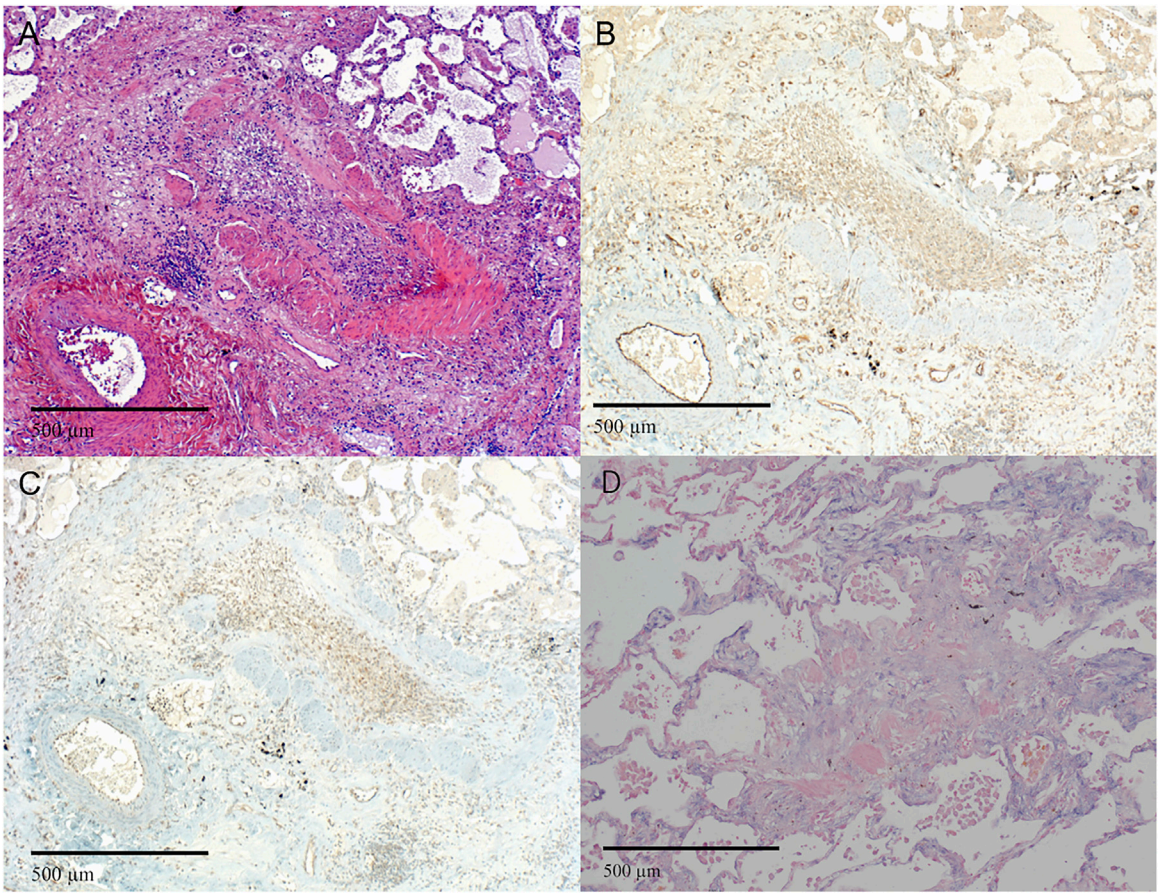


FIGURE 3 | Representative images of bronchiolitis obliterans in BOS. **(A–D)** Completely obliterated bronchioles. **(A)** H&E staining of a bronchiole showing luminal loose fibrous connective tissue with scattered chronic inflammatory cells and fibroblasts. **(B and C)** Immunohistochemistry of the same area as in **(A)** showing **(B)** NOTCH1 and **(C)** pSMAD2,3 expression of the lesion. **(D)** miR-21 ISH shows expression of miR-21 in fibroblasts. (A-D original magnification ×10). H&E = Hematoxylin and Eosin; miR-21 = microRNA-21, ISH = *in situ* hybridization.

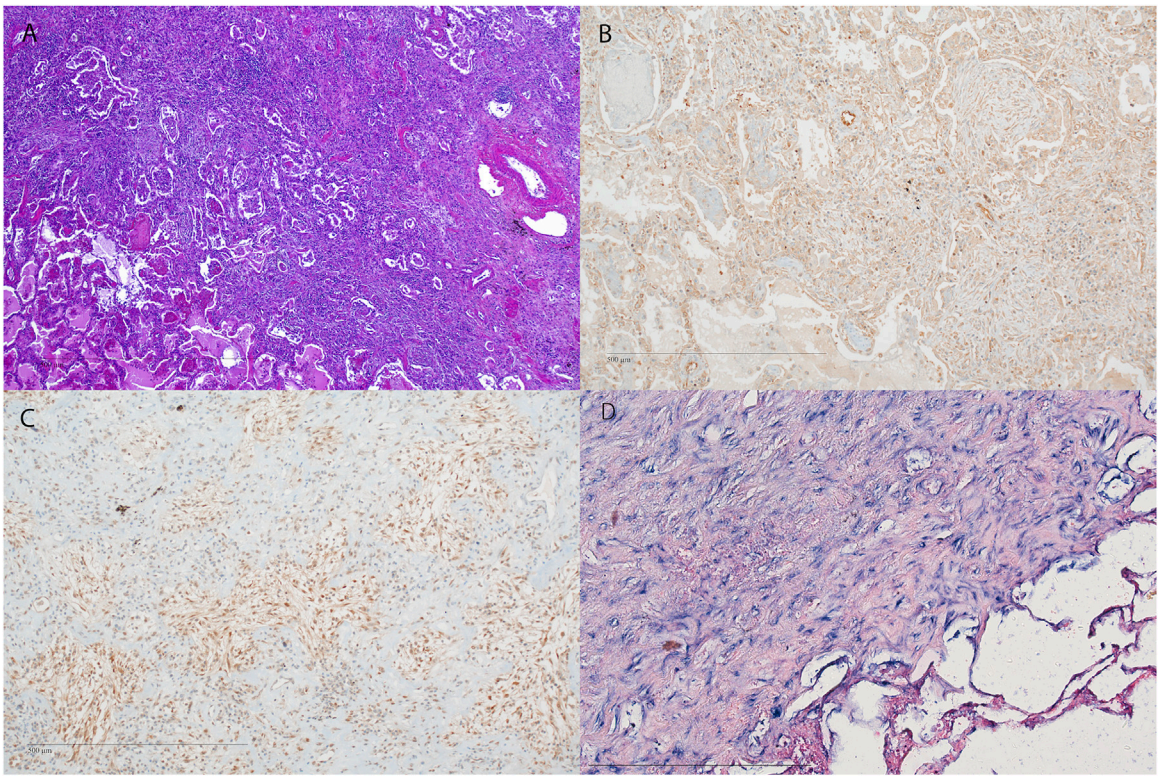


FIGURE 4 | Representative images of fibrosis in RAS. **(A–D)** H&E staining of the pleura and subpleural parenchyma with fibrosis (original magnification ×40). **(B–D)** Immunohistochemistry and *in situ* hybridization of the corresponding HE **(A)** showing **(B)** NOTCH1, **(C)** pSMAD2,3 expression. **(A–D)** original magnification ×10). H&E = Hematoxylin and Eosin; miR-21 = microRNA-21, ISH = *in situ* hybridization.

TABLE 3 | Correlations.

Variables		MiR-21			
		BOS[a] (*n* = 17)		RAS[b] (*n* = 7)	
		r	*p*-value	*r*	*p*-value
Interstitium	Vimentin	0.564	0.023	0.000	1.000
	NICD[c]	0.417	0.096	0.837	0.019
	p-SMAD[d] 2/3	0.649	0.006	0.230	0.620
Intra/Peribronchiolar	Vimentin	0.224	0.404	0.270	0.558
	NICD	0.827	0.0001	0.842	0.018
	p-SMAD 2/3	0.374	0.154	–0.258	0.576

[a]*Bronchiolitis obliterans syndrome.*
[b]*Restrictive allograft syndrome.*
[c]*Notch intracellular domain.*
[d]*Sma and Mad-reated protein.*

patients received rabbit anti-thymocyte globulins and 4 (17%) alemtuzumab. Fifteen (63%) patients were treated by azithromycin at time of CLAD onset, and twelve (50%) underwent extracorporeal photopheresis.

Clinical characteristics of the 51 patients included in the serum analysis are detailed in **Table 1**. Gender and age were equally distributed between the CLAD and control group with 10 (40%) vs 14 (53%) females and 49 ± 14 vs 46 ± 13 years. In both groups, the most frequent underlying diagnosis was COPD (16, 64% vs 13, 50%), followed by cystic fibrosis (4, 16% vs 5, 19%). All twelve patients with RAS received azithromycin compared to nine (69%) patients with BOS ($p = 0.036$). Extracorporeal photopheresis was more often used in BOS (6, 46%) than in RAS (1, 8%) patients ($p = 0.035$).

MiR-21 Expression in CLAD and Control Tissue

MiR-21 was significantly upregulated in CLAD tissue ($2^{-\Delta\Delta Ct}$: 10.1 ± 9.2 vs 4.3 ± 4.4, $p = 0.002$) (**Figure 1**). RT-PCR data were supplemented by *in situ* hybridization of miR-21, in order to

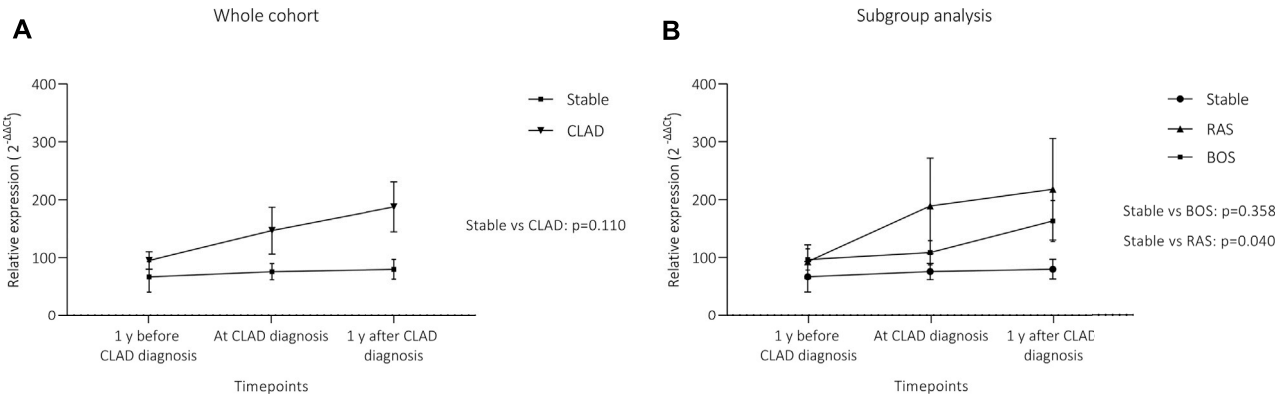


FIGURE 5 | Differences in circulating miR-21 expression over time. **(A)** depicts the comparison between the whole CLAD cohort and stable patients, **(B)** shows results for CLAD subgroups. Although there was a visible trend towards higher miR-21 levels in the whole CLAD cohort, this did not reach the level of significance. However, when the two phenotypes were analyzed separately, RAS patients showed a significant increase in circulating miR-21 over time. MiR-21 expression is presented as $2^{-\Delta\Delta Ct}$ values (Mean + Standard error). Statistical significance was tested using a 2-way repeated measure ANOVA. **(A)** CLAD (n = 25) vs stable (n = 26); **(B)** BOS (n = 13), RAS (n = 12) and stable (n = 26).

identify distinct expression patterns within the lung parenchyma. Five (30%) BOS samples and 6 (86%) RAS samples showed positive ISH staining for miR-21 (**Figure 2**; **Table 2**). In BOS samples, positivity of miR-21 was mainly prevalent in peribronchiolar fibroblasts, in the bronchiolar epithelium as well as in the myofibroblasts of bronchiolar obliterative lesions. In RAS lesions, miR-21 staining was most commonly found in parenchymal fibroblasts and the extracellular matrix (ECM) as well as in the interlobular septa. In both BOS and RAS samples, macrophages showed a positive miR-21 staining (**Supplementary Figure S1**). Of note, miR-21 was mostly absent in control lung specimens, however slight positivity of miR-21 was observed in bronchiolar epithelium.

Expression of EMT Markers

Both, BOS (17, 100%) and RAS (6, 86%) specimens, showed strong expression of vimentin in peribronchiolar myofibroblasts, suggesting mesenchymal differentiation (**Table 2**; **Figures 3**, **4**, **Supplementary Figure S2**). Notch-intracellular domain was positive in 12 (71%) BOS cases, predominantly in the cytoplasm of peribronchiolar myofibroblasts and bronchiolar epithelium, and in 4 (57%) RAS cases in the cytoplasm of interstitial fibroblasts. Finally, staining of p-SMAD 2/3 was positive in 7 (42%) BOS and 4 (57%) RAS specimens. pSMAD-2/3 expression was prevalent in interstitial fibroblasts as well as in the peribronchiolar myofibroblasts and bronchiolar epithelium in BOS allografts while in RAS allografts it was mainly present in interstitial fibroblasts (**Table 2**). β-catenin and E-cadherin stainings were negative in specimens of both phenotypes (**Supplementary Figure S3**). All four markers were negative in control tissue from donor lungs (**Supplementary Figure S4**). Correlations between IHC stainings of EMT markers and miR-21 ISH were calculated with Pearson correlation coefficients (**Table 3**). In BOS specimens, a strong positive correlation was found between miR-21 and p-SMAD 2/3 expression in the interstitium (r = 0.649, p = 0.006) as well as between miR-21 and NICD in the bronchiolar epithelium and myofibroblasts in the BO lesions (r = 0.827, p < 0.001). In RAS specimens, a positive correlation was found between miR-21 staining and NICD expression both in the fibroblasts in the interstitium (r = 0.837, p = 0.019) and peribronchial (r = 0.842, p = 0.018).

Circulating miRNA-21 Before and After CLAD Onset

Figure 5 summarizes analysis of miR-21 expression difference over time between the groups. CLAD patients showed a non-significant trend towards higher miR-21 expression over time compared to stable patients (Panel A, p = 0.110). Panel B shows results of subgroup analysis with RAS patients having a significant increase in serum concentration of miR-21 overtime as compared to stable patients (p = 0.040). No difference was observed between BOS patients and control patients (p = 0.358).

DISCUSSION

Lung transplantation is a well-established treatment for end-stage lung disease; however, long-term success is still impaired by chronic lung allograft dysfunction with a cumulative incidence of about 50% at 5 years after transplantation (1). CLAD pathophysiology is characterized by several stimuli which trigger graft remodeling and irreversible allograft fibrosis (11). Current evidence suggests the activation of TGF-β dependent and independent mechanisms as well as the role micro-RNAs acting as central regulators of EMT in CLAD (3, 6). Our study aimed to investigate the expression of miR-21 in CLAD and to correlate it with a set of key transcription factors of fibroproliferative processes. MiR-21 was expressed in most of the explanted CLAD allografts. Histologic miR-21 data were validated by serum analyses showing that RAS patients tend to have higher levels of circulating miR-21.

Micro-RNAs belong to the group of small non-coding-RNAs. They control post-translational gene expression by binding to mRNA and inducing its degradation or inhibiting its translation. They play a fundamental role in key biological processes including cell development, regulation of immunity and apoptosis (12). The pathogenic role of miR-21 in fibrotic processes has recently been highlighted. MiR-21 was found to be upregulated in lung tissue as well as serum of patients with idiopathic pulmonary fibrosis (13). In addition, miR-21 has been found to play a central role in the development of cardiac and renal fibrosis (14, 15). The transcription of miR-21 is under the control of several transcription factors (e.g., AP-1, SRF, p53, STAT3) (16), targets of miR-21 are manifold. It suppresses cell growth and invasiveness, induces cell cycle stop, inhibits matrix metalloproteases and other proteases, inhibits angiogenesis, cellular branching and migration (16). Moreover, it seems to be involved in an amplifying circuit to enhance TGF-β1 signaling and thus promote the progression of fibrotic lung diseases (6). Only recently, miRNAs were studied in lung transplantation. Xu et al. found a dysregulated set of miRNAs and their target genes in lung recipients with BOS and recipients who developed donor-specific antibodies (17). In mouse models of bronchiolitis obliterans, miR-21, miR-146, miR-20, miR-302, miR-19, miR-98, let-7a, miR-15a were altered in affected animals (18, 19). In 20 BOS patients, analysis of miR-144 showed a 4-fold increase in BOS patients with a parallel reduction of its target, TGF-β–induced factor homeobox 1 (TGIF1) (20). In our study, miR-21 expression was evaluated in 17 BOS and seven explanted RAS allografts. It was found upregulated both in fibroblasts of BO lesions as well as in the interstitial myofibroblasts of RAS specimens. Moreover, macrophages showed strong miR-21 staining. Levels of circulating miR-21 were then longitudinally investigated in lung recipients. Although not significantly significant, miR-21 levels tend to be higher in patients with an established CLAD diagnosis. Taken together, miR-21 may play a role in the fibrotic derangements of CLAD allografts and it might be used as a non-invasive diagnostic marker or serve as a therapeutic target.

Parenchymal injury and inflammation activate stromal fibroblasts, recruit circulating mesenchymal progenitor cells and induce EMT. Inflammatory milieu is the main contributor to excessive tissue remodeling and fibrosis. Macrophages are a potent source of TGF-β, which is one of the main contributors of EMT and fibroblast activation. EMT is the transdifferentiation process of epithelial cells into motile mesenchymal cells. It plays a role in embryonic development and wound healing but also contributes to pathological processes such as fibrosis and cancer progression. EMT is a complex phenomenon, which includes a crosstalk between signaling pathways and transcriptional, translational and post-translational regulation (21). Downregulation of E-cadherin destabilizes adherens junctions and promotes loss of the epithelial barrier function (21). The intermediate filament composition changes with the repression of cytokeratin and the activation of vimentin expression. This could also be confirmed in our study. Immunohistochemical stainings showed a complete absence of E-cadherin and a diffuse expression of vimentin in all explanted CLAD allografts. EMT is regulated by several signaling pathway. The most studied pathway is the TGF-β-SMAD pathway (22). TGF-ß1 up-regulation and SMAD3 activation have been previously described in BO lesion (18, 23, 24). Moreover, miR-21 plays an important role in SMAD-dependent TGF-β1 signal amplification (25). These findings could be confirmed in our patient cohort. High expression of phosphorylated form of SMAD 2/3 was found in the majority of CLAD samples. Concomitant miR-21 expression was confirmed by ISH. MiR-21 was also strongly stained in alveolar macrophages, known source of TGF-β. Alveolar macrophages were found to produce high levels of miR-21 containing liposomes, which induced EMT in tracheal epithelial cells through TGF-β1/Smad signaling pathway (26). Therefore, it is reasonable to hypothesize that alveolar macrophages play a central role in promoting fibroproliferative mechanisms by secreting exosomes containing fibrosis inducers such as cytokines and miRNAs. Though less studied, Wnt/β-catenin and Jagged-Notch signaling pathways also seem to be important in EMT induction (27-29). In our analysis, the active form of Notch was expressed in 69% of BOS and 42% of RAS allograft. This data suggests an active role of the Notch pathway in CLAD pathogenesis and further support the hypothesis that EMT is relevant in CLAD. On the contrary, expression of β-catenin was absent in both, BOS and RAS specimens.

We acknowledge there are several limitations of our study. Despite a relatively high number of re-transplantations performed in our institution, the sample size of lung specimens of RAS patients was small. Second, miR-21 was not measured in lung parenchyma of early-stages CLAD. Transbronchial biopsies have a low sensitivity for bronchiolitis obliterans or interstitial fibrosis (30), thus, they are not routinely performed in these patients. A panel investigation of other profibrinogenic and antifibrogenic microRNAs could have improved our mechanistic understanding of CLAD. Finally, miR-21 positive cells were only defined by their histomorphological appearance. Immunohistological co-stainings would have been required to confirm miR-21 expressing cells types.

In conclusion, this study could show that mir-21 is expressed both in tissue and serum in a large cohort of CLAD patients and its expression significantly increased in RAS patients over time. Moreover, its expression correlates with key markers of EMT. Further research is necessary to elucidate role miR-21 as a therapeutic target of CLAD.

CAPSULE SUMMARY SENTENCE

Long-term outcomes after lung transplantation are still hampered by the development of chronic lung allograft dysfunction (CLAD). Despite all efforts, the pathogenetic mechanisms of CLAD are not fully understood, however, fibroproliferation and epithelialto-mesenchymal transition have recently been described as important factors. Micro-RNAs are post-translational regulators of a variety of pathologic processes and miR-21 has been previously linked

to fibrosis. To the best of our knowledge, our manuscript describes for the first time miR-21 expression in CLAD. We analyzed tissue samples of BOS and RAS allografts, as well as serum samples and could show that this miRNA is highly expressed in both CLAD subtypes. Of note, RAS patients had a significant increase in serum concentration of miR-21 overtime as compared to stable patients. Based on our results, miR-21 could serve as a future therapeutic target for CLAD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of the Medical University of Vienna, Austria (EK-No.2106/2017). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Designed research/study: NM, AB, AI, and KH. Performed research/study: NM, AB, and FO. Contributed important reagents: AB, FO, AI, MH, PJ, WK, and KH. Collected data: NM, AB, FO, VL, and BD. Analyzed data: NM, AB, FO, and KH. Wrote paper: NM, AB, AI, WK, and KH.

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CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10184/full#supplementary-material

Supplementary Figure S1 | Representative images of miR-21 ISH staining of alveolar macrophages (arrow) in BOS patients.

Supplementary Figure S2 | Representative images of Vimentin staining in **(A)** BOS patients and **(B)** RAS patients [**(A,B)** original magnification 10×].

Supplementary Figure S3 | Representative images of β-catenin and E-cadherin staining. in **(A–C)** β-catenin and E-cadherin in BOS patients and **(B–D)** β-catenin and E-cadherin in RAS patients [**(A,B)** original magnification 10×].

Supplementary Figure S4 | Representative images of positive controls. **(A)** pSMAD2,3 **(B)** NOTCH1 **(C)** β-catenin **(D)** E-cadherin **(E)** Vimentin [**(A,B)** original magnification 10×].

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ORIGINAL RESEARCH
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Adult Combined Heart-Liver Transplantation: The United States Experience

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Background: We aimed to review the indications and outcomes of adults undergoing combined heart-liver transplantation (CHLT) in the US using national registry data.

Methods: Adult (≥18 years) CHLT recipients in the United Network for Organ Sharing database were included (09/1987–09/2020; era 1 = 1989–2000, era 2 = 2001–2010, era 3 = 2011–2020). Survival analysis was conducted by means of Kaplan-Meier method, log-rank test, and Cox regression.

Results: We identified 369 adults receiving CHLT between 12/1989–08/2020. The number of adult CHLT recipients (R^2 = 0.75, p < 0.001) and centers performing CHLT (R^2 = 0.80, p < 0.001) have increased over the study period. The most common cardiac diagnosis in the first two eras was restrictive/infiltrative cardiomyopathy, while the most common in era 3 was congenital heart disease (p = 0.03). The 1-, 3-, and 5-years patient survival was 86.8, 80.1, and 77.9%, respectively. In multivariable analysis, recipient diabetes [adjusted hazard ratio (aHR) = 2.35, 95% CI: 1.23–4.48], CHLT between 1989-2000 compared with 2011–2020 (aHR = 5.00, 95% CI: 1.13–22.26), and sequential-liver first CHLT compared with sequential-heart first CHLT (aHR = 2.44, 95% CI: 1.15–5.18) were associated with increased risk of mortality. Higher left ventricular ejection fraction was associated with decreased risk of mortality (aHR = 0.96, 95% CI: 0.92–0.99).

Conclusion: CHLT is being increasingly performed with evolving indications. Excellent outcomes can be achieved with multidisciplinary patient and donor selection and surgical planning.

Keywords: liver transplantation, combined heart-liver transplantation, heart transplantation, United Network for Organ Sharing, patient survival

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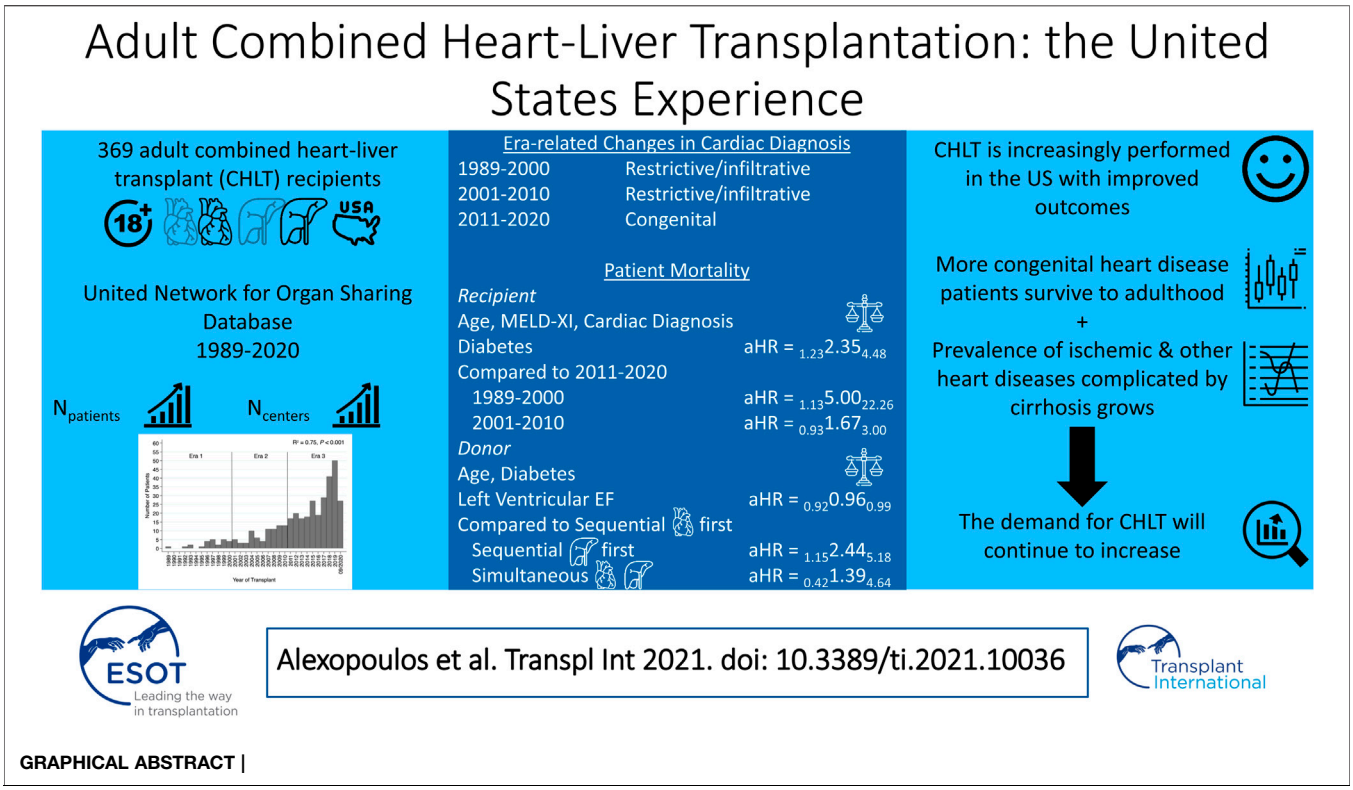
Abbreviations: CHD, congenital heart disease; CHLT, combined heart-liver transplantation; CI, confidence interval; CIT, cold ischemia time; eGFR, estimated glomerular filtration rate; HR, hazard ratio; QR, interquartile range; MELD-XI, model for end-stage liver disease excluding international normalized ratio; UNOS, United Network for Organ Sharing; US, United States.



GRAPHICAL ABSTRACT |

INTRODUCTION

Since the first combined heart-liver transplant (CHLT) in 1984, its indications, patient demographics, and outcomes have evolved significantly [1, 2]. Once a rare and herculean endeavor, CHLT is now being practiced with increasing regularity and improved outcomes [2–7].

The growing practice of CHLT is partially credited to the poor outcomes associated with isolated heart transplantation in the context of concurrent end-stage liver disease. Mortality has been reported to be as high as 50% for patients with known cirrhosis undergoing isolated heart transplantation [8]. In such cases, dual organ transplantation remains the only definitive therapy that can achieve long-term survival. Recent graft survival after CHLT has been found to be similar to that of isolated heart and isolated liver transplantation in carefully selected patients [3].

In the contemporary era, increasing heterogeneity of indications for CHLT as well as variability in listing practices, patient selection, and perioperative management exist. Recipients' complex pathologies vary broadly—from congenital, ischemic, or infiltrative heart diseases with associated congestive hepatopathy, to liver-based metabolic disorders with systemic complications, to cirrhotic cardiomyopathy [3, 9]. As CHLT becomes an increasingly frequent practice, renewed analyses and review of current practices are necessary to optimize patient selection, perioperative practices, and outcomes. Here, we present a comprehensive retrospective review of adult patients undergoing CHLT in the United States (US) between 1989 and 2020 using national registry data.

PATIENTS AND METHODS

Data Source, Patient Identification, Data Encoding

The United Network for Organ Sharing (UNOS) database administers the Organ Procurement and Transplantation Network under contract with the US Department of Health and Human Services. This database contains data on all transplant candidates listed for solid organ transplantation in the US since October 1987. All data are de-identified, and thus no Institutional Review Board approval was required.

We included all adult (≥18 years) patients undergoing CHLT between September 30th, 1987, and September 4th, 2020, in the US. Patient pre-transplant, transplant, and follow-up data were obtained from the UNOS Standard Transplant Analysis and Research data file (released on September 4th, 2020).

For candidates on dialysis, creatinine was set to 4.0 mg/dl [10]. Estimated Glomerular Filtration Rate (eGFR) was estimated by the 4-variable Modification of Diet in Renal Disease Study equation [eGFR = 175 × (serum creatinine in mg/dL)$^{1.154}$ × (age in years)$^{-0.203}$ × 1.212 (if black) × 0.742 (if female)] [11]. Model for End-stage Liver Disease excluding International Normalized Ratio (MELD-XI) score was calculated as MELD-XI = 11.76*ln(serum creatinine in mg/dL) + 5.112*ln(total bilirubin in mg/dL) + 9.44 [12]. Patients were grouped in the

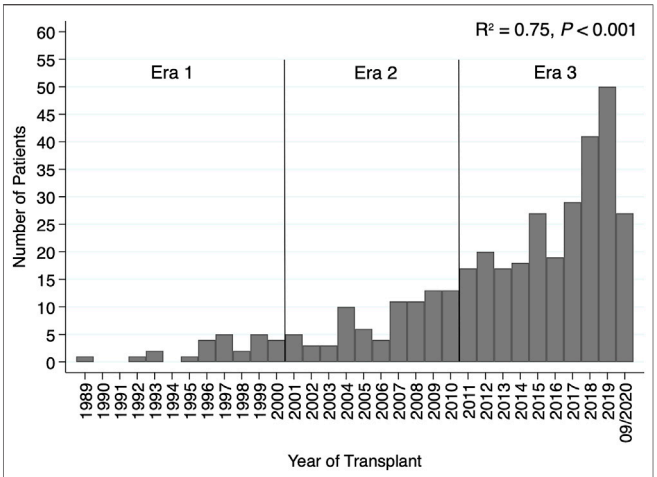


FIGURE 1 | Bar plot demonstrating an increase in the number of patients receiving combined heart-liver transplantation by transplant year.

following five cardiac diagnosis subgroups: 1) restrictive/infiltrative cardiomyopathy, 2) ischemic heart disease, 3) congenital heart disease, 4) dilated non-ischemic cardiomyopathy, and 5) other. Transplant sequence was determined by subtracting the cardiac cold ischemia time (CIT) from the liver CIT (<−30 min: sequential-liver first; −30 to 30 min: simultaneous; >30 min: sequential-heart first). Transplant era groups were generated by decade as follows: era 1 = 1989–2000, era 2 = 2001–2010, and era 3 = 2011–2020.

Statistical Analysis

Categorical variables are described as frequencies and percentages and compared with the chi-square or Fisher's exact test, while continuous variables are presented as medians [interquartile ranges (IQR)] and compared with the Kruskal-Wallis test. Univariable linear regression was used to assess the number of patients receiving and the number of centers performing CHLT over the study period. Patient survival was the main outcome of interest and was determined as the duration from the date of CHLT until the date of last patient contact or death. Survival analysis was performed using the Kaplan-Meier method, and between-group comparisons were performed with the log-rank test. Cox proportional hazards regression models were fitted to estimate the hazard ratio (HR) and 95% confidence interval (CI). The variables incorporated in the multivariable model were pre-specified to avoid the inferential limitations around selecting covariates for multivariable analysis based on stepwise procedures or univariable comparisons [13]. The multivariable Cox model investigating risk factors of patient mortality in the total cohort included recipient age at transplant, diabetes at listing, MELD-XI score at transplant, cardiac diagnosis, transplant era, donor age, donor diabetes, donor left ventricular ejection fraction, and transplant sequence. The median follow-up time was calculated using the reverse Kaplan-Meier method [14]. To determine the potential impact of annual isolated heart transplant and isolated liver transplant center volume on CHLT outcomes, all centers performing CHLT were classified in tertiles (low, medium, high) based on their isolated heart transplant and isolated liver transplant volume for each given year that each CHLT was performed. All statistical analyses were conducted using Stata IC 16.0 (StataCorp LLC, College Station, TX). All p-values were based on two-sided statistical tests, and significance was set at <0.05.

RESULTS

Patient Demographics and Clinical Characteristics

A total of 369 adult patients who received CHLT between December 1989 and August 2020 were identified. No CHLT recipients were identified between October 1987 and December 1989. The median follow-up time was 49.2 months (95% CI: 42.7–60.9) for the whole cohort. Both the number of adult patients receiving CHLT (R^2 = 0.75, p < 0.001; **Figure 1**) and the number of centers performing CHLT (R^2 = 0.80, p < 0.001) increased significantly over the study period. The number of centers performing CHLT was 10 between 1989 and 2000, 20 between 2001 and 2010, and 46 between 2011 and 2020.

Several differences in patient characteristics were identified among the three transplant eras. The median MELD score at transplant was higher in patients transplanted between 2011 and 2020 compared with those transplanted between 2001 and 2010 (16.0 vs. 13.5; p = 0.007). On the other hand, median MELD-XI was lower in patients transplanted in more recent eras (17.5 vs. 12.4 vs. 11.5; p = 0.007). Among the three eras, median waitlist time was significantly shorter in the most recent era (era 1: 86 days vs. era 2: 128 days vs. era 3: 82 days; p = 0.045). Cardiac diagnosis was also significantly different among the three eras (p = 0.03); the most common cardiac diagnosis in the first two eras was restrictive/infiltrative cardiomyopathy, while the most common cardiac diagnosis in the most recent era was congenital heart disease (CHD). During the first two eras, nearly all CHLTs were sequential-heart first (100 and 97.1%, respectively), while in the most recent era 79.9% were sequential-heart first, 13.8% sequential-liver first, and 6.3% simultaneous (p = 0.001). Two donor livers in era 3 underwent machine perfusion. A detailed comparison of patient characteristics among the three transplant eras is depicted in **Table 1**.

Several differences in patient characteristics were identified among the cardiac diagnosis groups. The majority of patients in the restrictive/infiltrative cardiomyopathy, ischemic heart disease, and dilated non-ischemic cardiomyopathy groups were male, while the sex proportions were more equally distributed in the CHD and other groups (p < 0.001). The CHD group had lower median age (p < 0.001) and MELD-XI at transplant (p = 0.01) compared with the other diagnosis groups, and together with the restrictive/infiltrative cardiomyopathy group had longer median waitlist times compared with the other three diagnosis groups (p < 0.001). Additionally, as compared with other cardiac diagnosis groups, a higher proportion of the CHD group had undergone prior cardiac surgery at transplant (p < 0.001) and had received sequential-liver first and simultaneous

TABLE 1 | Patient baseline demographic and clinical characteristics by era.

Characteristics	Total (*n* = 369)	1989–2000 (*n* = 25)	2001–2010 (*n* = 79)	2011–2020 (*n* = 265)	*p*-Value
Recipient					
Sex					
Female	113 (30.6%)	8 (32.0%)	19 (25.1%)	86 (32.5%)	0.36
Male	256 (69.4%)	17 (68.0%)	60 (76.0%)	179 (67.6%)	
Age at listing (years)					
Median (IQR)	49.0 (37.0–58.0)	45.0 (27.0–56.0)	48.0 (36.0–56.0)	50.0 (38.0–58.0)	0.30
Age at transplant (years)					
Median (IQR)	49.0 (37.0–58.0)	45.0 (28.0–57.0)	48.0 (36.0–57.0)	50.0 (38.0–58.0)	0.34
Waitlist time (days)					
Median (IQR)	96.0 (36.0–244.0)	86.0 (31.0–350.0)	128.0 (56.0–295.0)	82.0 (35.0–212.0)	0.045
Laboratory MELD score at transplant (*n* = 339)					
Median (IQR)	16.0 (11.0–20.0)	-	13.5 (10.0–19.0)	16.0 (12.0–20.0)	0.007
MELD-XI score at transplant (*n* = 368)					
Median (IQR)	11.9 (8.2–16.3)	17.5 (11.6–22.0)	12.4 (9.0–16.8)	11.5 (7.7–15.5)	0.007
Serum creatinine at transplant (mg/dl) (*n* = 368)					
Median (IQR)	1.2 (0.9–1.6)	1.2 (1.0–2.1)	1.3 (1.0–1.6)	1.2 (0.9–1.6)	0.64
Diabetes at listing (*n* = 355)					
No	295 (83.1%)	17 (89.5%)	65 (87.8%)	213 (81.3%)	0.31
Yes	60 (16.9%)	2 (10.5%)	9 (12.2%)	49 (18.7%)	
Dialysis the week prior to transplant (*n* = 361)					
No	345 (95.6%)	17 (94.4%)	77 (97.5%)	251 (95.1%)	0.62
Yes	16 (4.4%)	1 (5.6%)	2 (2.5%)	13 (4.9%)	
eGFR at transplant (ml/min/1.73 m^2) (*n* = 368)					
Median (IQR)	61.2 (45.2–81.6)	63.7 (28.7–83.5)	58.9 (45.1–81.9)	62.0 (45.8–81.4)	0.89
CKD stage at transplant (*n* = 368)					
Stage 1	70 (19.0%)	5 (20.8%)	14 (17.7%)	51 (19.3%)	0.01
Stage 2	117 (31.8%)	7 (29.2%)	24 (30.4%)	86 (32.5%)	
Stage 3a	82 (22.3%)	3 (12.5%)	20 (25.3%)	59 (22.3%)	
Stage 3b	62 (16.9%)	2 (8.3%)	11 (13.9%)	49 (18.5%)	
Stage 4	20 (5.4%)	5 (20.8%)	8 (10.1%)	7 (2.6%)	
Stage 5	17 (4.6%)	2 (8.3%)	2 (2.5%)	13 (4.9%)	
BMI at transplant (kg/m^2) (*n* = 367)					
Median (IQR)	24.5 (21.9–28.3)	23.7 (20.6–27.2)	24.1 (21.1–27.3)	24.9 (21.9–28.6)	0.20
On ventilator at transplant					
No	348 (94.3%)	22 (88.0%)	71 (89.9%)	255 (96.2%)	0.03
Yes	21 (5.7%)	3 (12.0%)	8 (10.1%)	10 (3.8%)	
ICU at transplant (*n* = 365)					
No	201 (55.1%)	15 (60.0%)	53 (67.1%)	133 (51.0%)	0.04
Yes	164 (44.9%)	10 (40.0%)	26 (32.9%)	128 (49.0%)	
Cardiac diagnosis					
Restrictive/infiltrative cardiomyopathy	109 (29.5%)	9 (36.0%)	29 (36.7%)	71 (26.8%)	0.03
Ischemic heart disease	42 (11.4%)	2 (8.0%)	6 (7.6%)	34 (12.8%)	
Congenital heart disease	98 (26.6%)	3 (12.0%)	13 (16.5%)	82 (30.9%)	
Dilated non-ischemic cardiomyopathy	80 (21.7%)	6 (24.0%)	18 (22.8%)	56 (21.1%)	
Other	40 (10.8%)	5 (20.0%)	13 (16.5%)	22 (8.3%)	
Prior cardiac surgery at transplant (*n* = 319)					
No	175 (54.9%)	-	41 (68.3%)	134 (51.7%)	0.02
Yes	144 (45.1%)	-	19 (31.7%)	125 (48.3%)	
VAD at transplant (*n* = 333)					
No	309 (92.8%)	-	67 (93.1%)	242 (92.7%)	0.92
Yes	24 (7.2%)	-	5 (6.9%)	19 (7.3%)	
Cigarette use at listing (*n* = 322)					
No	209 (64.9%)	-	29 (50.0%)	180 (68.2%)	0.009
Yes	113 (35.1%)	-	29 (50.0%)	84 (31.8%)	
Liver diagnosis					
Amyloidosis	74 (20.1%)	7 (28.0%)	19 (24.1%)	48 (18.1%)	<0.001
Cardiac cirrhosis	123 (33.3%)	1 (4.0%)	15 (19.0%)	107 (40.4%)	
NASH	9 (2.4%)	0 (0.0%)	0 (0.0%)	9 (3.4%)	
Alcoholic liver disease	12 (3.3%)	2 (8.0%)	2 (2.5%)	8 (3.0%)	
Other	151 (40.9%)	15 (60.0%)	43 (54.4%)	93 (35.1%)	
Donor					
Age (years)					
Median (IQR)	28.0 (21.0–38.0)	24.0 (17.0–34.0)	30.0 (21.0–43.0)	28.0 (22.0–38.0)	0.04
Donor-to-recipient height ratio (*n* = 366)					

(Continued on following page)

TABLE 1 | (*Continued*) Patient baseline demographic and clinical characteristics by era.

Characteristics	Total (*n* = 369)	1989–2000 (*n* = 25)	2001–2010 (*n* = 79)	2011–2020 (*n* = 265)	*p*-Value
Median (IQR)	1.00 (0.96–1.04)	1.01 (0.97–1.08)	0.99 (0.94–1.03)	1.00 (0.96–1.04)	0.04
Left ventricular ejection fraction (%) (*n* = 343)					
Median (IQR)	62.0 (59.0–65.0)	60.0 (52.5–62.5)	65.0 (56.0–65.0)	61.5 (60.0–65.0)	0.50
Diabetes (*n* = 361)					
No	352 (97.5%)	21 (100.0%)	78 (98.7%)	253 (96.9%)	0.82
Yes	9 (2.5%)	0 (0.0%)	1 (1.3%)	8 (3.1%)	
Liver CIT (hours) (*n* = 352)					
Median (IQR)	7.0 (5.3–8.0)	7.2 (6.7–9.0)	6.7 (6.0–8.0)	7.0 (5.0–8.0)	0.06
Heart CIT (hours) (*n* = 359)					
Median (IQR)	3.0 (2.3–3.8)	2.8 (2.3–3.1)	2.5 (1.9–3.2)	3.1 (2.5–3.9)	<0.001
Transplant sequence (*n* = 344)					
Simultaneous	17 (4.9%)	0 (0.0%)	1 (1.4%)	16 (6.3%)	0.001
Sequential-heart first	291 (84.6%)	20 (100.0%)	68 (97.1%)	203 (79.9%)	
Sequential-liver first	36 (10.5%)	0 (0.0%)	1 (1.4%)	35 (13.8%)	

Abbreviations: CIT, cold ischemia time; CKD, chronic kidney Disease; eGFR, estimated glomerular filtration rate; ICU, intensive care unit; INR, international normalized ratio; IQR, interquartile range; MELD, model for end-stage liver disease; MELD-XI, model for end-stage liver disease excluding INR; NASH, nonalcoholic steatohepatitis; VAD, ventricular-assist device.

Note: Continuous variables are presented as median (interquartile range) and categorical variables as frequencies (%).

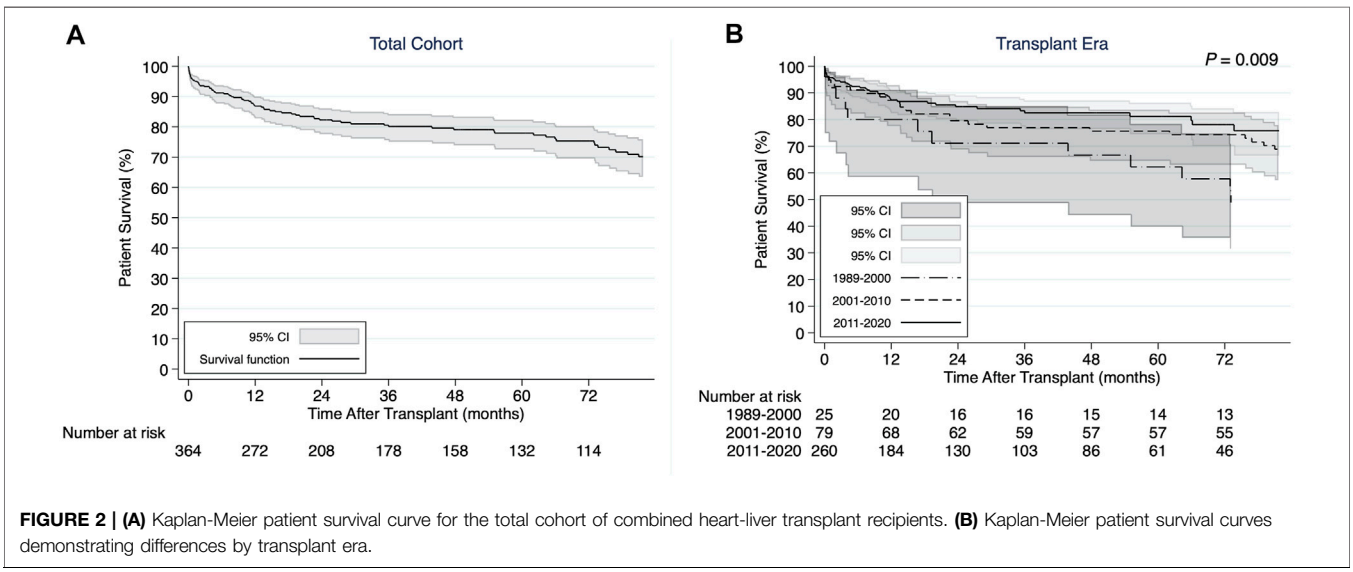


FIGURE 2 | (A) Kaplan-Meier patient survival curve for the total cohort of combined heart-liver transplant recipients. **(B)** Kaplan-Meier patient survival curves demonstrating differences by transplant era.

CHLT ($p < 0.001$). A detailed comparison of patient characteristics among the five cardiac diagnosis groups is depicted in **Supplementary File S1**.

Survival Outcomes

The 1-, 3-, and 5-years cumulative patient survival point estimates after CHLT for the total cohort were 86.8, 80.1, and 77.9%, respectively (**Figure 2A**). For those who survived at least 1 year after CHLT ($n = 286$), the 3- and 5-years cumulative patient survival point estimates were 92.6 and 90.3%, respectively. Six patients required liver retransplant over a median post-CHLT period of 19 days (IQR: 13.0–441.0) with indications being hepatic artery thrombosis ($n = 2$), acute rejection ($n = 1$), primary graft failure ($n = 1$), severe preservation injury ($n = 1$), and unknown ($n = 1$). One patient required heart retransplant 12 days post-CHLT due to primary nonfunction. In the total cohort, statistically significant differences in unadjusted patient survival were observed between the three transplant eras ($p = 0.009$; **Figure 2B**). More specifically, patients undergoing CHLT between 1989 and 2000 demonstrated 2.5 times higher risk of mortality (95% CI: 1.37–4.53; $p = 0.003$) compared with those undergoing CHLT between 2011 and 2020, while no statistically significant differences in survival were observed between those undergoing CHLT between 2001 and 2010 and between 2011 and 2020 (HR = 1.38, 95% CI: 0.85–2.25; $p = 0.19$) (**Supplementary File S2**). No statistically significant differences were observed in unadjusted patient survival among the cardiac diagnosis groups ($p = 0.85$; **Figure 3**). In univariable Cox regression analysis

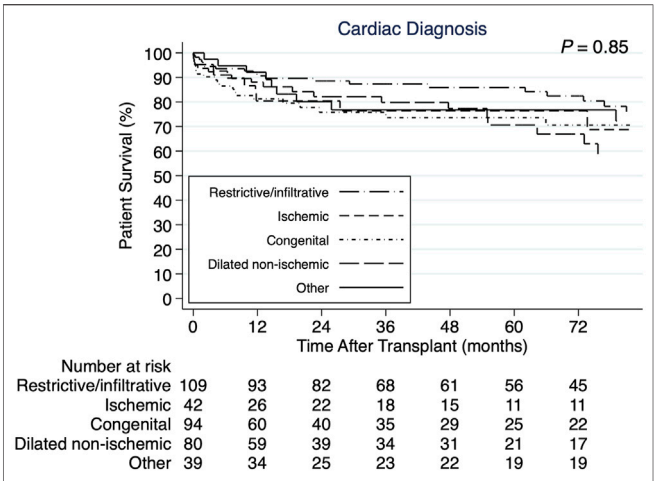


FIGURE 3 | Kaplan-Meier patient survival curves demonstrating no statistically significant difference by cardiac diagnosis.

TABLE 2 | Multivariable analysis for association among recipient and donor characteristics with patient survival.

Characteristics (n = 312)	Hazard ratio (95% CI)	*p*-value
Recipient		
Age at transplant (years)	0.98 (0.96–1.01)	0.19
Diabetes at listing (ref: no)	2.35 (1.23–4.48)	0.009
MELD-XI score at transplant	1.01 (0.97–1.05)	0.56
Cardiac diagnosis (ref: restrictive/infiltrative cardiomyopathy)		
Ischemic heart disease	0.83 (0.34–1.98)	0.67
Congenital heart disease	0.81 (0.36–1.83)	0.61
Dilated non-ischemic cardiomyopathy	0.88 (0.42–1.84)	0.74
Other	0.57 (0.22–1.49)	0.25
Transplant era (ref: 2011–2020)		
1989–2000	5.00 (1.13–22.26)	0.03
2001–2010	1.67 (0.93–3.00)	0.09
Donor		
Age (years)	1.00 (0.98–1.03)	0.70
Left ventricular ejection fraction (%)	0.96 (0.92–0.99)	0.01
Diabetes (ref: no)	0.97 (0.23–4.13)	0.97
Transplant sequence (ref: Sequential-heart first)		
Simultaneous	1.39 (0.42–4.64)	0.59
Sequential-liver first	2.44 (1.15–5.18)	0.02

Abbreviations: CI, confidence interval; MELD-XI, model for end-stage liver disease excluding INR.

(**Supplementary File S2**), recipient diabetes at listing was associated with an increased risk of patient mortality (HR = 1.72, 95% CI: 1.01–2.94; p = 0.047) and higher donor left ventricular ejection fraction with a decreased risk of patient mortality (HR = 0.96, 95% CI: 0.93–0.99; p = 0.02). Nevertheless, no statistically significant difference between the groups was determined, when classifying each center performing CHLT as low, medium, or high volume based on either their annual isolated heart transplant (p = 0.18) volume or their annual isolated liver transplant volume (p = 0.87).

In multivariable Cox regression analysis (**Table 2**), recipient diabetes at listing (adjusted HR = 2.35, 95% CI: 1.23–4.48; p = 0.009), receiving CHLT between 1989 and 2000 compared with 2011–2020 (adjusted HR = 5.00, 95% CI: 1.13–22.26; p = 0.03), and receiving sequential-liver first CHLT compared with sequential-heart first CHLT (adjusted HR = 2.44, 95% CI: 1.15–5.18; p = 0.02) were associated with an increased risk of patient mortality after CHLT. Higher donor left ventricular ejection fraction was associated with a decreased risk of patient mortality after CHLT (adjusted HR = 0.96, 95% CI: 0.92–0.99; p = 0.01).

DISCUSSION

The annual number of adult CHLT in the US has risen sharply, with more CHLT performed during the past 2 years than during either of the previous 2 decades, and a more than four-fold increase over time in the number of centers offering this therapy. While our data demonstrate progressive, era-related improvements in outcomes after CHLT, an appreciation for evolving patient characteristics and indications for CHLT, as well as best practices in surgical techniques, will be critical to ensure appropriate patient selection and favorable outcomes going forward.

Among the most significant changes in CHLT in recent decades has been an evolution in the indications for this procedure. Although restrictive/infiltrative cardiomyopathies secondary to diseases such as amyloidosis and hemochromatosis were the most common indication for CHLT in the early era, CHD is now the most common indication, accounting for nearly one third of CHLT. This trend corresponds to the rising prevalence of liver disease among children with single-ventricle physiology palliated with Fontan. Current life-expectancy post-Fontan exceeds 25 years, by which point many patients develop advanced heart failure and Fontan-associated liver disease manifesting with peri-central and peri-sinusoidal hepatic fibrosis which may progresses to cirrhosis, with increased risk of hepatocellular carcinoma [15, 16]. Even in hemodynamically well-compensated patients, isolated liver transplantation in this population has been ill-advised due to inability to manage elevated right-sided pressures during the anhepatic and reperfusion phases [15]. Despite a progressive era-related increase in use of a sequential-liver first approach for patients with CHD, our data suggest that this approach is associated with worse outcomes.

As would be expected, we identified significant differences in the characteristics of patients undergoing CHLT, based on cardiac diagnosis. Interestingly, however, cardiac diagnosis in and of itself was not associated with differences in post-transplant survival, nor was recipient age, prior tobacco use or MELD-XI score at transplant. Conversely, recipient diabetes, liver-first surgical sequence, and lower donor left ventricular ejection fraction were each independently associated with worse post-CHLT outcomes. These findings underscore the importance of appropriate patient and donor selection for CHLT based on individual patient and donor characteristics, as well as the need for thoughtful pre-operative planning among surgeons from both heart and liver disciplines [12, 17–20]. Despite all of these challenges, the reported survival outcomes of CHLT are similar to those of heart transplant alone [4, 21].

The issue of identification of specific donor factors impacting outcomes persists and may be confounded by changes in donor selection criteria over the years to identify excellent donors, but also a change towards more lenient selection criteria as experience grows. This is supported by the higher donor age, liver and

heart CIT, proportion of diabetic donors, as well as by the more optimal donor-recipient height matching and the lower left ventricular ejection fraction in the most recent era. At our center, candidates for CHLT are evaluated jointly by our heart and liver transplant teams and discussed in a multidisciplinary forum that includes transplant cardiologists and hepatologists, adult (and sometimes pediatric) surgeons and, when appropriate, members of the adult CHD team. Upon listing of patients and prior to transplant, surgeons agree on a peri-operative strategy. Team members of both organ programs take part in donor selection. Future research on the optimization of donor selection would enable improved donor-recipient matching. Additionally, the advent and increasing utilization of donor liver machine perfusion may be particularly useful in CHLT as it can mitigate the effects of increased liver graft preservation time while allowing the heart transplant to occur without time pressure constraints [22].

Although the present analysis represents the largest, most comprehensive review of US patients undergoing CHLT during recent decades, certain limitations should be considered when interpreting the results of our study. Due to its retrospective nature, the present study imparts a degree of selection bias regarding patient selection and management. Additionally, there is inconsistency or lack of reporting of parameters that may influence patient survival (i.e., anatomical complexity and number of prior surgeries of the CHD patients, pathologic degree of liver involvement, rationale of performing sequential-liver first CHLT, biliary complications, abortion of liver transplant because of heart transplant induced issues). Lastly, the statistically insignificant results in certain variables may be attributed to lack of power to detect the presence of a potential association.

In conclusion, as more CHD patients survive to adulthood and the prevalence of ischemic and other heart diseases complicated by cirrhosis increases, CHLT will be increasingly necessary to help extend lives. Our data suggest that in the contemporary era, appropriate patient selection for CHLT combined with thoughtful surgical planning and donor selection allow for excellent patient outcomes.

CAPSULE SENTENCE SUMMARY

The aim of this paper was to present a comprehensive retrospective review of adult patients undergoing combined heart-liver transplantation (CHLT) in the United States between 1989 and 2020 using national registry data. According to our findings, CHLT is being increasingly performed with evolving indications as more congenital heart disease patients survive to adulthood and the prevalence of ischemic and other heart diseases complicated by cirrhosis increases. Additionally, in the contemporary era, appropriate patient selection for CHLT combined with thoughtful surgical planning and donor selection allow for excellent patient outcomes. Overall, we believe that our work is of increased interest and educational value to the readership of Transplant International and anticipate to decisively influence current perspectives in the field of CHLT.

AUTHOR'S NOTE

Orally presented at the American Transplant Congress—June 4–9, 2021.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the Standard Transplant Analysis and Research file from the United Network for Organ Sharing. Requests to access the datasets should be directed to https://optn.transplant.hrsa.gov/data/request-data/.

AUTHOR CONTRIBUTIONS

SA: conception and design, analysis and interpretation of data, drafting of the manuscript, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. WW: analysis and interpretation of data, drafting of the manuscript, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. IZ: conception and design, analysis and interpretation of data, drafting of the manuscript, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. LM: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. MR: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. MI: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. RP: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. KS: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. JM: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work. AS: analysis and interpretation of data, revising the manuscript critically for important intellectual content, final approval of the manuscript submitted, agreement to be accountable for all aspects of the work.

AUTHOR DISCLAIMER

The data reported here have been supplied by the United Network for Organ Sharing as the contractor for the Organ Procurement and Transplantation Network. The interpretation and reporting of these data are the responsibility of the author(s) and in no way should be seen as an official policy of or interpretation by the OPTN or the U.S. Government.

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.2021.10036/full#supplementary-material

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ORIGINAL RESEARCH
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A Novel Strategy for Preventing Posttransplant Large-For-Size Syndrome in Adult Liver Transplant Recipients: A Pilot Study

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There are two causes of graft compression in the large-for-size syndrome (LFSS). One is a shortage of intra-abdominal space for the liver graft, and the other is the size discrepancy between the anteroposterior dimensions of the liver graft and the lower right hemithorax of the recipient. The former could be treated using delayed fascial closure or mesh closure, but the latter may only be treated by reduction of the right liver graft to increase space. Given that split liver transplantation has strict requirements regarding donor and recipient selections, reduced-size liver transplantation, in most cases, may be the only solution. However, surgical strategies for the reduction of the right liver graft for adult liver transplantations are relatively unfamiliar. Herein, we introduce a novel strategy of HuaXi-*ex vivo* right posterior sectionectomy while preserving the right hepatic vein in the graft to prevent LFSS and propose its initial indications.

Keywords: large-for-size syndrome, reduced-size liver transplantation, *ex vivo* right posterior sectionectomy, size mismatch, right anteroposterior vertical distance, graft-recipient weight ratio

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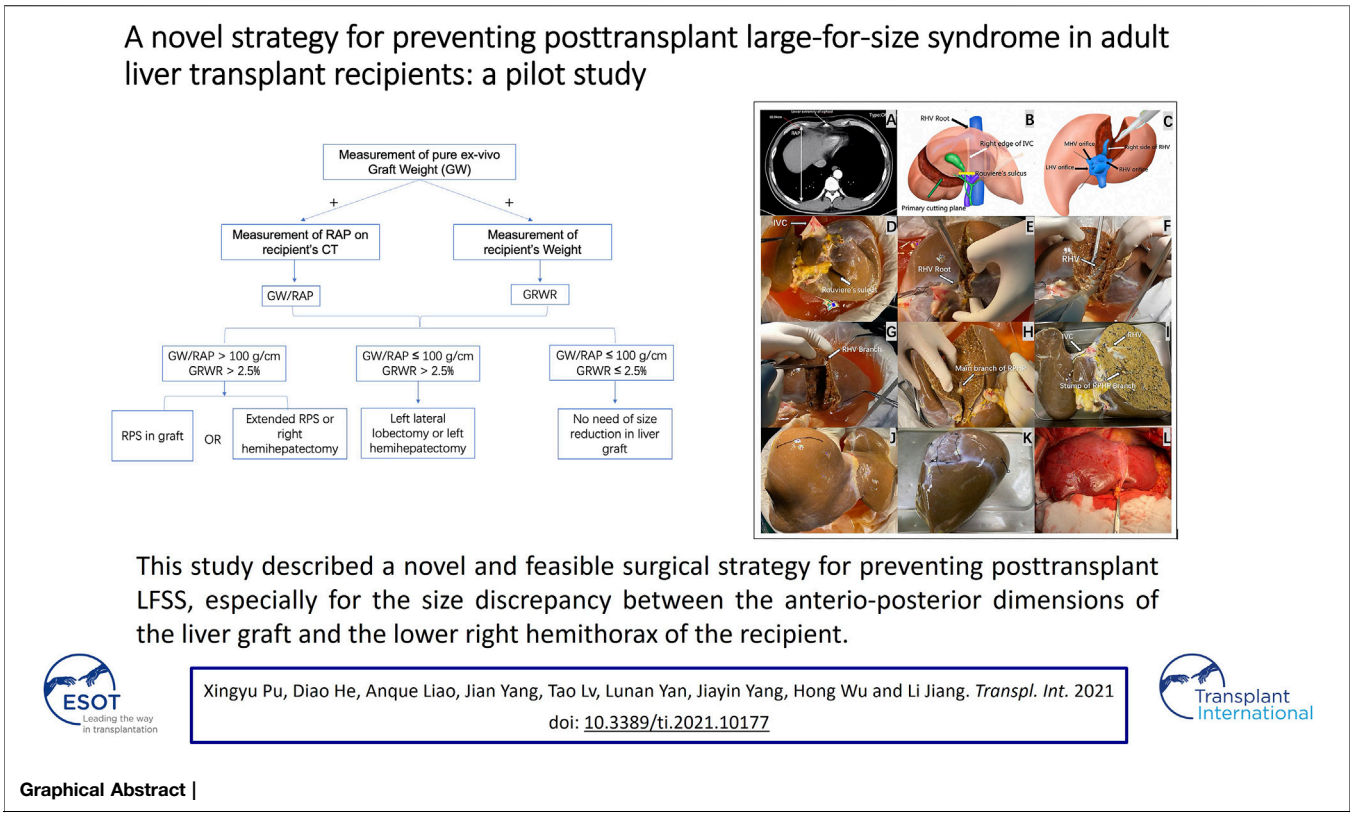
INTRODUCTION

Large-for-size syndrome (LFSS) usually occurs in paediatric liver transplantation (LT) due to the implantation of an excessively large liver graft into a small recipient cavity, resulting in poor graft or recipient outcomes.(1, 2) However, in recent years, with the increased prevalence of obesity epidemic among the donor pool, the incidence of LFSS tends to increase in adult LTs.(3) In addition, the present organ-allocation system is mainly based on scores reflecting the severity of liver disease without any consideration of the morphological parameter mismatch between the donor and recipient.(4) Therefore, transplant surgeons can encounter graft-recipient size mismatch in adult LTs.

Abbreviations: LFSS, large-for-size syndrome; LT, liver transplantation; SLT, split liver transplantation; RSLT, reduced-size liver transplantation; eRPS, *ex vivo* right posterior sectionectomy; RHV, right hepatic vein; RAP, right anteroposterior; GRWR, graft-recipient weight ratio; GW, graft weight; IVC, inferior vena cava; RPHP, right posterior hepatic pedicle; CUSA, cavitron ultrasonic surgical aspirator; HuaXi-eRPS, HuaXi-*ex vivo* right posterior sectionectomy; FHB, fulminant hepatitis B; SFSS, small-for-size syndrome.



Graphical Abstract |

TABLE 1 | A short review of the literature regarding graft reduction.

Author	Year	Recipient age (year)	Recipient gender	GRWR (%)	Reduced-size method	Surgery time (min)	Blood loss (ml)	PHS (day)	Outcome
Kim et al. (6)	2019	44	Female	3.49%	*in vivo* left lateral sectionectomy	NA	NA	45	IVC stenosis and liver and kidney dysfunction
Nagatsu et al(7)	2017	58	Female	2.74%	*in vivo* right posterior sectionectomy	554	935	21	No complication
Kim et al(8)	2015	36	Female	3.98%	*in vivo* right hemihepatectomy	386	14,000	NA	No complication
Eldeen et al(9)	2013	49	Female	NA	*ex vivo* left lateral segmentectomy	NA	NA	NA	Death due to sepsis and multiorgan failure

GRWR, graft-recipient weight ratio; IVC, inferior vena cava; NA, not available; PHS, postoperative hospital stay.

There are two causes of graft compression in LFSS. One is a shortage of intra-abdominal space for the graft, and the other is the size discrepancy between the anteroposterior dimensions of the graft and the lower right hemithorax of the recipient. The former could be treated using delayed fascial closure or mesh closure; however, the latter may only be treated by reduction of the right liver graft to increase space. Given that split liver transplantation (SLT) has strict requirements for donor and recipient selections,(5) reduced-size liver transplantation (RSLT), in most cases, may be the only solution. A short review of the literature(6-9) regarding the standard techniques used for graft reduction is listed in **Table 1**. Herein, we introduce a novel strategy of *ex vivo* right posterior sectionectomy (eRPS) while preserving the right hepatic vein (RHV) in the graft to prevent LFSS and propose its initial indications.

METHODS

It is dangerous for donors to undergo computed tomography (CT) examinations during organ maintenance in the intensive care unit (ICU), although CT is the most accurate method to measure the graft's right anteroposterior (RAP) vertical distance and the largest horizontal distance. Hence, in our centre, we do not perform CT imaging on donors to ensure the safety of donors during organ maintenance in the ICU. eRPS was performed in five grafts between January 2019 and November 2020.

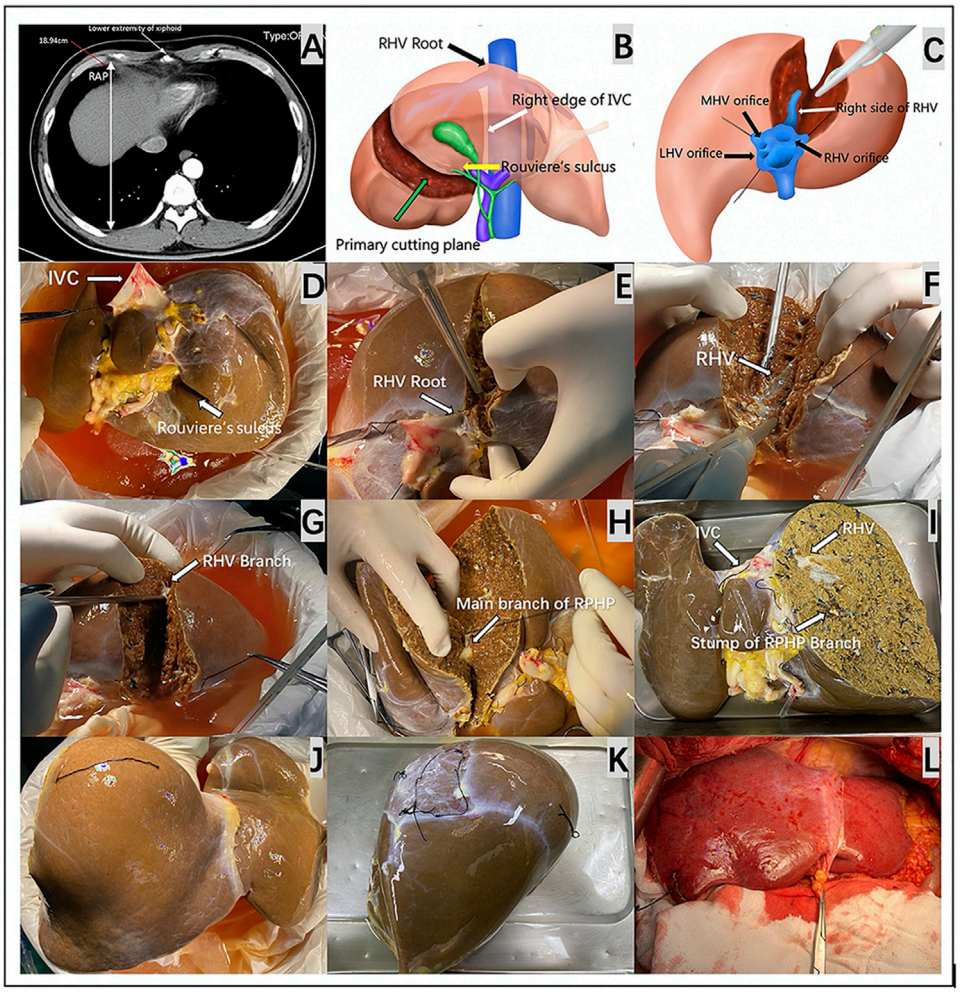


FIGURE 1 | The key preoperative assessment and surgical procedures for HuaXi-eRPS. **(A)** The longest RAP vertical distance between the anterior and posterior parts of the ribs at the lower extremity of the xiphoid process is preoperatively measured on a CT scan for the recipient. **(B)** The primary cutting plane for HuaXi-eRPS is designed according to the right side of the RHV root (black arrow) entering into the suprahepatic IVC, right edge of the retrohepatic IVC (white arrow), and Rouviere's sulcus (yellow arrow). **(C)** Parenchymal transection is designed to be started from the cranial side of the main RHV to the caudal direction, and the right side of the RHV (white arrow) is used as the surgical marker to navigate the intrahepatic transection. **(D)** The view on the visceral surface of the whole liver graft. IVC (long arrow); Rouviere's sulcus (short arrow). **(E)** Parenchymal transection is started from the cranial side of the main RHV root (arrow) to the caudal direction. **(F)** The right side of the RHV (arrow) is used as the surgical marker to navigate the intrahepatic transection. **(G)** Dissection of the RHV branch (arrow) entering into segment VI. **(H)** Dissection of the main branch of RPHP (arrow). **(I)** The view on the visceral surface of the remnant liver graft after HuaXi-eRPS. **(J)** The view on the diaphragmatic surface of the remnant liver graft after HuaXi-eRPS. **(K)** The view on the diaphragmatic surface of the resected right posterior sector. **(L)** Implantation of the reduced-size liver graft into the recipient. HuaXi-eRPS, HuaXi-*ex vivo* right posterior sectionectomy; IVC, inferior vena cava; LHV, left hepatic vein; MHV, middle hepatic vein; RAP, right anteroposterior; RHV, right hepatic vein; RPHP, right posterior hepatic pedicle.

Regarding the recipients, we defined the longest RAP vertical distance between the anterior and posterior parts of the ribs at the lower extremity of the xiphoid process on a CT scan (**Figure 1A**). Both graft-recipient weight ratio (GRWR) > 2.5% and graft weight (GW)/RAP > 100 g/cm indicated the need for reduction of the right liver graft. The estimated mean volume of the right posterior sector was approximately 27.9% of the total liver volume.(10) Based on these parameters, we can estimate the weight of the remnant graft after eRPS and if both new GRWR and GW/RAP could be reduced to normal values (≤2.5% and 100 g/cm, respectively). Therefore, it was considered acceptable to perform the eRPS. A detailed flow chart is shown in **Figure 2**.

All organs were donated after death, and no organs were obtained from executed prisoners. eRPS was performed on the back table. The primary cutting plane was designed according to the right side of the RHV root into the suprahepatic inferior vena cava (IVC), right edge of the retrohepatic IVC, and Rouviere's sulcus (**Figure 1B**). Parenchymal transection was started from the cranial side of the main RHV to the caudal direction, which was similar to the cranial approach in laparoscopic anatomic liver resection (**Figure 1C**). We mainly used the right side of the RHV as the surgical marker to navigate the intrahepatic transection. The cutting point for the main branch of the right posterior hepatic pedicle (RPHP) was in Rouviere's sulcus and was distant

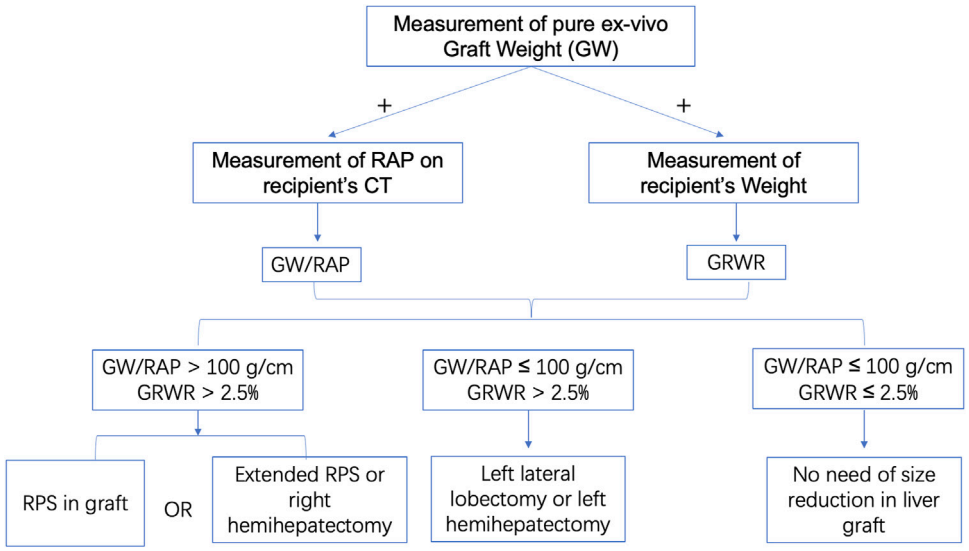


FIGURE 2 | The flow chart of using GW/RAP and GRWR. First, we calculate the GW/RAP and GRWR. Subsequently, if GW/RAP > 100 g/cm and GRWR > 2.5%, RPS or extended RPS or right hemihepatectomy will be considered in graft; if GW/RAP ≤ 100 g/cm and GRWR > 2.5%, left lateral lobectomy or left hemihepatectomy will be considered in graft; if GW/RAP ≤ 100 g/cm and GRWR ≤ 2.5%, no size reduction will be considered in graft. GRWR, graft-recipient weight ratio; GW/RAP, graft weight/right anteroposterior vertical distance; RPS, right posterior sectionectomy.

from the porta hepatis, which may prevent damage to the right anterior hepatic pedicle (**Figures 1D–I**). Cavitron ultrasonic surgical aspirator combined with a harmonic scalpel was used to dissect the liver parenchyma, and intrahepatic larger ducts of more than 3 mm were ligated or clipped. The main branch of the RPHP was clipped or transected using a linear stapler. Hemostasis was achieved using the Aquamantys System (Medtronic Advanced Energy, United States). Any potential leaks were carefully detected via repeated organ perfusion and sutured before implantation, and the bile leak test was completed at the back table by injecting indocyanine green into the graft's bile duct. Finally, the right posterior sector and remnant grafts were weighed separately. (**Figures 1J,K**). All reduced grafts were implanted using the piggyback method (**Figure 1L**). Owing to the innovation of this technology, we named it HuaXi-eRPS (HuaXi is the acronym of our hospital name, West China Hospital of Sichuan University). This study was approved by the West China Hospital Ethics Committee and was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

RESULTS

In this study, HuaXi-eRPS was performed in five grafts. The five donors did not meet the criteria for split candidates utilised by UNOS(5); thus, SLTs were not considered. All data regarding the recipients and donors are summarised in **Table 2**. It took much time to separate the abdominal adhesions for three recipients with recurrent hepatocellular carcinoma (HCC) (Cases 1, 4, and 5) after liver resection. One recipient (Case 3) with fulminant hepatitis B had portal vein thrombosis and had undergone thrombectomy. In addition, meticulous hemostasis on the graft cutting face is a critical procedure for RSLT. Based on the reasons mentioned above, the total operation time was longer than that of non-RSLT.

The 30-days mortality was zero. Postoperative complications occurred in two patients (40%); however, complications higher than those in Clavien-Dindo grade II(11) were not observed in all patients. No patient experienced biliary leakage or postoperative haemorrhage, and no infection-related complications, including liver abscess or pulmonary infection, were identified in this series. During the follow-up period (range, 2.1–14.2 months), all patients were alive with normal daily activities, and three patients with HCC did not experience tumour recurrence with a normal alpha-fetoprotein level. All five recipients did not experience posttransplant rejection and biliary complications, such as bile leakage and biliary stricture, were not observed in any of the recipients.

DISCUSSION

The morphology of the right upper abdominal cavity may differ among individuals. To date, four formulas have been proposed to predict the occurrence of LFSS.(2, 12-14) However, only one formula introduced an individualised morphological measurement (RAP value) on the recipient.(2) In the present case series, we selected GW/RAP combined with GRWR as new "LFSS predictors" for the following reasons. First, the GW/RAP considers the depth of the lower right hemithorax, which directly influences rib compression in the right liver. Second, both GRWR and GW/RAP do not rely on the donor's radiological examination, which is an almost impossible task when the

TABLE 2 | The related data of recipients and their allocated donors.

Parameters	Case 1	Case 2	Case 3	Case 4	Case 5
Recipient profiles					
Age, years	56	39	18	51	65
Gender	M	M	F	F	M
Height, cm	163	168	160	162	168
Weight, kg	67	53	59	53	54
BMI, kg/m^2	25.22	18.78	23.05	20.2	19.13
Indications for liver transplantation	HCC recurrence	FHB	FHB	HCC recurrence	HCC recurrence
MELD scores	22	25	28	26	27
Allocated DCD donor profiles					
Age, years	43	62	56	54	58
Gender	M	M	M	M	M
Height, cm	180	178	175	175	176
Weight, kg	99	80	83	80	81
BMI, kg/m^2	30.56	25.25	27.1	26.12	26.15
Death reason	Acute cerebral hernia	Acute cerebral hernia	Cerebral hemorrhage	Irreversible cerebral injury	Irreversible cerebral injury
Intraoperative data					
Procured GW, g	2060	1830	1750	1800	1850
Preoperatively measured RAP in recipients, cm	18.94	16.13	15.57	17.86	16.55
Calculated GRWR for whole graft, %	3.07	3.45	2.97	3.40	3.43
Calculated GW/RAP for whole graft, g/cm	108.8	113.5	112.4	100.8	111.8
Preoperatively estimated GRWR for the remnantt graft after eRPS, %	2.22	2.49	2.14	2.45	2.47
Preoperatively estimated GW/RAP for the remnant graft after eRPS, g/cm	78.4	81.8	81.0	72.7	80.6
Actual weight of the remanent graft after eRPS, g	1,526	1,250	1,320	1,295	1,300
Actual GRWR after *ex vivo* reduction, %	2.28	2.36	2.24	2.44	2.41
Actual GW/RAP after *ex vivo* reduction, g/cm	80.6	77.5	84.8	72.5	78.5
Duration for graft reduction, min	40	33	41	38	35
Total operation time for recipient, h	7.5	5.9	7.7	8.2	8.5
Anhepatic time for recipient, min	85	76	75	70	74
Cold ischemic time, min	359	402	300	414	383
Estimated total blood loss, ml	650	2,100	2,250	1,120	1,020
Estimated blood loss after anhepatic phase, ml	170	340	360	230	240
Amount of blood transfusion during operation, units	3	13	14	4	6
Postoperative course					
Delay the fascial closure after LT	No	No	No	No	No
The POD of extubation	1	1	1	1	2
ICU stay, days	5	9	4	5	5
Postoperative hospital stay, days	9	19	16	13	15
Postoperative complication grade according to Clavien-Dindo classification					
Grade I					$\surd^{\pi}$
Grade II			$\surd^{\phi}$		
Grade IIIa					
Grade IIIb					
Grade IVa					
Grade IVb					
Grade V					
Follow-up, months	14.2	10.1	8.2	7.2	2.1

M, male; F, female; BMI, body mass index; DCD, donation after citizen death; eRPS, ex vivo *right posterior sectionectomy; FHB, fulminant hepatitis B; GRWR, graft-recipient weight ratio; GW, graft weight; HCC, hepatocellular carcinoma; ICU, intensive care unit; LT, liver transplantation; POD, postoperative day; RAP, right anteroposterior;* $^{\phi}$ *need of blood transfusion;* $^{\pi}$ *wound infection.*

donor is in critical condition. Third, GRWR can predict the risk of LFSS and is also a commonly used index for evaluating the occurrence of the small-for-size syndrome (SFSS).

Compared to paediatric RSLT,(15, 16) the surgical strategies for graft reduction in adult LTs are relatively unfamiliar. In most cases, a limited resection, such as left lateral lobectomy or left

hemihepatectomy, is preferred because of its convenience.[2] However, it is very unlikely to solve some mismatch issues because compression, due to the ribs, mainly applies to the right liver. Right hemihepatectomy has been proposed as an alternative method, but the residual left liver may be insufficient for some recipients.(8) Compared to the *in vivo* method, the HuaXi-eRPS used in our series could be a unique method with the following advantages. First, the graft weight can be accurately measured on the back table to provide a precise parameter for determining the feasibility of eRPS. Second, because the *ex vivo* graft can be rotated 360-degree, it is easy and simple to perform eRPS using the cranial approach to the RHV. Although the demarcated area for the right posterior sector cannot be displayed easily after ligating the right posterior Glisson's sheath as an *in situ* graft, the main purpose of eRPS is to overcome size mismatch. It is not necessary to perform a precise anatomic right posterior sectionectomy, as required for hepatic malignancy. Third, eRPS in the graft before implantation is beneficial to reduce the difficulty of implantation and shorten the period for the anhepatic phase. In addition, compared to the whole right lobe, which accounts for 60–75% of the total liver volume, eRPS can ensure both the integrity of outflow and adequate residual graft volume to avoid SFSS while avoiding rib compression.

The present study had some limitations. GW/RAP combined with GRWR, as a new "LFSS predictor," is a preliminary formula whose optimal cutoff value or predictive validity still requires further confirmation by a well-designed trial with a large sample size. However, this is the first study to propose the initial indications for HuaXi-eRPS in grafts, and its initial outcomes in our five adult series are safe and encouraging, especially in decreasing the difficulty of implantation, avoiding delayed fascial closure, shortening ICU stay, and reducing posttransplant complications.

In conclusion, this study described a novel and feasible surgical strategy for preventing posttransplant LFSS, especially for the size discrepancy between the anteroposterior dimensions of the liver graft and the lower right hemithorax of the recipient.

CAPSULE SENTENCE SUMMARY

This study describes a novel and feasible surgical strategy for preventing posttransplant large-for-size syndrome, especially for the size discrepancy between the anteroposterior dimensions of the liver graft and the lower right hemithorax of the recipient.

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 Ethics Committee on Biomedical Research, West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XP, DH, and AL wrote the paper. DH, JianY, and TL collected data. LY designed study. JiayinY and HW performed study. LJ designed and performed study. XP and DH contributed equally to this study and are co-first authors.

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CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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The Tangential Extraperitoneal Retrorenal Approach in Kidney Transplant Biopsy: An Observational Study to Assess Complication and Adequacy Rates

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Introduction: Ultrasound-guided percutaneous kidney allograft biopsy is the gold-standard for pathology work-up. Recent studies postulate better safety and efficacy for tangential approaches, however, there is no recommendation regarding biopsy needle path. In this context, we previously described the unified tangential extraperitoneal retrorenal (TER) approach for standard allograft biopsy.

Methods: A single-center retrospective observational study evaluated safety and efficacy of the TER biopsy approach among 250 patients that underwent 330 ultrasound-guided kidney transplant biopsies between January 2011 and May 2020.

Results: The overall major complication rate was 0.56% per biopsy attempt (1.21% per biopsy) including blood transfusion, arterial embolization and bladder catheterization for gross hematuria in 0.28, 0.14 and 0.14% of biopsy attempts, respectively (0.61, 0.30 and 0.30% of biopsies, respectively). Minor complications included subcapsular and/or perinephric hematoma, superficial bleeding, arteriovenous fistula and gross hematuria in 12.6, 3.0, 2.5 and 1.4% of biopsy attempts, respectively (27.0, 6.4, 5.5 and 3.0% of biopsies, respectively). Sample adequacy rate was 86.7%, ranging from 82.2 to 94.1% if one or ≥two cores were analyzed, respectively. Residents and consultants yielded similar complication and adequacy rates.

Conclusion: According to current literature, ultrasound-guided TER kidney transplant biopsy is a safe and efficient approach eligible for nephrology training.

Keywords: percutaneous kidney transplant biopsy, tangential extraperitoneal retrorenal approach, ultrasound-guided biopsy, complication rate, adequacy rate

Abbreviations: AVF, arteriovenous fistulas; CT, computer tomography; G, gauge; TER, tangential extraperitoneal retrorenal.

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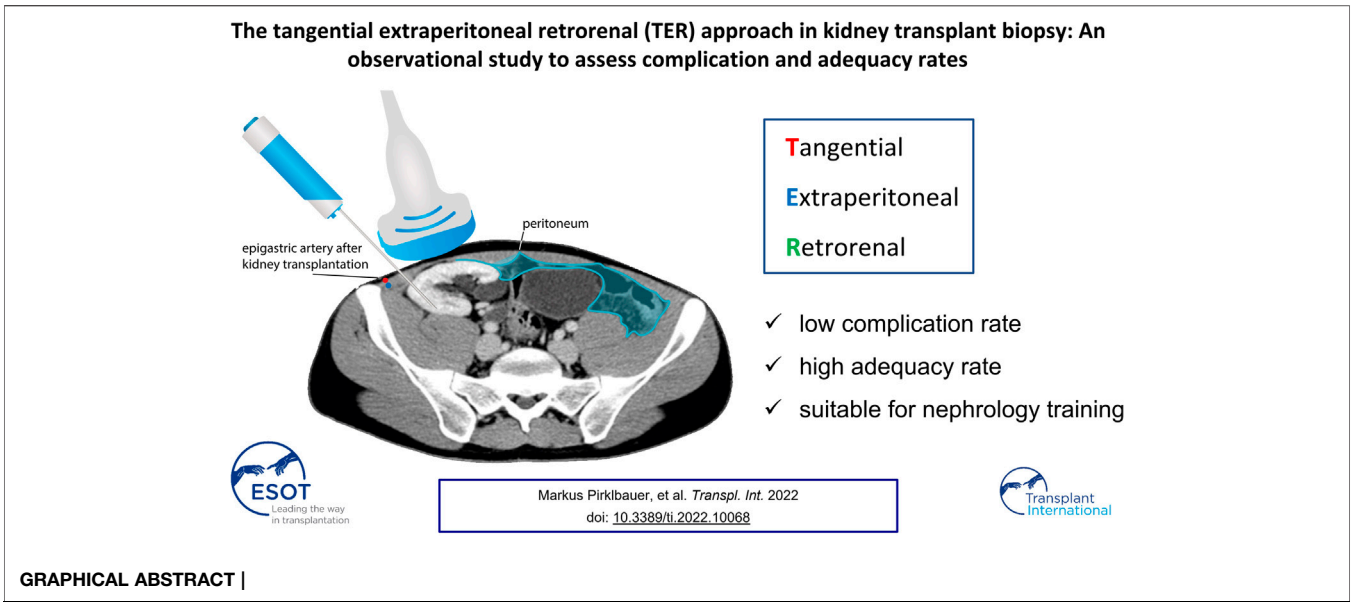

GRAPHICAL ABSTRACT |

INTRODUCTION

Ultrasound-guided percutaneous renal transplant biopsy is the gold-standard procedure for allograft pathology work-up. Recent studies, including previous research at our institution, postulate better safety and efficacy of tangential compared to radial approaches [1–3], however, there is no general consensus regarding biopsy needle path for this standard technique. A tangential biopsy allows to direct the needle tip away from the renal hilum, the ureter, and large vessels of the anastomosis region, thereby sparing these anatomical structures from potential injury. In this regard, we recently developed the so called tangential, extraperitoneal, retrorenal (TER) approach for standard allograft biopsy, that penetrates the allograft parallel to the renal capsule (tangential component, T), keeps safe distance to the peritoneal fold (extraperitoneal component, E), and targets the posterior side of the allograft (retrorenal component, R) in a lateral-to-medial approach. A pilot study among 104 patients already demonstrated excellent safety and efficacy of the TER approach in 127 kidney transplant biopsies [1]. In our present study we verify these results in a larger patient cohort by demonstrating excellent complication and adequacy rates among 250 patients undergoing 330 kidney transplant biopsies utilizing a conventional (96.1%) or modified (3.9%) TER approach. Furthermore, this is the first study to 1) assess both major and minor complications based on a standardized post-procedural ultrasound follow-up as well as to 2) confirm the eligibility of TER kidney transplant biopsy for nephrology training.

PATIENTS AND METHODS

A single-center retrospective observational study was conducted at our Department to assess safety and efficacy of TER kidney transplant biopsy. Between January 2011 and May 2020, 250 patients underwent at least one kidney transplant biopsy at our institution and were included in the present study. The TER approach is the standard technique for kidney transplant biopsy at our institution and was performed in 317/330 allograft biopsies (96.1%). A modified TER approach, which featured only two of the three components of the conventional TER approach (tangential, extraperitoneal, retrorenal), had to be conducted in 13/330 biopsies (3.9%) due to anatomical causes, e.g., dislocated inferior epigastric artery, orthotopic allograft transplantation or preexisting hematoma. 104 of 250 study patients were already included in our previous pilot study [1]. 6/330 allograft biopsies (1.8%) were protocol biopsies, the remaining 324 biopsies were based on indication. Patient data were available from the institutions' computerized clinical documentation systems.

Biopsy Protocol

Kidney transplant biopsy was exclusively performed in an inpatient setting where patients are admitted to hospital on the day of biopsy and discharged on the following day. Anti-platelet/anti-coagulant medication was halted from 7–14 days prior to 7–14 days after biopsy depending on the type of drug. Patients at high risk of thromboembolism were administered enoxaparin-sodium during that period; however, enoxaparin-sodium was administered no later than 24 h before biopsy. Blood pressure and heart rate were monitored peri-interventionally. Anti-hypertensive medication (e.g., nitroglycerine and/or urapidil and/or dihydralazine) was administered if blood pressure peri-interventionally exceeded 160/90 mmHg. Lorazepam sedation was available for episodes of anxiety and/or agitation; however, patients did not receive general anesthesia. Following biopsy, patients had to remain in a supine position and use an abdominal belt to minimize the risk of hematoma. Monitoring ended 5 h after biopsy if a post-biopsy urine void and no signs of gross hematuria, flank pain or other symptoms indicating a complication were reported. A blood count

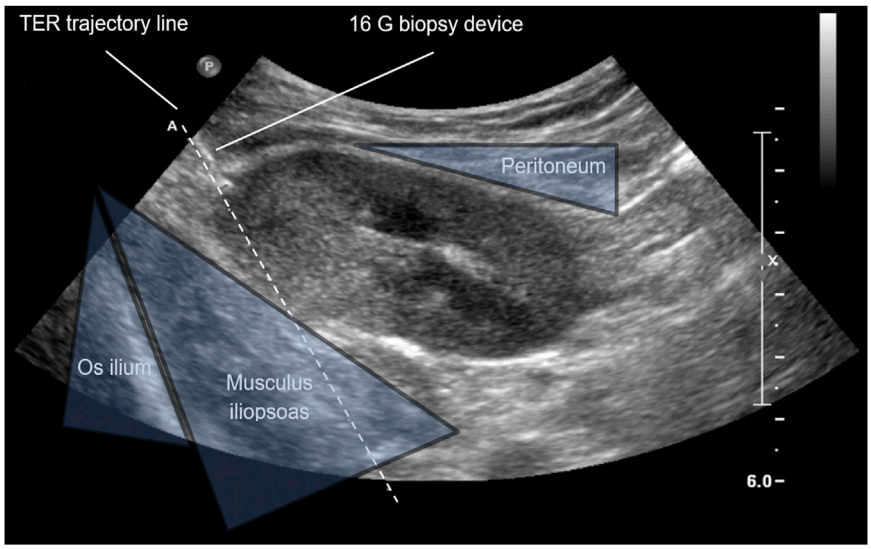

FIGURE 1 | Anatomic landmarks of real-time ultrasound-guided kidney allograft biopsy. Ultrasound image of the right iliac kidney allograft; TER, tangential, extraperitoneal, retrorenal; G, gauge.

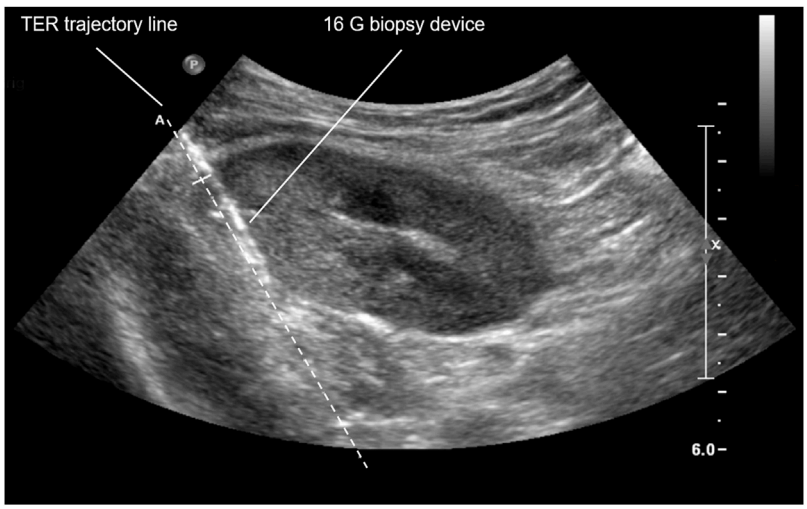

FIGURE 2 | TER biopsy of right iliac kidney allograft. TER, tangential, extraperitoneal, retrorenal; G, gauge.

as well as color-duplex ultrasound examination was performed on the next day to detect bleeding complications or arterio-venous fistulas (AVF). If both examinations yielded normal findings, patients were discharged from hospital and instructed to avoid weight-lifting >5 kg and contact sports for 14 days. Normal physical activity including running and cycling was encouraged to prevent thromboembolism. Patients were asked to immediately return to the hospital in case of discomfort after discharge.

Biopsy Technique

Real-time ultrasound-guided TER kidney transplant biopsy is performed in supine or—in case of obesity or pendulous abdomen—lateral decubitus position. A needle guidance system mounted to the ultrasound transducer optimizes needle handling and helps to visualize needle path. The ultrasound transducer is placed approximately 2 cm medial to the anterior superior iliac spine to determine optimal biopsy area. The latter allows to 1) penetrate the allograft parallel to the renal capsule (i.e., tangential component), 2) keep safe distance to the peritoneal fold (extraperitoneal component), and 3) target the posterior side of the upper pole or the most dorsal part of the lateral portion of the allograft (retrorenal component) (**Figure 1**). Local anesthesia with xylocaine 2% is administered as subcutaneous depot prior to skin incision as well as ultrasound-guided deep depot along the needle path up to the renal capsule. *Via* a small skin incision the biopsy device is then advanced towards the allograft from lateral to medial in a transverse

TABLE 1 | Patient characteristics.

Patients	250
Male	155 (62.0)
Female	95 (38.0)
No. of performed biopsies	330
On male patients	203 (61.5)
On female patients	127 (38.5)
Per patient	
1	194 (77.6)
2	39 (15.6)
3	11 (4.4)
4	5 (2.0)
5	1 (0.4)
No. of kidney transplant	1.41 ± 0.8 (1–7)
Age (years)	50 (18–78)
Body Mass Index (kg/m^2)	24.3 (15.6–42.0)
Arterial hypertension (≥140/90 mmHg)	291 (88.2)
Diabetes mellitus	76 (23.0)
Arterial hypertension and diabetes mellitus	69 (20.9)

Data are presented as numbers (percent), mean ± standard deviation (range), or median (range) for age and body mass index.
No., number.

TABLE 2 | Biopsy characteristics.

No. of performed biopsies	330
No. of biopsy attempts per biopsy	
1	28 (8.5)
2	237 (71.8)
3	57 (17.3)
4	4 (1.2)
5	4 (1.2)
No. of biopsy attempts	2.2 ± 0.6 (1–5)
Total	709
No. of samples recovered	1.9 ± 0.5 (1–5)
Total	637 (89.9)
No. of biopsy attempts without recovery of sample	72 (10.2)
Biopsy technique	
No. of performed biopsies	330
Using TER approach	317 (96.1)
Using modified TER approach	13 (3.9)
No. of biopsy attempts	709
Using TER approach	683 (96.3)
Using modified TER approach	26 (3.7)

Data are displayed as number (percent) or mean ± standard deviation (range).
ND, no data; No., number.

plane using real-time ultrasound guidance. Once the renal capsule is reached, the biopsy needle is fired tangentially into the outer third of the renal cortex (**Figure 2**). Bedside analysis of biopsy cores for adequacy was routinely done by using a magnifying glass for the crude assessment of glomerular number. Whenever feasible, at least two core samples measuring 1.3 mm in diameter and 22 mm in length are obtained using a 16 cm long, 16 Gauge (G) spring-loaded biopsy device (Bard® Monopty® Disposable Core Biopsy Instrument).

Definition of Complications

Major complications were defined as biopsy-related complications requiring invasive therapy and included bladder catheterization for gross hematuria, blood transfusion (following either a biopsy-related drop of hemoglobin or image confirmation of biopsy-related bleeding), interventional radiology procedure with or without arterial embolization, surgery, graft loss, or death. Minor complications were defined as any biopsy-related relevant medical condition not requiring invasive therapy. Complication rates were calculated per biopsy attempt and biopsy event.

Definition of Adequacy

According to the criteria of the Banff 97 working classification of renal allograft pathology [4], a biopsy core sample was considered 1) adequate if it contained at least 10 glomeruli and two arteries or 2) minimal if it contained a minimum of seven glomeruli and one artery in the pathologist`s assessment. Adequacy rates given in the present study represent the sum of samples deemed either minimal or adequate. Adequacy was calculated per biopsy as glomerular and arterial yield were reported per biopsy only.

Statistical Analysis

Descriptive statistics was performed using Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States). Results and baseline characteristics are presented as absolute frequencies or mean values ± standard deviation (range). Chi^2 statistics was performed with SPSS version 24.0 to assess potential associations between nominal parameters (i.e., training status, occurrence of complications and sample adequacy). The level of significance (p value) was set to 0.05.

Statement of Ethics

The study was conducted in accordance with the World Medical Association Declaration of Helsinki. The study protocol was reviewed and approved by the Innsbruck Medical University ethics committee prior to study initiation (approval number ECS 1106/2020). Patient information was managed entirely coded. All patient associated data are subject to privacy protection according to the current European General Data Protection Regulation. Based on the retrospective study design the Innsbruck Medical University ethics committee granted an exemption from requiring written informed consent.

RESULTS

Patient and Biopsy Characteristics

330 ultrasound-guided kidney transplant biopsies were performed among 250 patients between January 2011 and May 2020. 203 (61.5%) and 127 (38.5%) biopsies were performed on male and female patients, respectively. 194 (77.6%) patients underwent one biopsy, however, patients were subjected to kidney transplant biopsy up to five times. 2, 3, 4 and 5 biopsies were performed in 39 (15.6%), 11 (4.4%), 5 (2.0%) and 1 (0.4%) patient, respectively. Median age and body mass index at the time of biopsy was 50 years (range 18–78) and 24.3 (range 15.6–42.0), respectively. The mean number of kidney transplants per patient was 1.41 ± 0.8 (range 1–7). The total

TABLE 3 | Biopsy complications.

	TER + modified TER		TER only	
	709 biopsy attempts	330 biopsies	683 biopsy attempts	317 biopsies
Overall				
Minor	149 (21.0)	149 (45.2)	141 (20.6)	141 (44.5)
Major	4 (0.6)	4 (1.2)	2 (0.3)	2 (0.6)
Total	153 (21.6)	153 (46.4)	143 (20.9)	143 (45.1)
Periprocedural minor complications				
Drainage of serous fluid	2 (0.3)	2 (0.6)	2 (0.3)	2 (0.6)
Vasovagal reaction	5 (0.7)	5 (1.5)	5 (0.7)	5 (1.6)
Hypertensive urgency	1 (0.1)	1 (0.3)	1 (0.2)	1 (0.3)
Superficial bleeding	21 (3.0)	21 (6.4)	19 (2.8)	19 (6.0)
Postprocedural minor complications				
Gross hematuria	10 (1.4)	10 (3.0)	10 (1.5)	10 (3.2)
Arteriovenous fistula	18 (2.5)	18 (5.5)	18 (2.6)	18 (5.7)
Subcapsular hematoma	7 (1.0)	7 (2.1)	6 (0.9)	6 (1.9)
Perinephric hematoma	82 (11.6)	82 (24.9)	78 (11.4)	78 (24.6)
<3 × 1 cm	69 (9.7)	69 (20.9)	66 (9.7)	66 (20.8)
>3 × 1 cm	8 (1.1)	8 (2.4)	7 (1.0)	7 (2.2)
ND	5 (0.7)	5 (1.5)	5 (0.7)	5 (1.6)
Pain[a]	2 (0.3)	2 (0.6)	1 (0.2)	1 (0.3)
Deep vein thrombosis	1 (0.1)	1 (0.3)	1 (0.2)	1 (0.3)
Major complications				
Rinsing catheter for gross hematuria	1 (0.1)	1 (0.3)	1 (0.2)	1 (0.3)
Transfusion	2 (0.3)	2 (0.6)	1 (0.2)	1 (0.3)
Coiling/arterial embolization	1 (0.1)	1 (0.3)	0 (0.0)	0 (0.0)

[a]*No ultrasound correlate.*
Data are displayed as number (percent).
ND, no data; No., number.

number of biopsy attempts was 709, yielding 637 core samples. Mean number of biopsy attempts per biopsy and recovered core samples was 2.2 ± 0.6 (range 1–5) and 1.9 ± 0.5 (range 1–5), respectively. TER biopsy was performed in 317/330 biopsies (96.1%) and 683/709 biopsy attempts (96.3%). In 13 biopsies (3.9%) and 26 biopsy attempts (3.7%), a modified TER approach had to be applied due to anatomical causes, e.g., dislocated inferior epigastric artery, orthotopic allograft transplantation or preexisting hematoma. Tangential, extraperitoneal or retrorenal biopsy could not be performed in four, two and seven biopsies, respectively. Though, at least two components of the conventional TER approach were performed in these 13 cases (**Tables 1**, **2**).

Major Complications

Among 709 biopsy attempts (330 biopsies), four major complications (0.6% of biopsy attempts and 1.2% of biopsies) were documented among 3 patients throughout the study period. Considering conventional TER approach only with 683 biopsy attempts (317 biopsies), 2 complications (0.3% of biopsy attempts and 0.6% of biopsies) were classified as major complications. One patient was subject to rinsing catheterization of the bladder (0.1% of biopsy attempts and 0.3% of biopsies) due to gross hematuria following biopsy. Transfusion of blood products (0.3% of biopsy attempts and 0.6% of biopsies) was required in two patients. One of these patients underwent conventional TER kidney transplant biopsy and received two units of packed red blood cells on the day after biopsy on account of suspected bleeding in abdominal ultrasound examination and CT scan. The other patient underwent a modified TER approach (radial biopsy) and experienced aggravated pain immediately after biopsy. Instant ultrasound examination revealed arterial bleeding involving the upper pole renal artery. Emergency coiling (0.1% of biopsy attempts and 0.3% of biopsies) was conducted and four units of packed red blood cells and platelet concentrates were administered for low hemoglobin and platelet count. No patient required surgical treatment. No graft losses or deaths occurred (**Table 3**).

TABLE 4 | Sample adequacy.

No. of performed biopsies	330
No. of analyzed samples	
0	2 (0.6)
1	174 (52.7)
2	150 (45.5)
3	2 (0.6)
ND	2 (0.6)
No. of biopsies considered	
Adequate	192 (58.2)
Minimal	94 (28.5)
Inadequate	42 (12.7)
ND	2 (0.6)
Adequate and minimal	286 (86.7)
If 1 sample analyzed	143 (82.2)
If 2 samples analyzed	141 (94.0)
If 3 samples analyzed	2 (100.0)
If 2 or 3 samples analyzed	143 (94.1)
No. of glomeruli	15.7 ± 9.3 (0–69)
No. of arteries	2.5 ± 1.6 (0–10)

Data are displayed as number (percent) or mean ± standard deviation (range).
ND, no data; No., number.

Minor Complications

Overall, 149 events were classified as minor complications (21.0% of biopsy attempts and 45.2% of biopsies). Considering TER approach only, 141 minor complications were documented (20.6% of biopsy attempts and 44.5% of biopsies). Routine ultrasound examination on day 1 after kidney transplant biopsy identified 82 perinephric hematomas, eighteen AVF, and seven subcapsular hematomas (i.e., 11.6, 2.5 and 1.0% of biopsy attempts, respectively and 24.9, 5.5 and 2.1% of biopsies, respectively). Of the perinephric hematomas, 69 were smaller than 3 × 1 cm, eight were bigger than 3 × 1 cm and five could not be categorized because of missing data (i.e., 9.7, 1.1 and 0.7% of biopsy attempts, respectively and 20.9, 2.4 and 1.5% of biopsies, respectively). All AVF had resolved spontaneously at follow-up ultrasound examination. 21 superficial bleedings, 10 episodes of gross hematuria, 5 vasovagal reactions requiring atropine administration, and one hypertensive urgency requiring administration of urapidil, dihydralazine and amlodipine, were detected after kidney allograft biopsy (i.e., 3.0, 1.4, 0.7, and 0.1% of biopsy attempts, respectively and 6.4, 3.0, 1.5 and 0.3% of biopsies, respectively). Abdominal pain (0.3% of biopsy attempts and 0.6% of biopsies) was reported in two patients. In both cases, no ultrasound correlate was detected and both patients were administered analgesic medication. One case of deep vein thrombosis (0.1% of biopsy attempts and 0.3% of biopsies) of the ipsilateral popliteal vein was documented (**Table 3**).

Sample Adequacy

Cores samples were evaluated according to the Banff 97 working classification of renal allograft pathology [4]. 192 (58.2%) and 94 (28.5%) biopsies were considered adequate and minimal, respectively. Thus, a total of 286 biopsies (86.7%) met the criteria for sample adequacy. 42 biopsies (12.7%) were considered inadequate and data from two biopsies (0.6%) were lacking. Adequacy rate increased to 94.1%, if two or more core samples were analyzed. Mean number of glomeruli and arteries was 15.7 ± 9.3 glomeruli (range 0–69) and 2.5 ± 1.6 arteries (range 0–10), respectively (**Table 4**).

Complications and Sample Adequacy of Training Biopsies

116 (35.2%) and 214 (64.9%) biopsies were performed by nephrology residents and consultants, respectively. Major and minor complications occurred in 1.2 and 20.5% of resident biopsy attempts, respectively (2.6 and 44.0% of resident biopsies, respectively) and 0.2 and 21.3% of consultant biopsy attempts, respectively (0.5 and 45.8% of consultant biopsies, respectively). $p = 0.094$ and $p = 0.798$ for association of resident status with major and minor complications, respectively. Considering TER approach only, major complication rate per biopsy attempt was 0.4 and 0.2% among 249 (35.1%) resident and 460 (64.9%) consultant biopsy attempts, respectively (i.e., 0.9 and 0.5% among 111 resident and 206 consultant biopsies, respectively). Overall adequacy rate was 87.9 and 86% for biopsies performed by residents and consultants, respectively ($p = 0.619$ for association of resident status with sample adequacy) (**Supplementary Tables S1, S2**).

DISCUSSION

The present study reinforces the results of a recent pilot study [1] demonstrating excellent safety and efficacy of TER kidney transplant biopsy and corroborates previous findings showing low major complication and high adequacy rates with the use of tangential kidney allograft biopsy [2, 3, 5]. With a major complication rate of 0.3% per biopsy attempt (0.6% per biopsy) the TER approach is among the safest allograft biopsy approaches according to current literature (**Supplementary Table S3**). Major complication rates have been previously demonstrated to be up to 5.6% [6]; however the latter study did not report a specific biopsy region or needle path. Comparable studies utilizing a tangential biopsy technique reported major complication rates ranging between 0.0% [5] and 3.6% [7]. While the former study first described the so called "cortex-only" view among 188 biopsies, the latter study used a computer tomography (CT)-guided approach among 28 biopsies. While small patient number is a substantial limitation of both studies, CT-guided approaches implicate additional risk from radiation exposure. The most comprehensive studies assessing ultrasound-guided tangential allograft biopsy yielded major complication rates of 0.7 [2], 0.3 [3] and 1.9% [8]. Minor complications, such as AVF and hematomas, are best detected through standardized post-procedural ultrasound examination and/or blood count; however, most of the available studies, including comparable studies with tangential biopsy techniques [2, 3, 5, 8], did not routinely perform post-procedural ultrasound and/or blood count. Thus, it is likely to speculate that these studies might not reflect the true incidence of minor complications. By performing routine ultrasound and blood count on the day after biopsy, our study is the first to comprehensively assess both symptomatic and asymptomatic complications. Based on these substantial differences in post-procedural management, however, the minor complication rates found in the present study are not comparable to previous studies in the field. AVF are usually asymptomatic and rarely require specific therapy; however, centers performing ultrasound-based screening report AVF rates of up to 10.7% [9]. Generally, AVF rate seems to correlate with both the extent and timing of post-procedural ultrasound examination. Consequently, AVF rates are usually reported to be low in studies that do not routinely perform post-procedural imaging [7, 10–13] and tend to be higher in studies that perform ultrasound examination within hours [9, 14, 15] as compared to those performing immediate post-procedural ultrasound [16–18].

The low AVF rate (2.5% per biopsy attempt and 5.5% per biopsy) found with the TER biopsy approach is likely to result from targeting the outer third of cortical renal parenchyma and thus, from sparing larger vessels in the medullary region. All AVF spontaneously resolved at a 2 weeks follow-up examination. Nevertheless, screening might be beneficial for individual patients as AVF-associated severe complications, such as

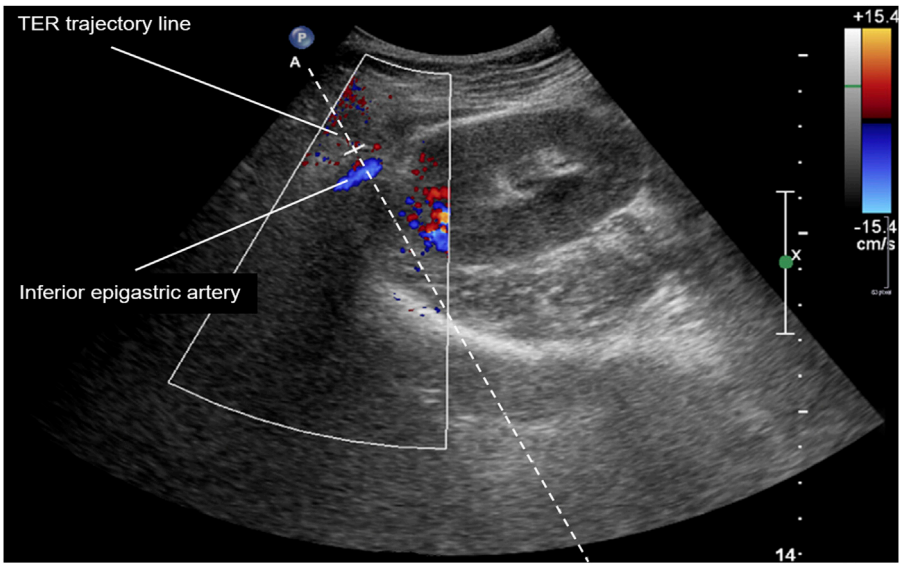

FIGURE 3 | Color-duplex ultrasound image of right iliac kidney allograft and dislocated inferior epigastric artery. TER, tangential, extraperitoneal, retrorenal.

arterial embolization and nephrectomy have been described in the literature [10, 15]. A recent retrospective Japanese study described an AVF rate of 2.6% after kidney allograft biopsy and proposed that embolization is a safe treatment for these AVF. However, the authors state that the study was likely to underestimate AVF rate as post-procedural management was not consistent among the study population [19].

As for AVF, hematoma detection rate correlates with the availability of post-procedural ultrasound examination and ranges from 0.0% in studies that did not perform ultrasound examination [20] and 13.4% [21] in studies that performed immediate postprocedural ultrasound. With a consequent post-procedural ultrasound examination, the present study reports an overall hematoma rate of 12.6% per biopsy attempt (27.0% per biopsy). A previous study performing comparable post-procedural management reported a similar hematoma rate of 11.1% [22]. While none of the reported perinephric (11.6% of biopsy attempt and 24.9% of biopsies) and subcapsular (1.0% of biopsy attempts and 2.1% of biopsies) hematomas required specific therapy in our study, individual patients might benefit from hematoma screening as large hematomas may profit from extended period of rest or—if applicable—extended period of anti-platelet/anticoagulant withdrawal post biopsy. Additionally, post-procedural screening might help to timely identify large hematomas that will require surgical evacuation in order to preserve kidney allograft function [6, 8, 13]. Nevertheless, substantial heterogeneity exists in post-procedural management of kidney transplant biopsy between facilities, partly due to reimbursement issues. While overnight in-hospital observation is clinical routine in many European and Japanese centers, others, including most U.S. facilities, perform shorter observation periods. In this regard, a recent study by Patel et al. both corroborated the low rate of major bleeding complications with ultrasound-guided renal transplant biopsy (0.2%) and presented evidence that a standardized 1-hour postprocedure observation protocol can be safely used. However, the authors state that more than half of these complications were not clinically apparent within 4 h of biopsy [23]. Overnight in-hospital observation is part of the routine post-procedural management at our facility, however, the present study does not advocate any specific post-procedural management strategy at this time.

The occurrence of gross hematuria following kidney allograft biopsy ranges from 0.0% [24] and 9.0% [13] in the literature. Our finding of a rather low gross hematuria rate (1.4% per biopsy attempt and 3.0% per biopsy) is consistent with the low rate (0.7%) found in another tangential allograft biopsy study [2]. Other minor complications, such as superficial bleedings, vasovagal reactions, hypertensive urgency, seroma drainage, and aggravated pain, are rarely reported in the literature, and thus, occurrence rates are difficult to compare. Deep vein thrombosis that is normally associated with immobilization might not be considered as direct biopsy complication. Previous studies reported that renal allograft biopsy within 30 days after transplantation, deep puncture (i.e., high percentage of medulla) and the number of biopsy attemps per biopsy increase the risk of AVF [25, 26]. In our study, 47/330 biopsies (14%) were performed within 30 days after kidney transplantation, however, we did not find a significant association with major and/or minor complications. Interestingly, the latter did also not significantly correlate with the number of biopsy attempts per biopsy. While major complications exclusively occurred among patients that were subject to 2 biopsy attempts, AVF rate did not increase with the number of biopsy attempts per biopsy (up to 5). Albeit not statistically significant, hematoma rate nominally increased from 25% (with up to 4 biopsy attempts) to 50% (with 5 biopsy attempts) ($p = 0.24$). Data regarding the percentage of medulla

in biopsy specimen is not available for the present study, however, deep puncture would be a rare finding with adequate TER biopsy as parallel orientation of the biopsy needle to the renal capsule should avoid any deep puncture.

Sample adequacy rates range from 52.9 [20] to 99.5% [8] in the literature (**Supplementary Table S4**). However, some of the previous studies [5, 20, 27] regarded glomerular yield only, and thus, might overestimate adequacy. With an overall sample adequacy rate of 86.7% our study is among the top 6 studies reporting adequacy according to Banff classification. Adequacy rate increased to 94.1% in our study, if two or more core samples were analyzed. However, the latter applied to only 46.1% of biopsies due to frequent electron microscopic work-up of a second core sample. It is likely to speculate that adequacy rates would have exceeded 90% once these samples were analyzed. Based on this finding we now obtain three core samples in case of planned electron microscopic work-up. Biopsy technique and needle size vary among different studies, however, it has been previously stated that adequacy rates rather correlate with biopsy technique than needle size [24, 28] (**Supplementary Table S4**). As we found no significant difference between resident status and complication as well as adequacy rates (**Supplementary Tables S1, S2**), the novel TER biopsy approach can be considered appropriate for training biopsies. This is in contrast to a previous study stating a possible association of major complications with lesser operator experience in tangential allograft biopsy [2]. Previous studies evaluating tangential biopsy approaches [2, 3] did not stipulate a particular biopsy region or needle paths. However, transperitoneal needle paths are more likely to cause intraperitoneal hematoma and medial-to-lateral biopsy approaches are prone to injure both the rectus sheath and inferior epigastric artery with subsequent development of rectus sheath hematoma. Both complications have been previously reported with tangential biopsy approaches [2, 5]. With the TER approach these complications are less likely to occur as an exclusive extraperitoneal as well as lateral-to-medial biopsy approach keeps safe distance to both the rectus sheath, inferior epigastric artery, and the peritoneal fold. However, as the inferior epigastric artery might not be located in its usual position along the rectus abdominis muscle but dislocated further lateral due to mobilization during transplantation, the TER biopsy approach should be modified to avoid vascular injury in that case (**Figure 3**). While tangential cortex biopsy has been previously demonstrated to convey substantial advantages in terms of safety and adequacy [2, 3, 5], our results do not support the hypothesis that retrorenal biopsy approaches may lead to uncontrollable bleeding [5]. In contrast, the surrounding iliopsoas muscle as well as the dorsal pelvis rather serve as natural barriers against extended hematoma formation. Furthermore, the iliopsoas muscle is the only adjacent organ structure to be accidentally injured in case of a retrorenal biopsy approach. While previous studies inconsistently reported the number of biopsy attempts per biopsy and calculated complication rates per biopsy only, our study is the first to present complication rates per biopsy attempt and biopsy event. The present study is limited by a small sample size.

By demonstrating low major complication (<1%) and high sample adequacy rates (>90% when two or more samples are analyzed) our present study confirms high safety and efficacy of the novel TER approach for standard kidney transplant biopsy. Furthermore, this is the first study to 1) comprehensively report both major and minor complication rates based on a standardized post-procedural management and 2) confirm the eligibility of the TER approach for supervised training biopsies with respect to safety and efficacy.

CAPSULE SUMMARY SENTENCE

Ultrasound-guided percutaneous renal transplant biopsy is the gold-standard procedure for allograft pathology work-up. Recent studies, including previous research at our institution, postulate better safety and efficacy of tangential compared to radial approaches, however, there is no general consensus regarding biopsy needle path for this standard technique. In this context, we recently described a unified tangential, extraperitoneal, retrorenal (TER) approach for standard allograft biopsy and demonstrated excellent safety and efficacy in a pilot study (Transpl Int. 2017; 30: 947–50). By penetrating the allograft parallel to the renal capsule (tangential component), keeping safe distance to the peritoneal fold (extraperitoneal component) and targeting the posterior side of the allograft in a lateral-to-medial approach (retrorenal component), the TER approach aims at reducing the risk of intraperitoneal as well as rectus sheet hematoma. By verifying low major complication (<1%) and high adequacy (>90%) rates among 250 patients undergoing 330 kidney transplant biopsies our present study confirms safety and efficacy of the TER approach for standard ultrasound-guided allograft biopsy. Furthermore, this is the first study to (1) assess both major and minor complications based on a standardized post-procedural ultrasound follow-up as well as to (2) confirm the eligibility of TER kidney transplant biopsy for nephrology training.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Innsbruck Medical University ethics committee, Medical University Innsbruck, Innrain 43, 6020 Innsbruck, Austria; Approval number: ECS 1106/2020. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Research idea and study design: MT; data collection: MT, MB, and MDB; data analysis/interpretation: MP, MB, MDB, and MT; statistical analysis: MB, MDB, and MP; manuscript preparation, drafting, and approval of the final version: MP, MB, MDB, and MT.

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.2021.10068/full#supplementary-material

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Age at Time of Kidney Transplantation as a Predictor for Mortality, Graft Loss and Self-Rated Health Status: Results From the Swiss Transplant Cohort Study

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Introduction: The effect of age on health outcomes in kidney transplantation remains inconclusive. This study aimed to analyze the relationship between age at time of kidney transplantation with mortality, graft loss and self-rated health status in adult kidney transplant recipients.

Methods: This study used data from the Swiss Transplant Cohort Study and included prospective data of kidney transplant recipients between 2008 and 2017. Time-to-event analysis was performed using Cox' regression analysis, and -in the case of graft loss- competing risk analysis. A random-intercept regression model was applied to analyse self-rated health status.

Results: We included 2,366 kidney transplant recipients. Age at transplantation linearly predicted mortality. It was also predictive for graft loss, though nonlinearly, showing that recipients aged between 35 and 55 years presented with the lowest risk of experiencing graft loss. No relationship of age with self-rated health status was detected.

Conclusion: Higher mortality in older recipients complies with data from the general population. The non-linear relationship between age and graft loss and the higher scored self-rated health status at all follow-up time-points compared to the pre-transplant status

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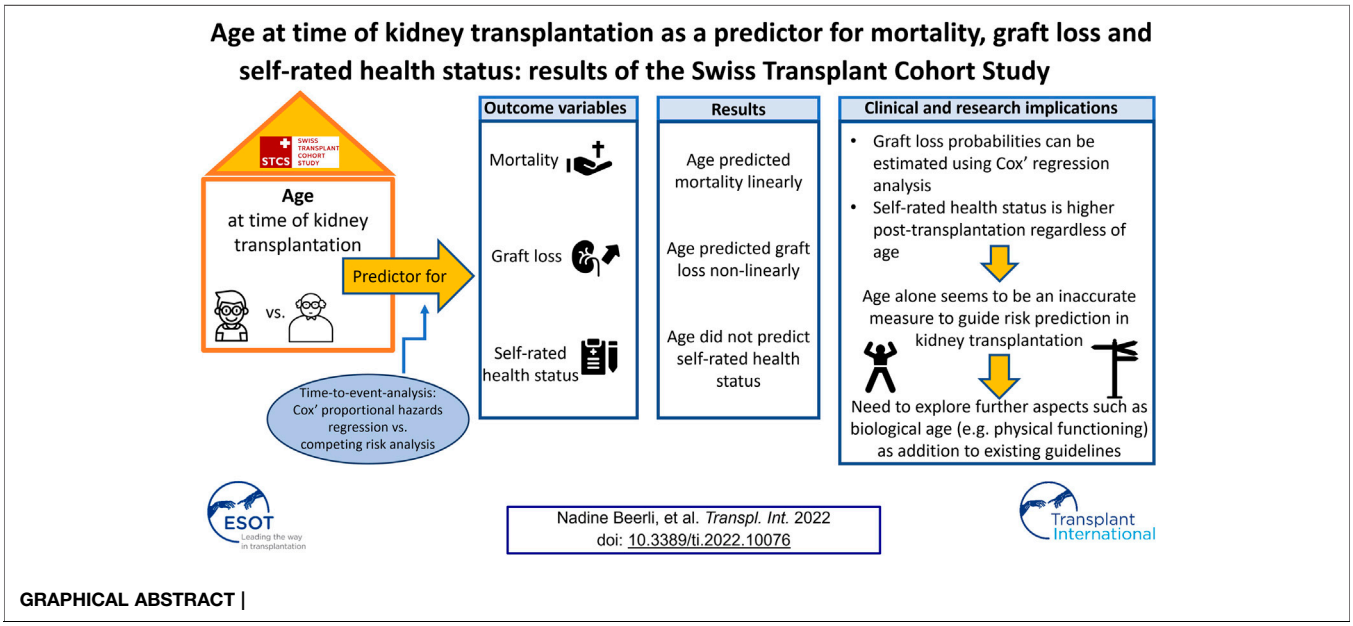
Abbreviations: Anti-CMV, anti-cytomegalovirus; BAASIS®, Basel Assessment of Adherence to Immunosuppressive Medications Scale; ESRD, end-stage renal disease; EQ-VAS, EuroQol Visual Analogue Scale; GF, allograft failure; HADS, Hospital Anxiety and Depression Scale; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; HR, hazard ratio; IQR, inter quartile range; KT, kidney transplantation; MICE, Multivariate Imputation by Chained Equations; PCKD, polycystic kidney disease; PROM, patient reported outcome measure; SD, standard deviation; STCS, Swiss Transplant Cohort Study.

-regardless of age- highlight that age alone might not be an accurate measure for risk prediction and clinical decision making in kidney transplantation.

Keywords: mortality, renal transplantation, age, graft loss, end stage renal disease, patient reported outcome measures



GRAPHICAL ABSTRACT |

INTRODUCTION

Ageing populations and a higher incidence of chronic conditions with advanced age have resulted in increasing numbers of older patients with end-stage renal disease (ESRD) [1, 2]. This trend is supported by a growing group of older adults considered eligible for and undergoing kidney transplantation (KT) [3–6]. According to records from the Swiss Transplant Cohort Study (STCS), 21% of all KT recipients in Switzerland—where there is no age limit prohibiting access to KT—are ≥65 years of age at time of transplantation [7]. In this context, age always refers to chronological age, i.e., the age counted in years since date of birth. Recently published guidelines recommend considering all patients with chronic kidney disease who are likely to progress to ESRD for KT regardless of their age [8]. KT is considered the preferred treatment option compared to hemodialysis, as it provides better results in terms of survival, cost effectiveness and patient reported quality of life [8–11]. The demand for KT at the same time significantly exceeds the number of available donor organs, thus, studies focusing on predictors for outcomes in older KT recipients present an important research area to support clinical decision and policy making.

Older patients often present with conditions such as disability, functional and cognitive decline and increased numbers of comorbidities such as cardiopulmonal diseases, diabetes or cancer, which can result in adverse health outcomes. Most studies point at an increased post-KT mortality in older KT recipients, an expected finding when comparing outcomes with study results from the general population [12–16]. On the contrary, a number of studies reported mortality rates similar to or lower than in adults of younger age [17–19]. Moreover, patients undergoing KT show a lower mortality risk compared to similar patients remaining on the waitlist and on dialysis [3, 20–22]. Inconsistencies also exist for graft loss, with studies showing higher rates in the older cohort [14, 17, 23] or alternatively a non-significant or protective effect by increasing age [15, 18, 24, 25].

Further, to better understand the effectiveness of healthcare interventions, the inclusion of patient reported outcome measures (PROMs) in addition to more commonly studied biomedical outcomes such as mortality and graft loss, is increasingly being acknowledged in transplant research [26]. In KT PROMs, like quality of life and self-rated health status, have been found to improve pre- to post-transplant in all age groups. Prospective longitudinal data from larger data sets, however, are scarce [9, 26]. Thus, studies in KT that include PROMs to better evaluate health outcomes over time are needed.

In previous studies, two methodological limitations in the field of KT point to the need to improve applied methods in future research. First, age has been frequently used as a categorical variable to facilitate interpretation of study findings, with varying age cut-offs across studies, thus assuming non-linear relationships. However, no one has investigated whether this holds true and where such a relationship would divert from linearity. Second, mortality and graft loss in KT are commonly analyzed using standard survival analysis (e.g., Kaplan-Meier survival curves and Cox' proportional hazards regression).

These methods only take into account one type of outcome per analysis, whereas KT recipients are simultaneously at risk for several adverse events. When graft survival is analyzed, a patient can experience death with a functioning graft, without altering the probability of graft loss, typically resulting in overestimated outcome probabilities [27].

Clinicians and policymakers have to rely on a limited body of evidence to guide organ allocation as well as pre- and post-KT management for older recipients. Thus, prospective multi-center research is essential to provide insights regarding causal relationships between patient's age and post-KT outcomes, with potential for generalizability [5, 6, 8, 11, 20, 28]. In particular, since age is still associated with lower odds to be waitlisted for and access to KT [12, 24, 29]. The aim of this study was to analyze the relationship between age at time of KT with mortality, graft loss incidence and post-transplant self-rated health status in adult KT recipients while controlling for bio-psychosocial risk factors.

PATIENTS AND METHODS

Design, Setting and Sample

This study used data from the STCS, a nation-wide prospective cohort study, which comprehensively assesses biomedical, psychosocial and behavioral risk factors [30]. Follow-up of a nationally representative sample of adult KT recipients from all six Swiss transplant centers occurs from pre-KT up to lifelong post-KT (6 months and 1 year post-KT, and yearly thereafter). Detailed information on the design of the STCS has previously been published elsewhere [30, 31]. The current study included data from patients enrolled between May 2008 (start of the cohort) and the end of 2017, who were aged ≥18 years at time of KT and had received a single-organ transplant. Follow-up of this cohort lasted until June 2019.

Data Collection, Management and Ethics

The STCS was approved by the ethical committees of all Swiss KT centers [EKBB 351/07, KEK 270/07, EKSG 07/122, EK 1487, CER 07-301 (NAC 07-117), Lausanne 284/07]. After providing written informed consent, patients completed the pre-KT STCS Psychosocial Questionnaire to collect selected socio-demographic, psychosocial and behavioral data [31]. Data on recipients' transplant outcomes (mortality and graft loss), age, and biomedical characteristics were collected from patient's charts by local data managers.

Variables and Measurement

Pre-KT covariates for the multivariable regression models, were based on evidence from the existing literature. We first determined the three controlling self-reported variables: depressive symptomatology, smoking and medication adherence of the STCS's psychosocial framework that were collected since the beginning of the STCS [32–39]. The multivariable regression models included covariates—donor age, donor type, specific types of comorbidities (diabetes mellitus, cardiopulmonary comorbidity, cancer history) preemptive KT and total number of HLA mismatches—that have been routinely assessed by the STCS [3, 5, 6, 8, 13, 16, 20, 24, 28].

Outcome Variables

Deaths recorded in the STCS were registered at the bedside by two physicians independently, and thereafter ascertained by the STCS endpoint committee. Graft loss as a primary cause of death is an unlikely event in the KT setting. To ensure correct classification of outcome events in patients with this primary cause of death registered in the STCS, their medical files were retrospectively re-analyzed by a physician of the transplant center where the patient was treated. Thereby, for patients who died due to multi-organ failure or a systemic infection (which secondary induced graft loss) a "*mortality*" *event* was considered as the first event. Mortality was recorded irrespective of previous graft loss, however, since graft survival cannot occur in patients already deceased, mortality was considered a competing risk of graft loss.

A *graft loss event* was defined as the absence of kidney function occurring at any time during follow-up, due to irreversible graft injury and requiring return to dialysis and/or re-KT. Death with a functioning graft was hereby not considered as graft loss.

Self-rated health status of the KT recipients was routinely assessed by the STCS at the time of listing, six, 12 months post-KT and then on a yearly base using the EQ VAS instrument, a PROM. The EQ VAS instrument is part of the EuroQol 5D instrument (EQ-5D), which is a preference-based measure of health status [40]. At each time point the EQ VAS score was collected by asking the KT-recipients to rate their self-perceived health today on a scale numbered from 0 to 100, where 0 represents the worst and 100 the best health they could imagine (continuous variable, presented as percentage). The EQ VAS instrument provides a quantitative measure of the patient's perception of their overall health and therefore represents the patient perspective.

Socio-Demographic, Behavioral and Psychosocial Characteristics

Socio-demographic characteristics extracted from the STCS baseline database included sex, race, marital status and age in years at transplantation. Depressive symptomatology pre KT was assessed with the 7-item depression subscale of the Hospital Anxiety and Depression (HADS) scale. Each HADS depression-subscale item was answered on a 4-point Likert scale (0 = "not at all" to 3 = "most of the time"), the total score was calculated by summing the item scores and used as a continuous variable (range 0–21) [31]. To assess implementation of medication adherence pre-KT two self-report items (taking adherence and drug holidays) from the Basel Assessment of Adherence to Immunosuppressive Medications Scale (BAASIS®) instrument—in an adapted version for adherence to other medications pre-KT—were used [31]. Medication non-adherence (yes/no) was defined as any missed doses, having missed at least one dose of medication and/or having missed two or more consecutive doses over the past 4 weeks. Psychometric data of

TABLE 1 | Sample characteristics.

Variable	Specification variable	Total sample (n = 2366)
Outcomes		
Mortality events	n (%)	298 (12.6)
Mortality events of patients without graft loss	n (%)	234 (9.9)
Graft loss events	n (%)	198 (8.4)
Time to death in months (n = 298)	Mean (SD)	45.9 (33.0)
	Median (IQR)	42.9 (54.0)
	Min—max	0.1–120.4
Time to graft loss in months (n = 198)	Mean (SD)	34.5 (31.6)
	Median (IQR)	28.6 (52.8)
	Min—max	0.0–118.0
Length of follow-up in months	Mean (SD)	72.0 (34.1)
	Median (IQR)	70.1 (61.2)
	Min—max	0.1–120.4
Socio-demographic recipient characteristics		
Age at transplantation	Mean (SD)	52.9 (13.6)
	Median (IQR)	55.0 (19.0)
	Min—max	18.0–82.0
Sex	Female, n (%)	848 (35.8)
Race	Caucasian, n (%)	2153 (91.7)
Marital status	Single, n (%)	378 (17.9)
	Married/living together, n (%)	1406 (66.7)
	Divorced/separated, n (%)	246 (11.7)
	Widow(er), n (%)	79 (3.7)
Psychological and behavioral recipient characteristics		
Depressive symptomatology[1]	Mean (SD) HADS score	4.5 (3.7)
	Median (IQR) HADS score	4 (4)
	Min—max	0–21
Medication non-adherence[2]	Yes, n (%)	677 (28.6)
Current smoking	Yes, n (%)	418 (19.6)
Biomedical recipient characteristics KT and donor characteristics		
Etiology of renal disease	Cause unknown, n (%)	136 (5.8)
	Congenital, n (%)	57 (2.4)
	Diabetic nephropathy, n (%)	195 (8.3)
	Glomerulonephritis, n (%)	561 (23.9)
	HIV nephropathy, n(%)	3 (0.1)
	Hereditary non PCKD, n (%)	76 (3.2)
	Interstitial nephropathy, n (%)	79 (3.4)
	Nephrosclerosis, n (%)	265 (11.3)
	Other, n (%)	283 (12.1)
	PCKD, n (%)	454 (19.3)
	Previous GF, n (%)	118 (5.0)
	Reflux/Pyelonephritis	120 (5.1)
Type of renal replacement therapy	None, n (%)	411 (17.4)
	Peritoneal dialysis, n (%)	319 (13.5)
	Haemodialysis, n (%)	1631 (69.1)
Years on dialysis	Mean (SD)	4.0 (5.0)
	Median (IQR)	3.0 (41.0)
	Min—max	1.0–42.0
Anti-CMV status	Seropositive, n (%)	1459 (61.9)
Cancer history	Yes, n (%)	258 (10.9)
Diabetes mellitus[3]	Yes, n (%)	651 (27.5)
Cardiopulmonary comorbidity[4]	Yes, n (%)	1180 (49.9)

(Continued on following page)

TABLE 1 | (*Continued*) Sample characteristics.

Variable	Specification variable	Total sample (n = 2366)
KT and donor characteristics		
Type of KT	Deceased-donor, n (%)	1392 (58.8)
	Living-donor, n (%)	974 (41.2)
Extended criteria donation[5] (n = 864)	Yes, n (%)	311 (36.0)
Total number of HLA mismatches[6]	Mean (SD)	3.8 (1.5)
	Median (IQR)	4 (2)
	Min—max	0–6
Donor age[7]	Mean (SD)	52.4 (16.1)
	Median (IQR)	55.0 (18)
	Min—max	0–88

[1]*Each HADS depression-subscale item was answered on a 4-point Likert scale (0="not at all" to 3="most of the time"), the total score was calculated by summing the item scores and used as a continuous variable (range 0–21).*
[2]*Medication non-adherence (yes/no) was defined as any missed doses, having missed at least one dose of medication and/or having missed two or more consecutive doses over the past 4 weeks.*
[3]*Defined as having diabetes mellitus 1 or 2 according to STCS definitions.*
[4]*Defined as having coronary heart disease, cerebral vascular disease, peripheral vascular disease, left ventricular dysfunction according to STCS definitions.*
[5]*Defined as a KT from a donor aged ≥60 years or aged ≥50 years with at least two of the following conditions: history of hypertension, serum creatinine >1.5 mg/dl or cerebrovascular accident as cause of death; 6 min 0; max 6.*
[6]*Count of HLA mismatches.*
[7]*Continuous variable in years since birth.*
SD, standard deviation; IQR, interquartile range; HADS, Hospital Anxiety and Depression Scale; KT, kidney transplantation; STCS, Swiss Transplant Cohort Study; HIV, human immunodeficiency virus; PCKD, polycystic kidney disease; GF, allograft failure; Anti-CMV, anti-cytomegalovirus; HLA, Human Leukocyte Antigen.

FIGURE 1 | Flow chart of the sample composition.

the BAASIS® were previously reported [41–44]. We assessed smoking through one self-report item on smoking status (yes/no).

Biomedical Recipient, KT and Donor Characteristics

The STCS biomedical variables reflecting the recipient's clinical status immediately pre-KT were: etiology of renal disease, type of renal replacement therapy received, years on dialysis, Anti-CMV status and pre KT comorbidities (cancer, diabetes mellitus or cardiopulmonary disease). Transplant-related variables were: type of KT (living, deceased-donor) date of KT (day/month/year), the total number of HLA mismatches, donors' age in years and sex (female/male), delayed graft function (yes, no), reason for graft loss, described immunosuppressant (**Table 1**). Extended criteria donation was not reliably captured as a controlling variable for the models, as data collection was not conclusive.

Data Analysis

Descriptive statistics were applied to describe the sample characteristics, perceived health status over time and the incidence of graft loss as well as mortality. Time to event analysis was performed by Cox' proportional hazards regression analysis for mortality and graft loss, and—in the case of time to graft loss, also by competing risk analysis using *Fine and Gray's* regression model [45]. A competing risk is defined as "an event whose occurrence either precludes the occurrence of another event under examination, or fundamentally alters the probability of occurrence of this other event" [27, 46]. The competing risk model estimates the prognosis of graft loss in the presence of mortality as a

TABLE 2 | Results of the survival analyses.

Outcome	Pre-KT predictor	Hazard ratio Cox' regression (95% confidence interval)	*p*-value	Hazard ratio fine & gray model (95% confidence interval)	*p*-Value
Mortality	Unadjusted model	[1]			
	Age at KT	1.07 (1.06–1.08)	<0.0001	/	
	Adjusted model	[2]			
	Age at KT	1.07 (1.05–1.08)	<0.0001	/	
	Current smoking	1.48 (1.12–1.95)	0.0060	/	
	Medication adherence	0.88 (0.66–1.18)	0.4086	/	
	Cancer history	0.84 (0.60–1.17)	0.3012	/	
	Cardiopulmonary comorbidity	0.71 (0.26–1.94)	0.5024	/	
	Diabetes mellitus	1.40 (1.10–1.77)	0.0056	/	
	Depressive symptomatology	1.00 (0.97–1.03)	0.9541	/	
	Donor age	2.58 (0.92–7.17)	0.0704	/	
	Donor type[5]	0.69 (0.52–0.91)	0.0097	/	
	Preemtive KT	1.65 (1.04–2.64)	0.0344	/	
Graft loss	Unadjusted model	[3]			
	Age at KT	0.95 (0.89–1.01)	0.0949	0.95 (0.90–1.01)	0.1181
	$Age^{squared}$ at KT	1.00 (1.00–1.00)	0.0457	1.00 (1.00–1.00)	0.0724
	Adjusted model	[4]			
	Age at KT	0.93 (0.88–0.99)	0.0241	0.94 (0.88–0.99)	0.0367
	$Age^{squared}$ at KT	1.00 (1.00–1.00)	0.0224	1.00 (1.00–1.00)	0.0425
	Current smoking	1.33 (0.96–1.86)	0.0905	1.29 (0.93–1.81)	0.1335
	Medication adherence	0.88 (0.63–1.23)	0.4585	0.88 (0.63–1.23)	0.4696
	Cardiopulmonary comorbidity	1.15 (0.28–4.70)	0.8493	1.33 (0.34–5.18)	0.6843
	Diabetes mellitus	1.17 (0.86–1.60)	0.3145	1.14 (0.84–1.55)	0.4168
	Depressive symptomatology	1.00 (0.97–1.04)	0.8676	1.00 (0.97–1.04)	0.8673
	HLA mismatches	1.02 (0.92–1.13)	0.7752	1.02 (0.92–1.13)	0.7250
	Donor age	1.19 (0.28–4.90)	0.8131	1.00 (0.25–3.95)	0.9958
	Donor type[5]	0.55 (0.39–0.78)	0.0008	0.57 (0.40–0.80)	0.0013
	Preemptive KT	2.05 (1.13–3.71)	0.0184	2.03 (1.11–3.70)	0.0212

C-statistics (95%CI).
[1]0.72 (0.69–0.74).
[2]0.75 (0.72–0.77).
[3]0.55 (0.51–0.58).
[4]0.65 (0.61–0.69).
[5]better survival for living donor grafts.

competing risk. Analyses for mortality and graft loss were executed unadjusted and adjusted for aforementioned controlling variables and additionally included examination of possible non-linear relationships of age with both outcomes by testing higher-order terms and also by plotting martingale residuals [47]. Missing data generally did not exceed 10%. However, in the case of non-adherence to medication with missing values of 14% of the sample resulting from the fact that not all wait-listed patients stated to be taking prescribed medications, we applied multiple imputation *via* "Multivariate Imputation by Chained Equations" (MICE). In this case MICE was performed in order to calculate adjusted models on the same sample as the unadjusted ones. Five rounds of "fully conditionally specificated imputation" were executed on variables deemed appropriate by the algorithm.

To analyse the relationship between age and self-rated health status, we applied a random-intercept regression model, predicting recipient's repeatedly measured health status over time. Analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, United States); MICE was performed in R 3.6.2 (cran.r-project.org). Alpha was set at $p = 0.05$.

RESULTS

Sample Characteristics

For the current study, 2,553 KT recipients involved in the STCS were considered eligible, of whom 2,366 agreed and were included. A flowchart showing the sample selection process is provided in **Figure 1**. The sample's median follow-up time (which lasted until June 2019) was 70.1 months (IQR 61.2, range: 0.1–120.4). We lost 27 patients to follow-up prior the end of the study period (n = 27, 1.1%).

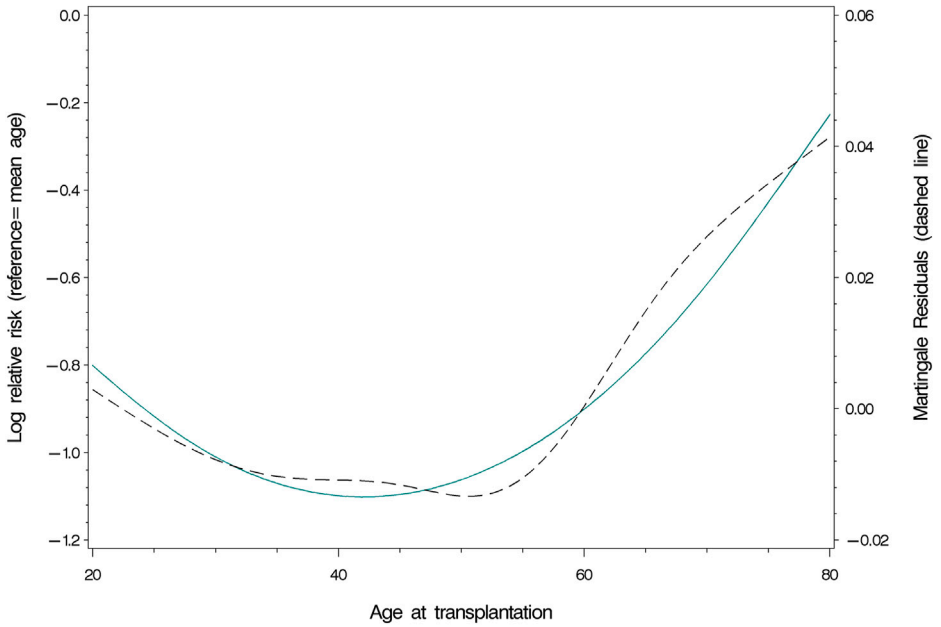


FIGURE 2 | Nonlinear relationship between the probability of graft loss and age at transplantation.

TABLE 3 | Self-rated health status.

Month of follow up	N	Mean (SD) EQ-VAS	Median (IQR) EQ-VAS
Baseline	2098	62.2 (20.6)	65.0 (30.0)
6	1776	74.0 (17.5)	80.0 (22.0)
12	1633	76.2 (17.0)	80.0 (21.0)
24	1336	75.6 (17.6)	80.0 (20.0)
36	1107	74.9 (17.7)	80.0 (25.0)
48	863	75.1 (17.8)	80.0 (24.0)
60	669	74.5 (17.5)	80.0 (25.0)
72	509	74.1 (16.8)	79.0 (21.0)
84	349	72.6 (17.9)	76.0 (24.0)
96	190	74.0 (17.2)	80.0 (20.0)

EQ-VAS, EuroQol Visual Analogue Scale; SD, standard deviation; IQR, interquartile range.

Table 1 provides an overview of the sample characteristics. The average recipient age was 52.9 years (SD 13.6, range: 18–82) at the time of transplantation, 35.8% (n = 848) of the recipients were female. In 58.8% (n = 1392) of cases grafts were received from deceased donors, and the average donor age was 52.4 years (SD 16.1, range: 0–88). In our sample 8.4% experienced graft loss (n = 198) during the study period, 12.6% died (n = 298) and 2.7% experienced both outcomes (n = 64). The etiology of renal disease was glomerulonephritis (23.9%) and polycystic kidney disease (19.3%) in majority of studied patients. A non-adherence to the pre-KT medication was reported by 28.6% (n = 677) of the KT recipients and 19.6% (n = 418) were smoking at the time of transplantation. Renal replacement therapy before transplantation was provided for 69.1% (n = 1631) by haemodialysis treatment while 17.4% (n = 411) of KT recipients received a preemptive transplantation. We found a median HADS score of 4 (IQR 4, range: 0–21).

Age at Time of Transplant and Mortality and Graft Loss

Age at the time of transplantation predicted mortality in a linear fashion [HR (Hazard Ratio) = 1.07; 95% CI: 1.06–1.08; p <0 .0001; **Table 2**]. The relationship remained intact when adjusting for covariates (HR = 1.07; 95% CI: 1.05–1.08; p <0 .0001), of which current smoking (p = 0.0060), diabetes mellitus (p = 0.0056) and donor type (p = 0.0002, better survival for living donor grafts) were significant. Concurrently, age at time of transplantation predicted graft loss, though in a non-linear way (p = 0.0224; **Table 2**). **Figure 2** displays the results of our examination of non-linear relationships and shows that patients between 35 and 55 years of age had a lower risk of experiencing graft loss, while the probability of those younger and older was higher. This non-linear relationship remained significant (p = 0.0224) after controlling for covariates. Recipients who received a transplant from a living donor (p = 0.0008) and preemptive KT (p = 0.0184) recipients experienced lower graft loss rates. **Table 2** displays the results comparing statistical models using Cox' regression and Fine and Grays' competing risk approach, showing only negligible differences between the two analysis methods.

A sensitivity analyses was performed applying modeling without imputations, finding that the quadratic term that predicted graft loss was insignificant (p = 0.18), for which the adherence variable was responsible. However, this was not because of a confounding relationship of adherence, but

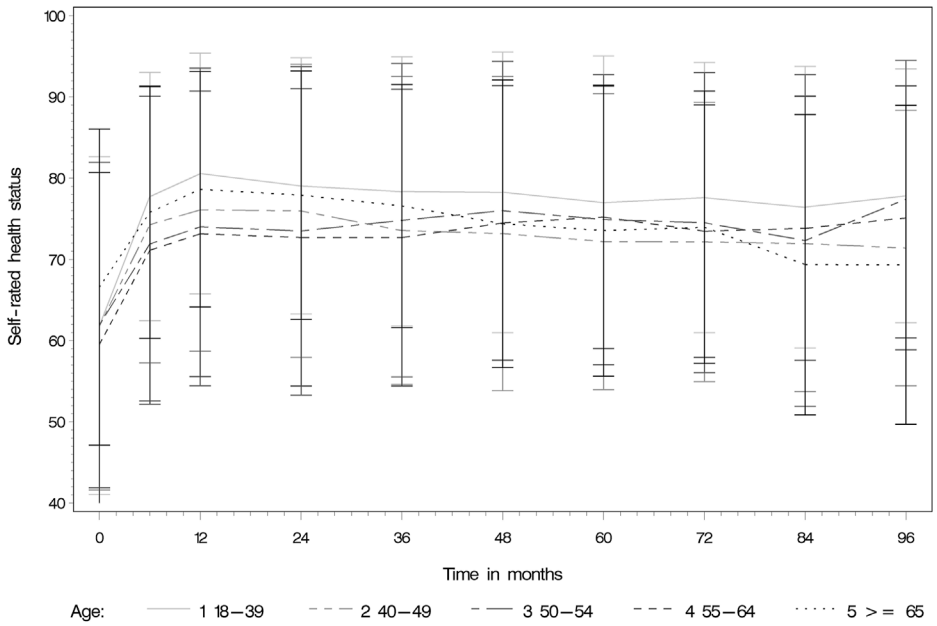


FIGURE 3 | Self-rated overall health during the assessment period from pre-KT up to 8 years post-KT.

TABLE 4 | Results of the linear mixed-model regression analysis, predicting (square-transformed) health status.

Effect	Estimate (95%Confidence interval)	Standard error	DF	t Value	Pr > |t|
(1) Intercept	6445.31 (6107.74–6782.88)	172.15	2376	37.44	<0.0001
(2) Follow up time in months	−7.26 (−9.56 to −4.96)	1.1711	1203	−6.20	<0.0001
(3) Measurements from month 12 on (yes/no)	−1883.03 (−1984.47 to −1781.58)	51.7514	8755	−36.39	<0.0001
(4) Interaction effect of follow up time in months (2) with the binary variable before/from month 12 on (3)	250.42 (234.05 to 266.79)	8.3534	7829	29.98	<0.0001
(5) Age in years	−5.29 (−11.31 to 0.72)	3.0660	2191	−1.73	0.0844

Note: Parameters (3) and (4) were functional in modeling the initial increase in the health status curve as shown in Figure.

resulting merely from the missing subjects, as omitting the same patients in the unadjusted model had the same effect. Results also show a collinearity between cardiopulmonary comorbidities and donor age; both were in all models statistically significant if included separately. Both variables were kept in the model, as they were not the primary aim of our analysis and were only needed as controlling factors.

Age at Time of Transplant and Self-Rated Health Status

The median pre-KT health status was rated at 65/100 (IQR 30). The median self-rated health status was assessed noticeably higher during the whole post-KT follow up time (e.g., 12 months post-KT median EQ-VAS 80/100 IQR 21). **Table 3** and **Figure 3** display the self-rated health status during the assessment period from pre-KT up to 8 years post-KT, showing higher scores at all follow-up time-points compared to the pre-KT status, regardless of age. Generally, younger and older KT patients rated their health status higher before and after KT compared to middle-aged. No relationship of age with health status could be detected (**Table 4**) (β = −5.29; 95% CI: −3.36 to 0.85; p = 0.0844).

DISCUSSION

The objective of this prospective nationwide cohort study was to analyze the relationship between age at time of KT with mortality, graft loss incidence and self-rated health status in adult KT recipients. Age at the time of KT predicted mortality in a linear fashion but a non-linear relationship between age and graft loss was detected. Our analysis indicates that by taking into account the competing risk of mortality in estimating probabilities of graft loss, the risk of both outcomes is fairly independent of each other. Thus, graft loss probabilities can be reasonably well estimated using Cox'

regression analysis without applying a competing risk analysis. It should be noted that only 2.7% of our sample experienced both outcomes, and a higher overlap may result in larger differences between the two analysis methods. The self-rated health status during the assessment period from pre-KT up to 8 years post-KT showed higher scores at all follow-up time-points compared to the pre-KT status, regardless of their age. No relationship between age at time of KT and post-KT self-rated health status was found, age therefore did not predict this outcome.

Our results support those of previous studies that reported increased mortality after KT in older recipients compared to younger ones; however, this is consistent with data from the general population [12–16]. Whereas the linear relationship between age and patient survival in the current study does not back the assumptions of previous studies that stepwise mortality risk changes across age groups exist. The result of our analysis does hence not support the use of age as a categorical variable to interpret study findings of patient survival in KT. In contrast, the nonlinear prediction of graft loss by age at the time of transplantation does not reflect conclusions of studies that found a linear increase in post-KT graft loss with older patients [14, 17, 23]. Patients between 35 and 55 years of age presented with a lower risk of experiencing graft loss in our study, whereas older and younger recipients showed a higher probability. Pre-transplant drug non-adherence is a proposed factor that can negatively influence adherence to immunosuppressive regimen in transplant candidates. Several studies reported that non-adherence to the immunosuppressive regimen has a negative effect on graft and patient survival in the population of KT recipients [36–38, 48–51]. Evidence shows that younger adults are at greater risk for drug non-adherence in KT [37]. Concurrently, mild cognitive impairment and the presence of additional comorbidities are common in ESRD and KT recipients. They are also found to be associated with older age in these populations [49, 50]. Furthermore, mild cognitive impairment is associated with decreased medication adherence as well as health literacy in KT recipients [51]. This evidence may support our findings that middle-aged adults after KT have a lower risk of experiencing graft loss than their younger and older counterparts.

Chronological ageing alone has been described as an inaccurate representation of patients' functional ability and individuals of similar age can show diverse physical and cognitive conditions [28, 52]. Biological age in turn was found to be a strong independent predictor for adverse health outcomes such as mortality and graft loss in KT recipients [5, 8, 28, 52]. Physical frailty is currently proposed as an indicator for biological age [5, 8, 28]. The inclusion of frailty measurements to determine the biological age of a KT recipient could hence be a valuable addition to the single determination of age counted in years in the KT population to predict adverse health outcomes. Relevant associations and organizations increasingly acknowledge the importance of the inclusion of frailty assessments in clinical practice guidelines for evaluating and managing candidates for KT [5, 8, 28, 53].

Besides biological age, psychosocial factors can independently predict poor post-KT outcomes and are increasingly valued in transplant research [31, 54]. International transplant societies endorse a comprehensive bio-psychosocial evaluation prior to transplantation and include them in their clinical guidelines [14, 20, 28]. With a low median HADS score of 4 points, our study participants reported fewer depressive symptoms than described in other studies [33, 55]. However, our study showed that 28.6% of the KT recipients were non-adherent to their medication before transplantation and 19.6% were smoking. These figures reflect the results of previous research [32, 34, 56] but only current smoking status was determined as a significant covariate for the mortality outcome event in our sample. No other psychosocial covariate was identified as significant in our analysis. These results could be due to the fact that in our current study only a limited number of psychosocial factors could be considered as covariates, since routine data collection of a comprehensive set of variables has only been added more recently. The STCS Psychosocial Questionnaire is self-administered and not conducted as a face-to-face interview.

Regardless of age, the self-rated health status during the whole follow-up period was rated notably higher post-KT. This shows that the effect of the intervention from a patient perspective was influencing their health status positively in a sustainable way and therefore KT presents a longtime advantage compared to the pre-transplant status. This finding concurs with previous smaller studies over shorter time periods showing an increase in quality of life and self-rated health post-KT [9, 26] and can be used in clinical practice for counselling particularly of older potential KT recipients. To include the patient perspective on health outcomes by assessing PROMs such as self-rated health status in the pre-KT evaluation and decision making should therefore be considered.

The strengths of this extensive study are its longitudinal, prospective design as well as the application of competing risk analysis. The nationwide multi center design in a European setting including a comprehensive sample of KT recipients with an extensive follow-up time, provides insights regarding relationships between patient's age and the post-KT outcomes of patient and graft survival. The application of competing risk analysis allows the prognosis of graft loss in the presence of mortality as a competing risk. Despite its' strong and rigorous study design, a notable weakness of this study is that only 2.7% of our sample experienced both outcomes of mortality and graft loss. A higher overlap may result in larger differences between the competing risk and standard survival analysis. Thus, in samples with a higher overlap, the probabilities of graft loss may not be sufficiently well estimated if only Cox' regression analysis is used without applying a competing risk analysis. A further weakness of our study is that we assessed only a limited number of pre-KT psychosocial factors.

CONCLUSION

This study revealed that age at the time of KT predicted mortality in a linear fashion concurring with records from the general population in the same country. In contrary, a non-linear relationship between age and graft loss was detected showing that KT recipients aged between 35 and 55 years presented with the lowest risk of experiencing a graft loss event. Taking into account the competing risk of mortality in estimating

probabilities of graft loss, the risk of both outcomes was fairly independent from each other. Thus, graft loss probabilities can be estimated using Cox' regression analysis. Self-rated health status during the follow-up period was indicated notably higher post-KT, regardless of age. No relationship between age at time of KT and post-KT self-rated health over the entire follow-up time was found. Therefore, age alone seems to be an inaccurate measure to guide risk prediction in KT. This underlines the importance of exploring further aspects such as biological age as a valuable addition to existing KT-guidelines aiming to provide pre-tailored and effective guidance particularly as individuals of similar age can show substantially diverse conditions.

CAPSULE SENTENCE SUMMARY

The numbers of older patients considered eligible for and undergoing kidney transplantation are increasing. However, the effect of age at time of transplantation on health outcomes in kidney transplantation remains inconclusive. The objective of our study was to analyze the relationship between age at time of kidney transplantation with mortality, graft loss and self-rated health status in adult kidney transplant recipients. We used data from the prospective Swiss Transplant Cohort Study and included data of 2366 kidney transplant recipients who received a single-organ kidney transplant between 2008 and 2017. Age at transplantation linearly predicted mortality. It was also predictive for graft loss, though nonlinearly, showing that recipients aged between 35 and 55 years presented with the lowest risk of experiencing graft loss. Self-rated health status during the follow-up period was indicated notably higher post-transplantation, regardless of age. No relationship of age with self-rated health status was detected. Therefore, age alone seems to be an inaccurate measure to guide risk prediction and clinical decision making in kidney transplantation. This underlines the importance of exploring further aspects such as biological age including cognition, psychosocial factors, PROMs and physical functioning as a valuable addition to existing KT-guidelines aiming to provide pre-tailored and effective guidance particularly as individuals of similar age can show substantially diverse conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because dataset cannot be shared due to current STCS policy. Requests to access the datasets should be directed to www.stcs.ch.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committees of all Swiss kidney transplant centers [EKBB 351/07, KEK 270/07, EKSG 07/122, EK 1487, CER 07-301 (NAC 07-117), Lausanne 284/07]. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OM, NB, KD, and SDG participated in research design, performance of the research, data analysis and interpretation. OM, NB, KD, IB, SD, MD, DG, KH, UH-D, AS, and SDG participated in the writing of the paper.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10076/full#supplementary-material

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ORIGINAL RESEARCH
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Urological Complications Associated With Pyeloureterostomy Without Ipsilateral Nephrectomy in Renal Transplant Recipients

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Background: The implications of ligating the native ureter without ipsilateral nephrectomy after primary kidney transplant pyeloureterostomy (PU) have been described previously.

Methods: This single-center retrospective cohort study including 4,215 kidney transplants performed between February 2010 and December 2014, analyzed urological complications following primary (P-PU) and secondary (S-PU) pyeloureterostomy used to treat urological leaks (UL-PU) and ureteral stenosis (US-PU) without concomitant ipsilateral nephrectomy, in a large cohort of patients.

Results: There were 495 (11.7%) pyeloureterostomy with native ureter ligation without nephrectomy, 409 P-PU (82.6%) and 86 S-PU (17.4%), of which 76 were UL-PU and 10 were US-PU. The median follow-up was 33.8 months. The incidence of native ipsilateral kidney complications requiring nephrectomy was 2.02% (n = 10). Urinary leak was diagnosed in 3.6% of patients after P-UP and 9.2% after UL-PU. Ureteral stenosis was diagnosed in 1.7% of patients after P-UP, 3.9% after UL-PU and 10% after US-PU.

Conclusion: This cohort analysis suggests that native ureter ligation during pyeloureterostomy without native nephrectomy is associated with low incidence of clinically indicated ipsilateral native nephrectomy. Caution and awareness should be emphasized in patients with history of ADPKD and neurogenic augmented bladders.

Keywords: kidney transplant recipients, pyeloureterostomy, nephrectomy, urinary complications, urinary leak, ureteral stenosis

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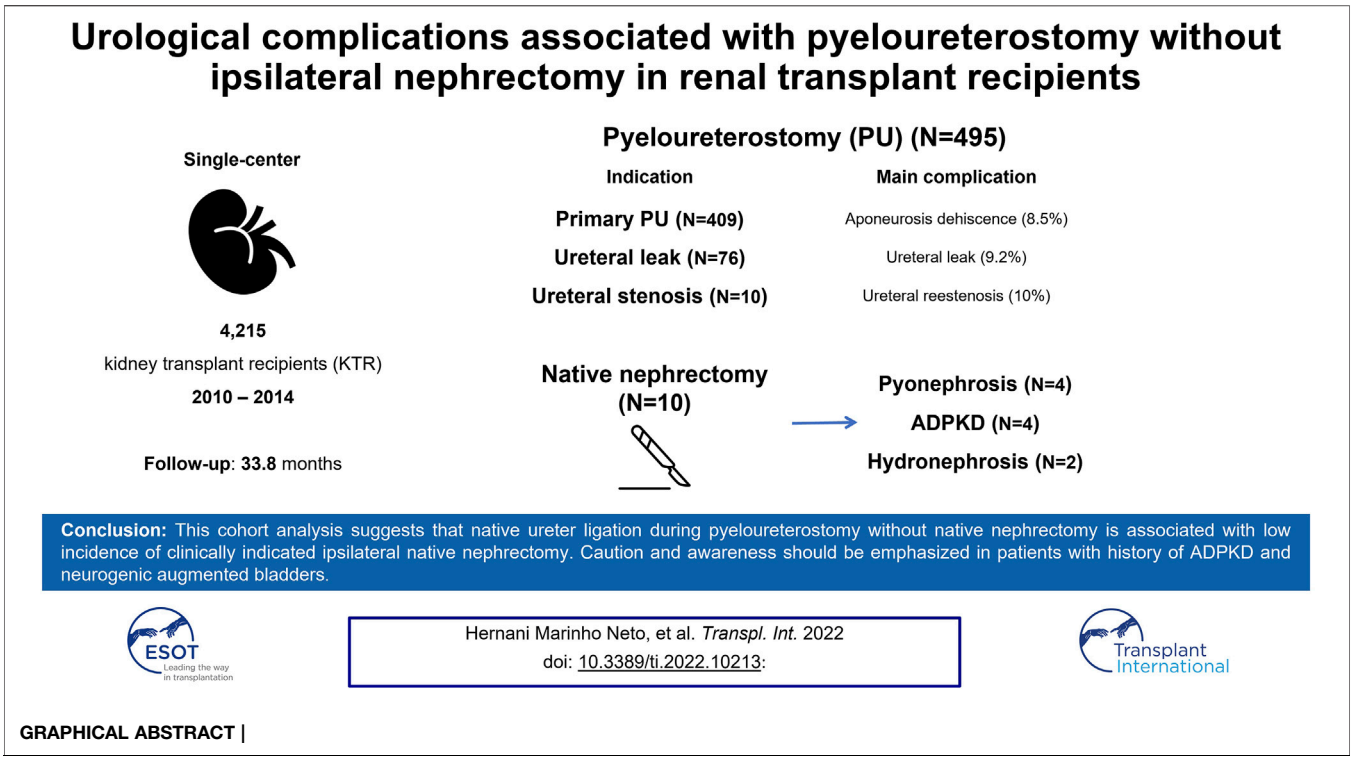
INTRODUCTION

Classical techniques for urinary tract reconstruction during a kidney transplant surgery include reimplantation of the kidney donor ureter with the recipient´s bladder (ureteroneocystostomy) or with the recipient's native ureter (pyeloureterostomy or ureteroureterostomy). While both techniques show similar urological complication rates, most transplant centers initially opt for a ureteroneocystostomy using the Lich-Gregoir technique (1–3), deferring the use of ureteroureterostomy, usually without ipsilateral nephrectomy, as a secondary option in case of complications in the ureteroneocystostomy



GRAPHICAL ABSTRACT |

TABLE 1 | Demographic characteristics of the study population.

Variable, n (%)	Total (n = 495)	P-PU (n = 409)	UL-PU (n = 76)	US-PU (n = 10)
Recipients				
Age, years	48.7 ± 13.2	49.8 ± 12.8	42.8 ± 13.9	46.4 ± 11.8
Gender, male	332 (67)	277 (68.2)	50 (64.9)	5 (50)
Ethnicity, white	269 (54.3)	233 (57.3)	39 (50.6)	7 (70)
CKD etiology				
Undetermined	201 (40.6)	161 (39.2)	35 (46)	5 (50)
Hypertension	83 (16.7)	71 (17.3)	10 (13.1)	2 (20)
Diabetes Mellitus	58 (11.7)	49 (11.9)	8 (10.5)	1 (10)
Glomerulopathy	69 (14.1)	58 (14.1)	11 (14.4)	0
ADPKD	33 (6.6)	28 (6.8)	3 (3.9)	2 (20)
Neurogenic bladder	11 (2.2)	9 (2.2)	2 (2.5)	0
Other	40 (8.2)	33 (8.0)	7 (9.2)	0
BMI, Kg/m^2	25 ± 4.3	25 ± 4.5	23 ± 5.6	23 ± 4.5
Diabetes Mellitus	82 (16.6)	68 (16.7)	13 (16.8)	1 (10)
Hemodialysis	443 (89.4)	372 (90.9)	65 (85.5)	6 (60)
Dialysis time, months	73,4 ± 60.3	80,0 ± 61.2	38 ± 32.4	46.8 ± 28.2
Residual diuresis, mL/day	221 ± 431	164 ± 347	524 ± 649	360 ± 337
Donor				
Deceased	429 (86.6)	367 (89.7)	52 (68.4)	10 (100)
Living	66 (13.3)	42 (8.6)	24 (31.6)	0

BMI, body mass index; CKD, chronic Kidney disease ADPKD, autosomal dominant polycystic kidney disease.

anastomosis, such as urinary leak and ureteral stenosis (3–10). Although some reports have shown that the native ureter ligation without nephrectomy is safe (3–8), this technique may cause hydronephrosis, primarily in patients with significant residual diuresis, and eventually discomfort or lumbar pain.

As a primary objective, we evaluated the risk of future nephrectomy in these patients, and the secondary objective was to assess other urological complications with the need for surgical intervention.

METHODS

This was a single-center, retrospective cohort study that included data from the electronic records of all patients who

TABLE 2 | Surgical complications.

Primary pyeloureterostomy	n = 409
Total, n (%)	107 (26.1)
Aponeurosis dehiscence	35 (8.5)
Isolated	23
With skin dehiscence	4
With surgical site infection	3
With hematoma	3
With internal hernia	1
With skin dehiscence and surgical site infection	1
Ureteral leak	15 (3.6)
Isolated	11
With hematoma	1
With surgical site infection	1
With aponeurosis dehiscence and hematoma	1
With aponeurosis dehiscence and surgical site infection	1
Perigraft hematoma	12 (2.9)
Surgical site infection	11 (2.6)
Ureteral stenosis	7 (1.7)
Isolated	4
With aponeurosis dehiscence	2
With lymphocele and incisional hernia	1
Venous thrombosis	7 (1.7)
Skin dehiscence	6 (1.5)
Lymphocele	6 (1.5)
Incisional hernia	6 (1.5)
Arterial thrombosis	1 (0.2)
Renal rupture	1 (0.2)
Ureteral leak treated with pyeloureterostomy (UL-PU)	n = 76
Total, n (%)	16 (21.0)
Urinary leak	7 (9.2)
Isolated	5
With skin dehiscence	1
With aponeurosis dehiscence	1
Ureteral stenosis	3 (3.9)
Surgical site infection	2 (2.6)
Skin dehiscence	2 (2.6)
Aponeurosis dehiscence	1 (1.3)
Lymphocele	1 (1.3)
Ureteral stenosis treated with pyeloureterostomy (US-PU)	n = 10
Total of complication	1 (10)
Ureteral restenosis	1 (10)

underwent kidney transplantation from February 2010 to December 2014 at Hospital do Rim, Brazil. The local Ethics Committee approved this study. Patients with missing demographic or surgical data were excluded. For this analysis, only urological complications that required new surgical procedures were analyzed. Continuous variables were presented as mean and standard deviation, and categorical variables were presented as frequencies.

Our routine reimplantation technique is a Lich-Gregoir procedure without stenting, saving the pyeloureterostomy (PU) for three key indications: 1. difficult access to the bladder; 2. doubtful graft ureter viability; 3. as a secondary anastomosis method to treat ureteroneocystostomy complications (urine leak or stenosis). All cases, the PU included a simple proximal native ureter ligation, leaving the obstructed kidney *in situ*. The anastomosis between the renal pelvis and the spatulated distal native ureter is performed in an end-to -end technique using running 6.0 polydioxanone sutures (PDS® II). A double-J ureteral stent (6 fr × 18 cm) was left for 28 days and an indwelling urinary 20 fr Foley catheter for 7 days.

RESULTS

Demographic Characteristics and Prevalence of Pyeloureterostomy

From December 2010 to February 2014, a total of 4,215 kidney transplants were performed in our institution. We excluded 264 (6.3%) patients due to incomplete data. Of the remaining 3,951 transplanted patients, 2,903 (73.5%) received a kidney from a deceased donor and 1,048 (26.5%) from a living donor. Of them, 495 (12.5%) patients underwent pyeloureterostomy, 409 (10.3%) as a primary procedure performed at the time of the transplant (P-PU) and 86 (2.2%) as a secondary technique to treat urinary leak (UL-PU, n = 76) or ureteral stenosis (US-PU, n = 10). Demographic characteristics of the study population are described in **Table 1.**

Urological Complications

Primary Pyeloureterostomy

Of 409 P-PU, 367 were performed in deceased (89.7%) and 42 (10.3%) in living donor kidney transplant recipients. All these cases were performed for two reasons: 1. difficult access to the bladder; 2. doubtful graft ureter viability.

As indicated in **Table 2**, urinary leakage occurred in 15 patients (3.6%) between 2 and 45 days after the P-PU. In 13 patients (87%), the pyeloureterostomy was remade over a double-J catheter, and five required two surgical procedures, including a protective nephrostomy. One of these patients developed a deep surgical site infection requiring graft nephrectomy 56 days after transplantation. Finally, one (6.6%) patient was treated with a single suture stitch, and another one (6.6%) was treated conservatively by retrograde insertion of a double-J ureteral catheter and an indwelling urinary catheter.

Seven patients (1.7%) developed pyeloureterostomy stenosis between 2 and 563 days of follow-up. Five patients (71.4%) received conservative treatment with double-J catheter replacement every 6 months. Of them, 2 (40%) developed recurrent urinary tract infections with acute renal dysfunction requiring hospital readmissions. One (14.3%) of these patients was submitted to a surgical correction, and the last one died due to urosepsis despite the use of culture-guided antibiotics and the location of a percutaneous nephrostomy (**Table 2**).

Pyeloureterostomy Secondary to Urinary Leak

Pyeloureterostomy was used to treat urinary leak (UL-PU) in 76 patients. Seven patients (9.2%) developed a recurrent urinary leak between 1 and 66 days after UL-PU, all successfully treated with subsequent surgical interventions. Patients were treated by a new pyeloureterostomy over a double-J catheter (n = 2), bladder suture of a previous Leadbetter-Politano ureterocystostomy (n = 2), suture at the leakage site (n = 1), and with nephrostomy (n = 1). The last patient was treated by a double-J catheter and indwelling vesical catheter insertion followed by protective nephrostomy and suture of the leakage area. All patients

TABLE 3 | Native kidney nephrectomies after ureteral ligation for pyeloureterostomy.

Age (years)	Sex	CKD etiology	Residual diuresis (ml/day)	Type of surgery	Time after ureteral ligation (months)	Symptoms	Pathology	Outcome
59	Male	Diabetes Mellitus	0	P-PU	3	Fever	Hydronephrosis	Graft Nephrectomy/Death
12	Female	Neurogenic Bladder	500	UL–PU	5	Fever	Pyonephrosis	resolution
47	Male	Neurogenic Bladder	0	P–UP	4	Fever	Pyonephrosis	resolution
31	Male	Neurogenic Bladder	0	P–UP	16	Fever	Pyonephrosis	Graft Nephrectomy
55	Male	Diabetes Mellitus	0	P–UP	12	Fever	Pyonephrosis	resolution
54	Male	ADPKD	700	UL–PU	26	Lumbar pain	ADPKD	resolution
33	Male	Undetermined	500	P–UP	48	Lumbar pain	Hydronephrosis	resolution
47	Male	ADPKD	500	P–UP	11	Lumbar pain	ADPKD	resolution
50	Male	ADPKD	300	P–UP	19	Lumbar pain	ADPKD	resolution
48	Male	ADPKD	200	P–UP	13	Lumbar pain	ADPKD	resolution

CKD, chronic kidney disease; ADPKD, autosomal dominant polycystic kidney disease.

progressed with urinary fistulae resolution. Three patients (3.9%) developed ureteral stenosis between day 28 and 336 post-UL-PU, and all were treated conservatively with double-J catheter replacement every 6 months.

Pyeloureterostomy Secondary to Stenosis

Pyeloureterostomy (US-PU) was used in 10 patients with ureteral stenosis (8 Lich-Gregoir and 2 Leadbetter-Politano) following percutaneous nephrostomy (n = 4), retrograde placement of the double-J catheter (n = 4), or as a primary procedure (n = 2). One patient developed recurrent stenosis and was treated with double-J catheter replacement every 6 months.

Native Kidney Obstruction Requiring Nephrectomy

After a median follow-up of 33.8 months, ranging from 7 to 67 months, 10 patients (2%) required native nephrectomy (**Table 3**). Symptoms were lumbar pain with fever (n = 5) and isolated lumbar pain (n = 5). Among them, eight were patients in the P-PU, and 2 were in the UL-PU group.

Of the five patients with fever, 3 (60%) had neurogenic bladder with prior bladder augmentation, and 2 (40%) had diabetes mellitus. The time between native ureter ligation and nephrectomy ranged from 3 to 16 months, and all but one patient had a final histological diagnosis of pyonephrosis. Two patients required graft nephrectomy due to associated infectious complications, and one of them subsequently died due to complications from an infected sacral ulcer. Five patients developed isolated lumbar pain 11–48 months after transplantation, and four of them (80%) had autosomal dominant polycystic kidney disease (ADPKD). All these patients showed favorable outcomes after the native nephrectomy.

Causes of native kidney nephrectomy were then hydronephrosis and pyonephrosis. Two demographic characteristics were associated with increased likelihood of native ipsilateral kidney complications requiring nephrectomy: ADPKD and augmented neurogenic bladder. In fact, the incidence of complications requiring nephrectomy was 13% among 31 patients with ADPKD (n = 4) and 27% among 11 patients with neurogenic bladder (n = 3).

DISCUSSION

Pyeloureterostomy is a well-known option for urinary tract reconstruction during kidney transplantation (3,4,8,24,26) as well as for the treatment of ureteroneocystostomy complications (10–13). At least two surgical techniques, end-to-end and end-to-side anastomosis, have been performed. Leadbetter *et al.* described the end-to-end reconstruction with native kidney nephrectomy in 1966 (26). Later, ipsilateral native nephrectomy was almost abandoned due to the low incidence of complications (6).

Despite the previous reports of the low incidence of major complications requiring nephrectomy, there are some concerns, mainly in those with more significant residual diuresis. For this reason, some surgeons advocate the use of end-to-side anastomosis to maintaining the urinary flow of the native kidney (27). Still, ureteral length and impaired endoscopic manipulation of the collecting system may offset the advantages of this surgical technique.

This single-center large cohort analysis revealed a low incidence (2%) of native ipsilateral kidney complications requiring nephrectomy in 495 kidney transplant recipients that underwent pyeloureterostomy without ipsilateral nephrectomy during the kidney transplantation or after urological complications. There were three graft losses (0.6%) and 2 deaths (0.4%) secondary to surgical complications.

A retrospective study including 278 kidney transplant recipients submitted to primary pyeloureterostomy with native ureter ligation without nephrectomy described an incidence of 2.2% (n = 6) of subsequent nephrectomy due to symptomatic hydronephrosis. Of these, 50% were in patients with chronic kidney disease due to ADPKD (6), findings similar to ours, in which 40% of the patients who underwent posterior nephrectomy had ADPKD. This increased risk is probably warranted by increased renal volume before the ureter ligation and more significant residual diuresis.

Guilter J *et al.* (25) observed a 3% incidence of native nephrectomy after ureter ligation and observed that high residual diuresis was a risk factor. However, in our study, this relation not observed since all the six patients who required posterior nephrectomy had less than 300 ml of urine output previously to the transplant, being four of them (80%) anuric.

One interesting observation of our cohort is that in patients who had previously undergone bladder augmentation, they had not only a higher risk of undergoing a future nephrectomy but also a more significant risk to unfavorable outcomes after the removal of their native kidney since 2 patients who had their nephrectomy indicated due to fever ended up losing their grafts, one of them dying soon after. We believe that colonization or infection of the urinary tract may predispose the occurrence of pyonephrosis in patients with hydronephrosis.

The urinary leak was diagnosed in 3.6% of patients after P-UP, an incidence similar to that reported in the literature (3–5%) for different urinary tract reconstruction techniques (2,16-19). On the other hand, in the UL-PU group, the incidence was 3 times higher (9.2%). A similar incidence (12.5%) was observed in other series (12), and this higher incidence is probably due to the inflammatory environment secondary to the urinary leakage. We chose to treat this complication according to the intraoperative findings, performing a new UP or locating a protective nephrostomy.

When the pyeloureterostomy anastomosis stenosis requires intervention, open correction using a surgical technique similar to that described by Anderson-Hynes for ureteropelvic junction (UPJ) stenosis may be considered. Yet, this procedure may be challenging due to the local hilar adherences. On the other hand, although the surgical risk associated with endourologic techniques low, the patency is approximately 60% after 5 years of follow-up (20–25). Given these caveats associated with both techniques, only one (10%) patient chose to undergo conventional surgical treatment while the remaining nine (90%) patients preferred periodic double-J replacement.

This analysis has limitations inherent to the retrospective nature of the study, potential selection bias in selecting the study population, and local surgical strategies that do not include the routine use of stenting for primary ureteroneocystostomy.

CONCLUSION

End-to-end pyeloureterostomy with proximal ligation of the native ureter is a versatile procedure, allowing the reconstruction of the urinary tract even when the graft ureter is short, devascularized or when the recipient's bladder is tiny and difficult to access. It is also an essential surgical technique to treat urinary leaks and stenosis, with complication rates similar to other types of reimplantation. The need for native nephrectomy was restricted to very few cases, occurring predominantly in patients with ADPKD and neurogenic augmented bladders, and was associated with low morbidity.

CAPSULE SUMMARY SENTENCE

This study aims to analyze the safety of the native ureter ligation without ipsilateral nephrectomy during pyeloureterostomy, used either as a primary surgical approach or as a secondary reconstructive technique after ureteral complications, in patients undergoing kidney transplantation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Research Board - UNIFESP. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HN - collected data and write the article. HS - reviewed the article and approved the final version. JP - reviewed the article and approved the final version. RF - reviewed the article and approved the final version. WA - reviewed the article and approved the final 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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Cytomegalovirus Exposure and the Risk of Overall Infection After Kidney Transplantation: A Cohort Study on the Indirect Effects Attributable to Viral Replication

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Previous reports hypothesized that cytomegalovirus (CMV) may predispose to non-CMV infection after kidney transplantation (KT). We analysed the incidence of non-CMV infection (overall, bacterial and opportunistic) in 291 KT recipients according to the previous development of any level or high-level (≥1,000 IU/ml) CMV viremia. Exposure to CMV replication was assessed throughout fixed intervals covering first the 30, 90, 180 and 360 post-transplant days (cumulative exposure) and non-overlapping preceding periods (recent exposure). Adjusted Cox models were constructed for each landmark analysis. Overall, 67.7 and 50.5% patients experienced non-CMV and CMV infection, respectively. Patients with cumulative CMV exposure had higher incidence of non-CMV infection beyond days 30 (p-value = 0.002) and 90 (p-value = 0.068), although these associations did not remain after multivariable adjustment. No significant associations were observed for the remaining landmark models (including those based on high-level viremia or recent CMV exposure), or when bacterial and opportunistic infection were separately analysed. There were no differences in viral kinetics (peak CMV viremia and area under curve of CMV viral load) either. Our findings do not support the existence of an independent association between previous CMV exposure and the overall risk of post-transplant infection, although results might be affected by power limitations.

Keywords: cytomegalovirus, kidney transplantation, indirect effects, infection, opportunistic infection

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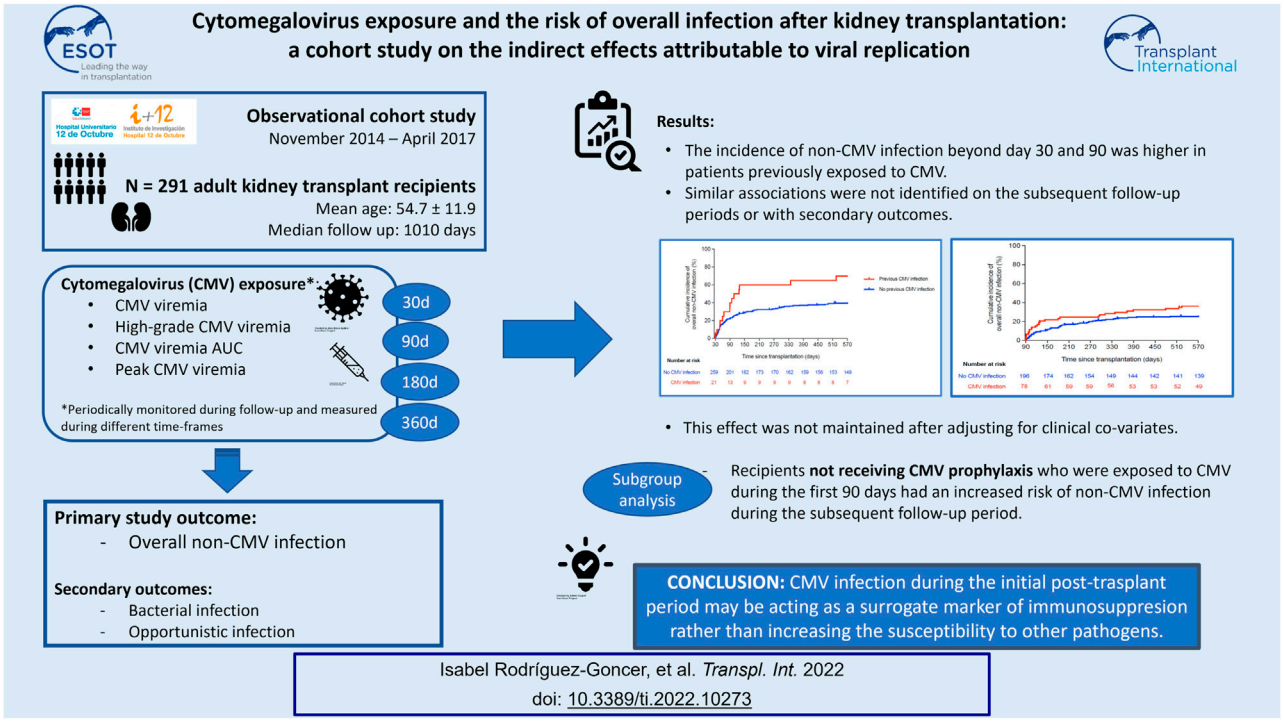
Abbreviations: allo-HSCT, allogeneic stem-cell transplantation; ATG, anti-thymocyte globulin; CI, confidence interval; CMV, cytomegalovirus; CMV-AUC, area under curve of CMV viral load; D, donor; HR, hazard ratio; HSV, herpes simplex virus; IQR, interquartile range; IV, intravenous; KT, kidney transplantation; PCR, polymerase chain reaction; R, recipient; SD, standard deviation; SOT, solid organ transplantation; VIF, variance inflation factor; VZV, varicella-zoster virus; WBC, white blood cell.



GRAPHICAL ABSTRACT |

INTRODUCTION

Despite notable advances in diagnosis, prevention and treatment, cytomegalovirus (CMV) remains as a leading cause of morbidity after solid organ transplantation (SOT) due to its direct pathogenic effects. In addition, CMV exposure is linked with a wide range of immune phenomena that would presumably exert a negative impact on the SOT population. (1, 2) These indirect effects attributable to CMV include decreased long-term graft survival (3, 4) graft rejection, (5-8) atherothrombotic events, (9, 10) new onset diabetes after transplantation (11) and a variety of bacterial and fungal infections (1, 12).

Cytomegalovirus has evolved multiple mechanisms to persist and replicate evading the host's immune system through the impairment of antiviral responses and the enhancement of local inflammation. (13) Such immune dysfunction is known to negatively affect innate (e.g., functionality of natural killer cells and tissue macrophages) and adaptive components (e.g., cytotoxic T-cell responses). (14, 15) Besides, CMV has been shown to modulate pathways mediated by toll-like receptor ligands (16) and to promote accelerated T-cell senescence. (17, 18) These immunomodulatory effects, maintained over time, are thought to underlie the deleterious consequences allegedly caused by CMV. It is controversial, however, whether reducing CMV replication with the use of antiviral prophylaxis would impact the incidence of post-transplant events (19-22).

Virus-induced immune dysregulation may explain the association reported between CMV exposure and infections due to other microorganisms after SOT. Previous studies have suggested that CMV replication increases the risk of bacterial infection (*Listeria monocytogenes* (23, 24) or *Clostridioides difficile* (21, 25, 26)), non-CMV viral infection (hepatitis C virus (27)) and, particularly, opportunistic events such as invasive aspergillosis, (28-30) nocardiosis (31) or *Pneumocystis jirovecii* pneumonia. (22, 32, 33) It should be noted that this evidence is mainly based on retrospective case-control studies with small sample sizes. The occurrence of non-CMV infection was not the primary study outcome, and monitoring strategies used to measure CMV exposure exhibited great heterogeneity. Misclassification bias in case-control studies cannot be excluded, as recipients that had previously experienced infectious complications might have been more closely monitored for CMV replication during the subsequent follow-up. Thus, it remains unclear whether the demonstration of CMV infection merely acts as a surrogate marker for over-immunosuppression.

With these research gaps in mind, we aimed to explore the potential impact of post-transplant CMV replication on the risk of non-CMV overall infection in a large cohort of kidney transplant (KT) recipients. To overcome the aforementioned limitations, CMV exposure was assessed by means of close monitoring of CMV viremia with real-time polymerase chain reaction (PCR) and by applying various methodological strategies.

PATIENTS AND METHODS

Study Population and Setting

We performed an observational cohort study with prospectively collected data at the University Hospital "12 de Octubre" (Madrid, Spain). All consecutive patients aged ≥18 years that

underwent KT between November 2014 and April 2017 were eligible for inclusion, including double organ (e.g., kidney-pancreas and liver-kidney) recipients. Patients experiencing primary graft non-function, death or graft loss within the first week were excluded, since they had no opportunity to be exposed to CMV viremia or to experience study outcomes. The study was performed in accordance with the ethical standards laid down in the Declarations of Helsinki and Istanbul. The local Ethics Committee approved the study protocol and written informed consent was obtained from all participants at study entry.

Study Design

All patients were enrolled at the time of transplantation and followed up until December 2018 or, alternatively, until graft loss or death. Patients were seen regularly at the outpatient transplant clinic at scheduled follow-up visits (baseline, every 2 weeks during the first 3 months, and monthly thereafter) or whenever clinically indicated. Clinical, laboratory, microbiological and histological features were prospectively collected in our institutional database by using a standardized case report form. CMV viral load was quantified by real-time PCR (as detailed below) fortnightly during the first 2 months, monthly through month 6, and every 2 months thereafter until completing the first year since transplantation, as well as at any time if clinical or laboratory manifestations suggestive of CMV disease were present.

The primary study outcome was the occurrence of non-CMV overall infection, as defined below, during the post-transplant follow-up period. Bacterial and non-CMV opportunistic infection were considered as secondary outcomes.

Study Definitions

The diagnosis of "post-transplant infection" was established by at least one of the following criteria: 1) positive culture of an unequivocally pathogenic microorganism (e.g., *Mycobacterium tuberculosis*) from any sample; 2) isolation of any microorganism from a sample obtained under sterile conditions; 3) isolation of a potentially pathogenic microorganism from any sample accompanied by signs of local or systemic infection; and/or 4) clinical data suggestive of infection without microbiological isolation and complete resolution under antimicrobial treatment.

Febrile episodes were not taken into account if no causative agent could be demonstrated and no antimicrobial treatment was needed to achieve clinical resolution. "Pneumonia" was defined by the presence of a new infiltrate on the chest X-ray or CT scan plus one or more compatible signs or symptoms (i.e., fever or hypothermia, new cough with or without sputum production, pleuritic chest pain, dyspnea, and/or altered breath sounds on auscultation). "Lower respiratory tract infection" denoted episodes of bronchitis and/or bronchiolitis with no new pulmonary infiltrates. "Digestive tract infection" included bacterial (e.g., *Clostridioides difficile*, *Salmonella* spp. or *Campylobacter* spp.), viral (e.g., norovirus) or parasitic (helminths or protozoa) infection producing colitis and/or diarrhea. "Non-CMV viral syndrome" included episodes with typical symptoms of viral infection (e.g., fever, headache or myalgia) accompanied with compatible laboratory findings and positive microbiological identification (e.g., influenza). "Presumptive BK polyomavirus-associated nephropathy" was defined by the presence of plasma viral loads >4 $\log_{10}$ copies/ml at two time points 3 or more weeks apart. (34) Episodes of asymptomatic bacteriuria, lower urinary tract infection (i.e., cystitis) or low-level BK polyomavirus viremia were excluded.

"Non-CMV opportunistic infection" was defined as that due to intracellular bacteria (e.g., *Listeria monocytogenes, Nocardia* spp. or mycobacteria), herpesviruses (herpes simplex virus [HSV], varicella-zoster virus [VZV] and Epstein-Barr virus-related post-transplant lymphoproliferative disease), yeasts (*Candida* spp. and *Cryptococcus* spp.), molds, *P. jirovecii*, and parasites (*Cryptosporidium*, *Toxoplasma gondii* and *Leishmania* spp.). (35) "Proven or probable invasive fungal disease" was defined based on the criteria proposed by the European Organisation for Research and Treatment of Cancer and the Mycoses Study Group. (36) Bloodstream, intraabdominal, surgical site and urinary tract infections due to *Candida* spp. were excluded from the definition of opportunistic infection as these episodes are usually related to previous surgery or indwelling catheters rather than impaired immune status.

"CMV infection" was defined by the demonstration of CMV DNAemia by real-time PCR regardless of the presence of attributable symptoms or other clinical manifestations. CMV disease comprised both viral syndrome and end-organ disease. "CMV viral syndrome" was defined by the presence of CMV infection plus fever plus at least one of the following: leukopenia (white blood cell [WBC] count $<3.50 \times 10^3$ cells/μL if baseline WBC count was $\geq 4.00 \times 10^3$ cells/μL or a decrease >20% if baseline WBC count was $<4.00 \times 10^3$ cells/μL); atypical lymphocytosis (≥5%); thrombocytopenia (platelet count $<100 \times 10^3$ cells/μL if baseline count was $\geq 115 \times 10^3$ cells/μL or a decrease >20% if baseline platelet count was $<115 \times 10^3$ cells/μL); or elevation of ALT or AST of more than 2 times the upper limit of normal. "CMV end-organ disease" included probable or proven categories, with the latter requiring the documentation of CMV replication in tissue specimens by viral culture, immunohistochemistry, histopathology, or DNA hybridization, in the presence of attributable clinical manifestations. (37) As previously stated, CMV infection (either asymptomatic replication or clinical disease), which constituted the explanatory variable of interest, was not included in the definition of study outcomes.

The graft function was assessed by estimated glomerular filtration rate using the abbreviated Modification of Diet in Renal Disease (MDRD-4) equation. (38) "Delayed graft function" was defined as the need for dialysis within the first two post-transplant weeks. Acute graft rejection was diagnosed by histological examination if possible or by response to empirical antirejection treatment. Graft loss was defined by the permanent return to dialysis and/or retransplantation.

Assessment of CMV Exposure

Plasma CMV DNA loads were quantified by means of a real-time PCR assay (RealStar® CMV PCR kit 1.0, Altona Diagnostics GmbH, Hamburg, Germany). DNA was extracted from 200 μL

TABLE 1 | Demographics and clinical characteristics of the study population (n = 291).

Variable	
Age of recipient, years [mean ± SD]	54.7 ± 11.9
Gender of recipient (male) [n (%)]	201 (69.1)
Prior or current smoking history [n (%)]	111 (38.1)
BMI at transplantation, kg/m^2 [median (IQR)][a]	25.3 (22.3–28.4)
Pre-transplant chronic conditions [n (%)]	
Hypertension	244 (83.8)
Diabetes mellitus	88 (30.2)
Coronary heart disease	29 (10.0)
Other chronic heart disease	47 (16.2)
Peripheral arterial disease	26 (8.9)
Cerebrovascular disease	24 (8.2)
Chronic obstructive pulmonary disease	7 (2.4)
Type of transplantation [n (%)]	
Single kidney	272 (93.5)
Simultaneous kidney-pancreas	13 (4.5)
Simultaneous liver-kidney	6 (2.1)
Previous solid organ transplantation [n (%)]	36 (12.4)
Underlying cause of end-stage kidney disease [n (%)]	
Glomerulonephritis	65 (22.3)
Diabetic nephropathy	58 (19.9)
Polycystic kidney disease	39 (13.4)
Nephroangiosclerosis	23 (7.9)
Congenital nephropathy	10 (3.1)
Reflux nephropathy	8 (2.7)
Lupus nephropathy	5 (1.7)
Vasculitis	5 (1.7)
Chronic interstitial nephropathy	2 (0.7)
Unknown	32 (10.9)
Other	38 (13.1)
CMV serostatus [n (%)]	
D+/R+	208 (71.5)
D-/R+	37 (12.7)
D+/R-	31 (10.7)
D-/R-	10 (3.4)
D unknown/R+	5 (1.7)
Positive EBV serostatus (anti-EBNA IgG) [n (%)]	258 (88.7)
Positive HCV serostatus [n (%)]	25 (8.6)
Positive HBsAg status [n (%)]	10 (3.4)
Positive HIV serostatus [n (%)]	3 (1.0)
Pre-transplant renal replacement therapy [n (%)]	261 (89.7)
Hemodialysis	216 (74.2)
Continuous ambulatory peritoneal dialysis	45 (15.5)
Time on dialysis, days [median (IQR)]	572 (287.5–1,085.5)
Age of donor, years [mean ± SD]	53.2 ± 16.6
Gender of donor (male) [n (%)]	165 (56.7)
Type of donor [n (%)]	
DBD donor	185 (63.9)
DCD donor	71 (24.4)
Living donor	31 (10.7)
Cold ischemia time, hours [median (IQR)]	17 (10.3–22.3)
Number of HLA mismatches [median (IQR)]	4 (3–5)
Intraoperative blood product transfusion [n (%)]	34 (11.7)
Induction therapy [n (%)]	
ATG	146 (50.2)
Total dose, mg [mean ± SD]	4.8 ± 2.4
Basiliximab	105 (36.1)
Methylprednisolone only	40 (13.7)
Primary immunosuppression [n (%)]	
Steroids	290 (99.7)
Tacrolimus	291 (100.0)
Mycophenolate mofetil/mycophenolic acid	279 (95.9)
Azathioprine	12 (4.1)
Everolimus	1 (0.3)
CMV antiviral prophylaxis [n (%)]	166 (57.0)
Duration of prophylaxis, days [median (IQR)]	96 (90–139)
Post-transplant complications [n (%)]	
Delayed graft function	140 (48.1)
Number of dialysis sessions [median (IQR)]	2 (1–3)
Reintervention within the first month	33 (11.3)
NODAT	39 (13.4)
Renal artery stenosis requiring revascularization	23 (7.9)
Acute graft rejection[b]	40 (14.1)
>2 episodes of acute rejection	8 (2.7)
Time to the first episode, days [median (IQR]	86.5 (15–182.5)
T-cell-mediated acute rejection	21 (7.2)
Antibody-mediated acute rejection	10 (3.4)

ATG: antithymocyte globulin; BMI: body mass index; CMV: cytomegalovirus; D: donor; DBD: donation after brain death; DCD: donation after circulatory death; EBV: Epstein-Barr virus; HCV: hepatitis C virus; HBsAg: hepatitis B virus surface antigen; HIV: human immunodeficiency virus; HLA: human leukocyte antigen; IQR: interquartile range; NODAT: new-onset diabetes after transplantation; SD: standard deviation; R: recipient.

[a]*Data on BMI, not available for 23 patients.*

[b]*Includes 7 patients with borderline acute rejection and 6 with empirically-treated episodes not confirmed by biopsy.*

of sample with the NucliSENS® easyMag® instrument (bioMérieux Diagnostics, Marcy l'Etoile, France), according to the manufacturer's instructions. Viral loads were $\log_{10}$-transformed for statistical analyses. "High-level CMV viremia" was defined as a viral load ≥1,000 IU/ml. The area under curve of CMV viral load (CMV-AUC) allows for capturing viral dynamics over time by considering not only peak viral loads but also persistent replication. Therefore, we calculated CMV-AUCs (expressed as $\log_{10}$ IU × day/ml) by means of the trapezoid rule (39) from the time of transplantation to days 30 ($AUC_{0\text{-}30}$), 90 ($AUC_{0\text{-}90}$), 180 ($AUC_{0\text{-}180}$) and 360 ($AUC_{0\text{-}360}$). The CMV-AUC value for a given interval could be estimated only if at least two viral load measurements were available. We also calculated peak CMV viral loads for each of these post-transplant periods.

Immunosuppression and Prophylaxis Regimens

Details on immunosuppressive regimens are provided in Supporting Material. All patients received preoperatively a single dose of intravenous (IV) cefazolin (or ciprofloxacin in those with ß-lactam hypersensitivity). Prophylaxis for *P. jirovecii* pneumonia was administered for 9 months with trimethoprim-sulfamethoxazole (160/800 mg three times weekly) or monthly intravenous pentamidine. In patients at high-risk for CMV infection, universal prophylaxis with oral valganciclovir (900 mg daily) was given for 3 months (seropositive recipients [R+] that received induction therapy with anti-thymocyte globulin [ATG]) or 6 months (serology mismatch [donor positive/recipient negative (D+/R–)] regardless of the type of induction therapy). Intermediate-risk patients (R+ without ATG induction) were managed by means of PCR-guided pre-emptive therapy, and IV ganciclovir (5 mg/kg/12 h) or oral valganciclovir (900 mg/12 h) for at least 2 weeks was initiated in the presence of

TABLE 2 | Clinical and microbiological description of all the episodes of non-CMV post-transplant infection occurring during the follow-up period (n = 424).

Clinical syndrome	**N (%)**
Acute graft pyelonephritis	147 (34.9)
Secondary bloodstream infection	48/147 (32.6)
Surgical site infection	46 (10.8)
Secondary bloodstream infection	4/46 (8.7)
Digestive tract infection	37 (8.7)
Secondary bloodstream infection	1/37 (2.7)
Skin and soft-tissue infection	35 (8.3)
Lower respiratory tract infection	35 (8.3)
Pneumonia	26 (6.1)
Secondary bloodstream infection	2/26 (7.7)
Viral syndrome	15 (3.5)
Intraabdominal infection	12 (2.8)
Secondary bloodstream infection	2/12 (16.7)
Catheter-related bloodstream infection	10 (2.4)
Prostatitis	4 (0.9)
CNS infection	1 (0.2)
Other	53 (12.5)
Isolated microorganisms	N (%)
Bacteria	
Escherichia coli	87 (20.5)
Klebsiella pneumoniae	59 (13.9)
Pseudomonas aeruginosa	37 (8.7)
Clostridioides difficile	20 (4.7)
Enterococcus faecalis	18 (4.2)
Enterococcus faecium	16 (3.8)
Other *Enterobacteriaceae*	11 (2.6)
Coagulase-negative staphylococci	7 (1.7)
Staphylococcus aureus	6 (1.4)
Enterobacter spp.	4 (0.9)
Campylobacter spp.	4 (0.9)
Streptococcus pneumoniae	2 (0.5)
Serratia marcescens	2 (0.5)
Non-typhoidal *Salmonella*	1 (0.2)
Nocardia spp.	1 (0.2)
Other	15 (3.5)
No microbiological diagnosis[a]	39 (9.2)
Viruses	
Influenza virus	16 (3.8)
HSV-1/2	13 (3.0)
Varicella-zoster virus	12 (2.8)
Respiratory syncytial virus	6 (1.4)
BK polyomavirus[b]	4 (0.9)
Human metapneumovirus	2 (0.5)
Norovirus	1 (0.2)
Erythrovirus B19	1 (0.2)
Other	13 (3.0)
Fungi	
Candida spp.	16 (3.8)
Aspergillus spp.	4 (0.9)
Mucorales	2 (0.5)
Cryptococcus neoformans	1 (0.2)
Pneumocystis jirovecii	1 (0.2)
Parasites	
Strongyloides stercoralis	1 (0.2)
Giardia lamblia	1 (0.2)

CNS: central nervous system; HSV: herpes simplex virus.
[a]*The presumptive diagnosis of bacterial infection was established by the complete clinical resolution with antibiotic therapy in the absence of an alternative cause.*
[b]*Presumptive BK, polyomavirus-associated nephropathy (i.e., plasma viral load >4 log^{10} copies/ml at two time points three or more weeks apart).*

high-level (≥1,000 IU/ml) or rapidly increasing viremia according to the criteria of the attending nephrologist. (Val)ganciclovir doses were adjusted according to renal function when necessary (1).

Statistical Analysis

Quantitative data were shown as the mean ± standard deviation (SD) or the median with interquartile range (IQR). Qualitative variables were expressed as absolute and relative frequencies. Categorical variables were compared using the χ (2) test. Student's t-test or U Mann-Whitney test were applied for continuous variables. Time-to-event curves were plotted by the Kaplan-Meier method and inter-group differences were compared with the log-rank test.

A series of landmark survival analyses were performed at days 30, 90, 180 and 360 after transplantation to evaluate the association between different approaches to CMV exposure (CMV viremia at any level, high-level CMV viremia, peak viremia and CMV-AUC) and the subsequent occurrence of non-CMV infection. Exposure to CMV was assessed within two different timeframes: throughout fixed intervals encompassing the first 30, 90, 180 and 360 days after transplantation (cumulative exposure); and through non-overlapping intervals covering the immediately preceding two-to-three-month periods (i.e., days 30–90, days 90–180, and days 270–360) (recent exposure). For each of these landmark analyses, Cox regression models were constructed with previous CMV exposure as the explanatory variable of interest and non-CMV infection as the dependant variable. Models were adjusted in a two-step process. First, a set of variables were initially tested at the univariable level. These variables encompassed demographic and clinical features of the recipient (i.e., comorbidities, causes of end-stage renal disease, previous transplantation), donor age and type (i.e., donation after brain or circulatory death, living donor), surgical and peri-operative variables (i.e., cold ischemia time, surgical complications, delayed graft function), laboratory results (i.e., graft function, leucocyte and lymphocyte count), immunosuppressive agents, occurrence of graft rejection, type of CMV prevention strategy used (antiviral prophylaxis or preemptive therapy), and the occurrence of non-CMV infection within the preceding period. Only variables achieving univariable p-values < 0.08 were next entered into the multivariable Cox models as potential covariates. Multicollinearity was analyzed with the variance inflation factor (VIF), with VIF values < 3 being considered acceptable. The administration of valganciclovir prophylaxis (versus preemptive therapy) was not significantly associated with the study outcome in the univariable analysis. Nevertheless, given the relevance of this variable and the potential interaction with CMV exposure we performed a set of sensitivity analyses by excluding those patients that received prophylaxis. Associations were expressed as hazard ratios (HRs) with 95% confidence intervals (CIs). Statistical analysis was performed using SPSS version 20.0 (IBM Corp., Armonk, NY) and graphs were generated with Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

TABLE 3 | Incidence, clinical characteristics and viral kinetics parameters of CMV events.

Asymptomatic CMV infection	
Number of patients with at least one episode	114
Cumulative incidence, % (95% CI)	39.2 (33.5–45.0)
Interval from transplantation to the first episode, days [median (IQR)]	71.0 (35.8–149.3)
Late-onset infection (beyond day 180), n (%)[a]	53/269 (19.7)
Requirement for pre-emptive therapy, n (%)	39/114 (34.2)
Patients with recurrent infection, n (%)[b]	27 (23.7)
Number of episodes of viremia	166
Peak viral load, log_{10} IU/ml [median (IQR)]	3.2 (2.7–3.8)
Episodes requiring antiviral therapy	42/166 (25.3)
Viral load, log_{10} IU/ml [median (IQR)]	3.5 (3.2–3.9)
Episodes not requiring antiviral therapy	124/166 (74.7)
Viral load, log_{10} IU/ml [median (IQR)]	2.9 (2.5–3.6)
CMV-AUC_{0-360}, log_{10} IU × days × ml^{-1} [median (IQR)]	4.7 (4.1–5.2)
CMV disease	
Number of patients with at least one episode	32
Cumulative incidence, % (95% CI)	11.0 (7.4–14.6)
Interval from transplantation to the first episode, days [median (IQR)]	50.0 (34.0–176.5)
Clinical syndrome [n (%)]	
Viral syndrome	27/32 (84.4)
Colitis	4/32 (12.5)
Hepatitis	1/32 (3.1)

CI: interval confidence; CMV: cytomegalovirus; CMV-AUC: area under curve of CMV viral load; IQR: interquartile range.

[a]*Percentage calculated on the basis of those KT, recipients that remained alive with a functioning graft by day 180 after transplantation (n = 269).*

[b]*At least two episodes separated by both a minimum 2-weeks interval and at least one negative sample for CMV DNA.*

RESULTS

Study Population and Outcomes

Overall, 291 KT recipients were included, whose clinical characteristics are summarized in **Table 1**. The median follow-up was 1,010 days (IQR: 715–1,246), totalling 276,239 transplant-days. Nineteen recipients (6.5%) died at a median interval of 446 days (IQR: 38–872), accounting for 1- and 2-year survival rates of 94.8 and 93.7%, respectively. Common causes of death were infection (4 patients), malignancy and cardiovascular events (3 patients each). Twenty-two patients (7.6%) experienced graft loss, yielding 1- and 2-year death-censored graft survival rates of 94.0 and 91.8%, respectively.

One-hundred and ninety-seven patients (67.7%) developed a total of 424 episodes of non-CMV infection (incidence rate of 1.54 episodes [95% CI: 1.39–1.69] per 1,000 transplant-days). Clinical syndromes and causative agents are detailed in **Table 2**. The median interval from transplantation to the first episode was 29.0 days (IQR: 13.0–73.5), and about one quarter of the episodes (25.2% [107/424]) occurred within the first month (mainly acute pyelonephritis, surgical site infection and other healthcare-associated infections). Regarding the secondary outcomes, 167 patients (57.4%) experienced 331 episodes of bacterial infection (incidence rate of 1.19 [95% CI: 1.07–1.33] per 1,000 transplant-days) and 34 patients (11.7%) had 41 episodes of non-CMV opportunistic infection (incidence rate of 0.15 [95% CI: 0.11–0.19] per 1,000 transplant-days), as detailed in **Supplementary Table S1** in Supporting Material. Fifty-three episodes did not meet the criteria for bacterial or opportunistic infection (namely influenza [30.2%], invasive candidiasis [20.8%] and other respiratory viral infections [18.9%]).

CMV Exposure

One hundred and sixty-six patients (57.0%) received antiviral prophylaxis with valganciclovir for a median of 96 days (IQR: 90–139), whereas the remaining of the cohort was managed with pre-emptive therapy. The total number of monitoring points for CMV DNAemia throughout the entire follow-up period was 3,177, with a median of 11 points per patient (IQR: 8–13).

Incidence, clinical characteristics and viral kinetics of CMV events are shown in **Table 3**. Overall, 146 patients (50.2%) experienced at least one episode of CMV infection, either as asymptomatic viremia (78.1% [114/146]) or clinical disease (21.9% [32/146]). About one third of the patients with asymptomatic CMV infection (34.2% [39/114]) actually received pre-emptive antiviral therapy with (val)ganciclovir at any time. The median load in episodes of viremia requiring or not requiring pre-emptive therapy was 3.5 log_{10} IU/ml (IQR: 3.2–3.9) and 2.9 log_{10} IU/ml (IQR: 2.5–3.6), respectively.

Association Between Cumulative CMV Exposure and Overall Non-CMV Infection

First, we explored the association between CMV infection throughout fixed intervals after transplantation (i.e., cumulative CMV exposure) and the subsequent development of non-CMV infection. The incidence of non-CMV infection beyond day 30 was significantly higher for patients with previous exposure to CMV infection at any level compared to those that had remained free from this event until day 30 (2-year incidence rates: 69.9 versus 40.8%, respectively; log-rank p-value = 0.002; crude HR: 2.27; 95% CI: 1.32–3.91; p-value = 0.003), whereas a near significant difference was observed beyond day 90 (2-year incidence rates: 36.1 versus 26.5%; log-rank p-value = 0.068; crude HR: 1.51; 95% CI: 0.97–2.36; p-value = 0.070). There were no significant differences at days 180 or 360 after transplantation (**Figure 1**). Similar trends were found when only high-level CMV viremia was considered, although none of the differences achieved statistical significance (**Supplementary Figure S1**).

After adjusting for those covariates that have been previously proven to achieve univariable p-values < 0.08 (listed in **Supplementary Table S2**) the exposure to CMV infection during the first 30 days was no longer associated with the occurrence of non-CMV infection beyond that point (adjusted HR: 1.45; 95% CI: 0.84–2.68; p-value = 0.172). No significant associations were found for the remaining landmark Cox models either (**Figure 2A** and **Supplementary Table S3**).

In the sensitivity analysis restricted to the subgroup of patients that did not receive CMV antiviral prophylaxis (n = 125), the development during the first 90 days of CMV infection at any level (adjusted HR: 2.54; 95% CI: 1.09–5.90; p-value = 0.030) or high-level viremia (adjusted HR: 2.37; 95% CI: 1.14–4.95; p-value

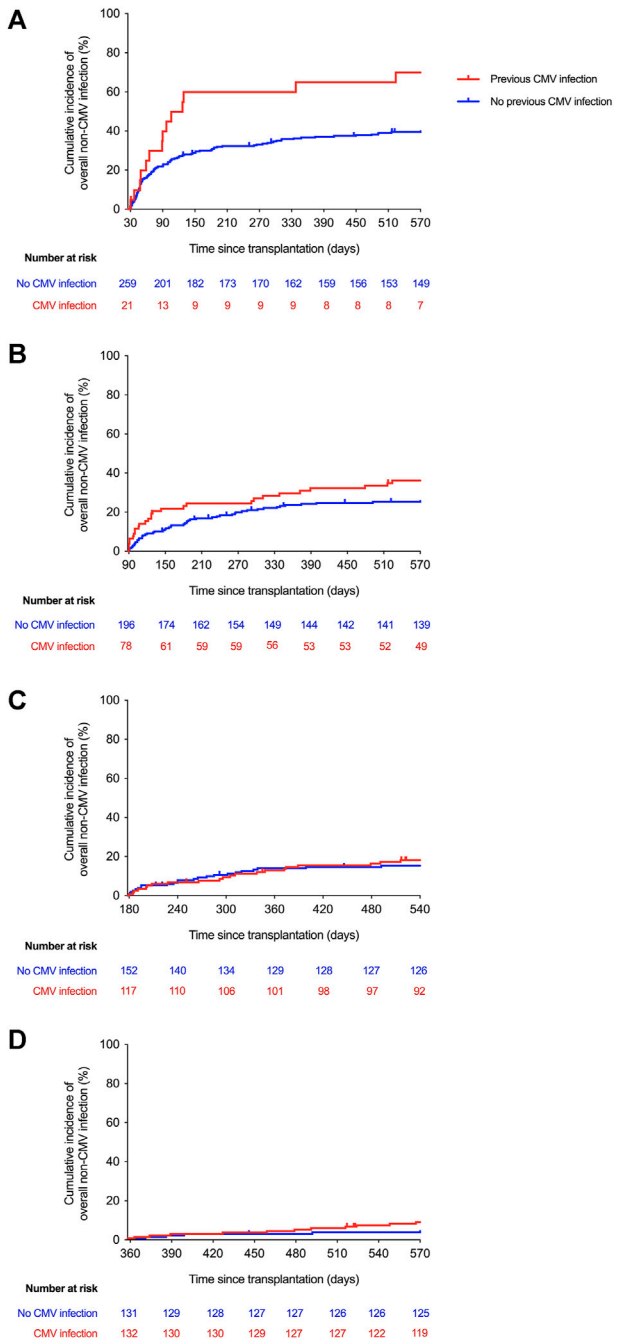


FIGURE 1 | Kaplan-Meier curves for the incidence of overall non-CMV infection according to the cumulative exposure to CMV infection at any level beyond day 30 (log-rank *p*-value = 0.002) **(A)**, day 90 (log-rank *p*-value = 0.068) **(B)**, day 180 (log-rank *p*-value = 0.727) **(C)**, and day 360 (log-rank *p*-value = 0.314) **(D)** after transplantation. CMV: cytomegalovirus.

= 0.021) was associated with subsequent non-CMV infection, with borderline significance. Similar associations were not observed for the remaining landmark analyses (**Figure 2B** and **Supplementary Table S3**).

Association Between Recent CMV Exposure and Overall Non-CMV Infection

Next, we exclusively considered CMV infection that occurred through non-overlapping 2- to 3-months intervals (days 30–90, days 90–180, and days 270–360) prior to the corresponding landmark time point (i.e., recent CMV exposure). There were no differences in the incidence of non-CMV infection beyond days 90, 180 or 360 between patients experiencing or not experiencing CMV infection at any level during the preceding period (**Supplementary Figure S2**). Accordingly, no significant associations were found in any of the adjusted Cox models (**Supplementary Figure S3A**). In the sensitivity analysis restricted to patients not receiving antiviral prophylaxis, however, recent high-level CMV exposure was associated (although with borderline significance) with the occurrence of non-CMV infection beyond day 90 (adjusted HR: 2.28; 95% CI: 1.06–4.70; *p*-value = 0.036) (**Supplementary Figure S3B**).

Kinetics of CMV Replication and Overall Non-CMV Infection

We also compared the kinetics of CMV DNAemia according to the subsequent occurrence of non-CMV infection. The peak CMV viral load through day 360 after transplantation was significantly higher among recipients that experienced non-CMV infection beyond that point as compared to those without this event (3.8 ± 1.3 versus 3.2 ± 0.8 $\log_{10}$ IU/ml, respectively; *p*-value = 0.010), with no differences for the remaining intervals (**Figure 3**). On the other hand, no significant differences were observed in the CMV-AUCs assessed through the first 30, 90, 180 and 360 days after transplantation (**Figure 4**).

Secondary Outcomes

There were no significant differences in the incidence of bacterial infection at the different landmark time points according to the cumulative exposure to CMV infection at any level (**Supplementary Figure S4**) or high-level CMV viremia (data not shown). Likewise, no differences were observed for non-CMV opportunistic infection either (**Supplementary Figure S5**).

DISCUSSION

Several reports have suggested that CMV would increase the risk of certain non-CMV infections in SOT and allo-HSCT recipients, (12, 22, 23, 25, 27, 29, 32, 33, 40, 41) which provided the clinical foundation for hypothesizing about its presumptive immunomodulatory effects. Nevertheless, these previous studies suffer from a number of methodological flaws, including the heterogeneous—and often imprecise—approaches to define the main explanatory variable (CMV exposure), such as donor or recipient serostatus, (12,30,42) viral culture, (28) pp65 antigenemia (22) or a combination of these approaches. (23, 29) Some studies only considered CMV clinical disease but not asymptomatic infection, (25, 31, 43) or

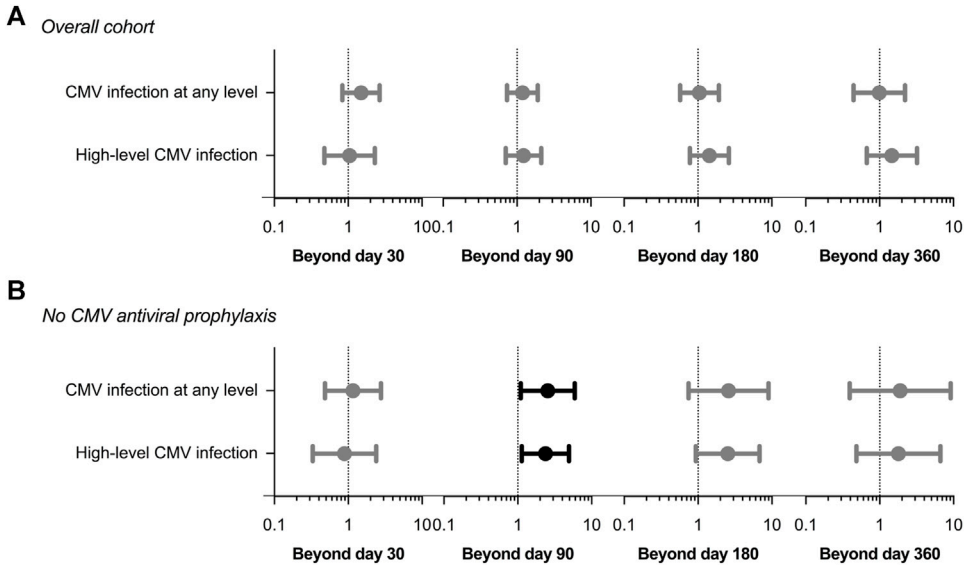


FIGURE 2 | Adjusted hazard ratios (circles) with 95% confidence intervals (whiskers) in landmark Cox regression models for the occurrence of overall non-CMV infection according to the previous development of CMV infection at any level or high-level CMV infection (>1,000 IU/ml): **(A)** entire study cohort; **(B)** patients not receiving CMV antiviral prophylaxis. Clinical covariates adjusted for are listed in **Supplementary Table S2**. CMV: cytomegalovirus.

were focused on specific opportunistic agents rather than capturing the entire spectrum of non-CMV infections. (22-24, 31-33) On the other hand, most of them did not attempt to explore the potential biological gradient between the amount and length of exposure to CMV and the incidence of non-CMV infection (28, 29, 32, 33, 40).

In the present single-center cohort study comprising 291 consecutive KT recipients, CMV replication was assessed by real-time PCR to investigate in a real-life scenario the potential impact of CMV exposure on the development of non-CMV infection. We applied a variety of methodological approaches (fixed intervals versus two-to three-month periods immediately prior to the landmark time point) to take into account not only the cumulative but also the recent CMV replication, and compared different viral parameters (any level and high-level viremia, peak CMV viremia and CMV-AUC) to capture changing replication kinetics. To align our work with prior research in the field and to allow for result comparison across studies, we used a rather inclusive definition for the primary outcome (i.e., "non-CMV overall infection" due to any potentially pathogenic microorganism). In addition, we separately analysed bacterial and opportunistic infection as secondary outcomes. In doing so we attempted to dissect the potential association between CMV replication and various forms of infection in whose pathogenesis different immune arms—innate, humoral and cellular—are involved. Therefore, our approach was relatively "hypothesis-free" regarding the specific type of infection to which CMV could eventually be contributing.

The most notable finding of our study was that KT recipients early exposed to CMV replication at any level during the first 30 days after transplantation exhibited a higher incidence of non-CMV infection over the following months. A similar trend, although non-significant, was also observed beyond post-transplant day 90. After adjusting for clinically relevant covariates, such as recipient age, comorbidities, type of donor, need of reintervention or graft function (as detailed in **Supplementary Table S2**), however, this effect was not sustained. Moreover, no significant associations were observed between cumulative CMV exposure and the subsequent occurrence of infection beyond days 180 or 360, or with bacterial and non-CMV opportunistic infections separately considered. We initiated landmark analyses by day 30, considering that most infections occurring earlier after transplantation were hospital-acquired (i.e., surgical site or catheter-related) and therefore hardly attributable to CMV, and that CMV infection typically occurs only after the first month. No apparent impact was observed when the causal relationship was temporally delineated in terms of recent CMV exposure either, by considering only the episodes of CMV infection that occurred in the preceding period. Finally, no evidence of dose-response gradient between the amount of CMV exposure—measured as high-level viremia, peak viral load or CMV-AUC—and the occurrence of non-CMV infection was found.

The results of the present study overall suggest that CMV replication would act as a surrogate marker of immunosuppression during the initial post-transplant period (first 30 and likely 90 days) rather than actually playing a causative role in the susceptibility to other pathogens. If CMV infection constitutes an independent risk factor for non-CMV infection, it would have been expected that this cause-and-effect relationship would have been evident throughout the entire follow-up and in particular beyond day 180, when drug-

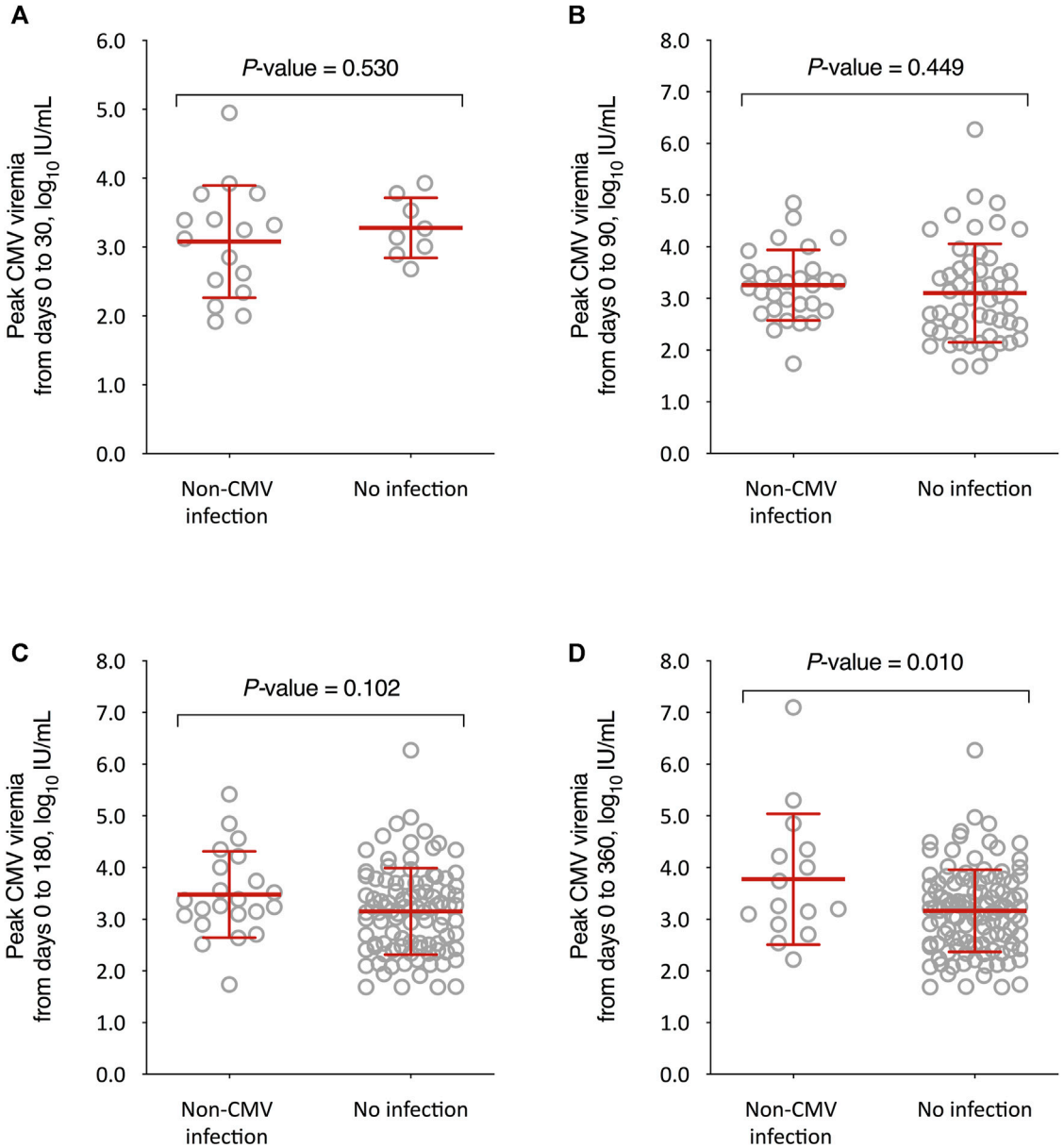


FIGURE 3 | Comparison of peak CMV viral loads between KT recipients developing or not developing overall non-CMV infection beyond day 30 **(A)**, day 90 **(B)**, day 180 **(C)**, and day 360 **(D)** after transplantation. Student's *t*-test for unpaired data was used. Bars represent mean values and whiskers the standard deviations. CMV: cytomegalovirus.

induced immunosuppression is usually reduced. No impact was observed for the specific outcome of opportunistic infection either, also supporting the notion that the immunomodulatory mechanisms deployed by CMV *in vitro* have no clinically meaningful effects. Nevertheless, the lack of apparent association for the late post-transplant period might be at least partially explained by the fact that most episodes of CMV viremia occurred during the first months after transplantation (with only 19.5% of patients experiencing late-onset infection), which could have contributed to dilute the potential effect (if any). On the other hand, it is unclear how long the alleged immunomodulatory actions resulting from active or recent CMV infection would last, although our results would be consistent with some type of effect at least early after KT.

The clinical impact of CMV antiviral prophylaxis on the risk of non-CMV infection after SOT or allo-HSCT remains controversial. A meta-analysis that compiled the results of 17 trials found a lower incidence of bacterial or fungal infections among patients that received prophylaxis, whereas no statistically significant reduction was observed with the pre-emptive approach. (44) Nevertheless, an updated meta-analysis performed by the same authors that included both direct and

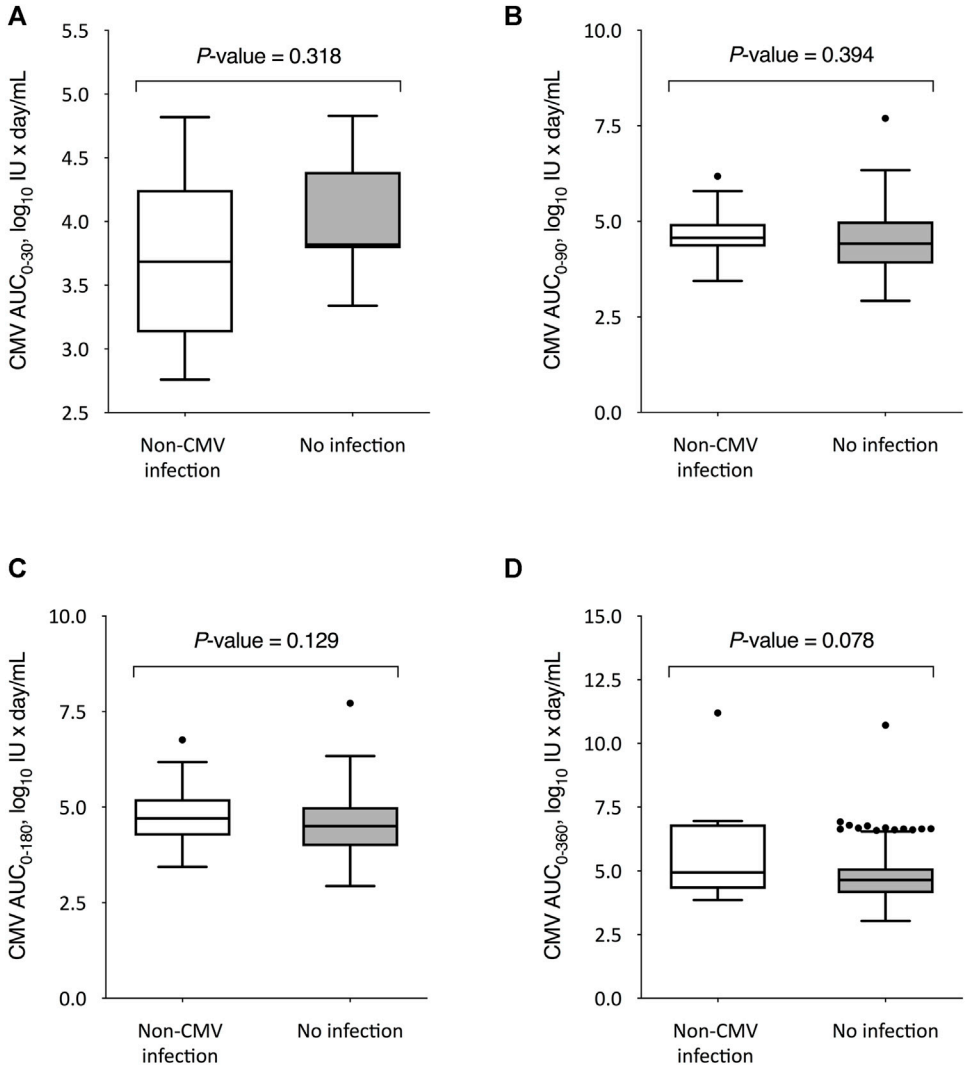


FIGURE 4 | Comparison of CMV-AUCs between KT recipients developing or not developing overall non-CMV infection beyond day 30 **(A)**, day 90 **(B)**, day 180 **(C)**, and day 360 **(D)** after transplantation. Student's *t*-test for unpaired data was used for all comparisons except for day 360, for which the U Mann-Whitney test was applied. The boxes present the interquartile distances, the horizontal lines the median, the whiskers the maximum and minimum values within 1.5 times the interquartile range, and the points the outliers. CMV-AUC: area under curve of cytomegalovirus viral load.

indirect comparisons across 20 studies reported no differences in the incidence of HSV, VZV, bacterial or fungal infection according to the strategy used. (45) A randomized clinical trial that compared 6-months valganciclovir versus PCR-guided CMV pre-emptive therapy in allo-HSCT recipients found no differences in the rates of bacterial or fungal infection between both arms. (46) In the same line, no apparent advantages of valganciclovir prophylaxis in terms of non-CMV infection were observed in a recently published trial in high-risk (D+/R−) liver transplant recipients. (47) One key finding of the present study was that, after multivariable adjustment, the development of any level or high-level CMV viremia through day 90 was marginally associated with subsequent non-CMV infection in the sensitivity analysis restricted to KT recipients that did not receive antiviral prophylaxis. A similar result was observed when only recent CMV exposure (from days 30 to 90) was considered. Again, such an association was not reproduced for the remaining periods, which supports the role of CMV replication as a marker of immunosuppression early after transplantation. Nevertheless, this subanalysis should be taken with caution due to the lower number of patients included. It should be noted that the frequency of monitoring points for CMV DNAemia in the group under prophylaxis was close to that of pre-emptively managed patients (median of 10 and 12 monitoring points, respectively). Interestingly, the use of antiviral prophylaxis per se (versus preemptive therapy) exerted no direct impact on the risk of non-CMV infection regardless of the landmark time point considered (data not shown). Thus, it is plausible that the presumed role of CMV infection as a surrogate marker of immunosuppression would only operate in those recipients managed by preemptive therapy, since

valganciclovir prophylaxis effectively abrogates viral replication even in the most severely immunocompromised patients. An alternative explanation resides in the fact that the main indication for antiviral prophylaxis was previous induction therapy with ATG, leading to a potential interaction between both variables. Indeed, CMV exposure would add little in recipients already experiencing ATG-induced long-term T-cell depletion. This modification of effect, on the contrary, would not be present in the group of preemptive therapy.

Our research has some limitations to be acknowledged. Firstly, this is a single-centre study and the differential impact of local monitoring practices cannot be excluded. Since half of the patients in our cohort received ATG as induction therapy, immunosuppression and prophylaxis regimens may not be applicable to other institutions. Due to the observational design, CMV monitoring was performed as usual clinical practice and may have not been as stringent as recommended in guidelines. (1) The number of episodes of non-CMV opportunistic infection—mainly due to HSV and VZV—was low, limiting the statistical power of this secondary analysis. Immunosuppressive drug levels at different time points were not available. Caution should be exercised when interpreting the comparison between CMV-AUCs, as the administration of antiviral therapy among preemptively managed patients alters viral kinetics. Finally, it should be noted that most of the non-CMV infection episodes occurred during the first 3–6 post-transplant months (median of 29.0 days to the first episode). Therefore, it is not possible to completely rule out a biologically relevant effect of CMV replication in later periods due to low statistical power.

On the other hand, the present study comprised a large and well-characterized cohort of consecutive KT recipients with a prolonged follow-up, allowing us to detect the occurrence of late events. In addition, the close monitoring of CMV viremia by molecular methods and the combination of different methods to measure viral dynamics strengthen our results. By performing different landmark survival analysis with separate Cox models for each period considered, we were able to test the potential impact of cumulative exposure to CMV across various post-transplant periods with changing immunosuppression load, (48) although alternative approaches—for instance, treating the variable of "CMV exposure" as a time-varying covariate in a single Cox model—would have been also reasonable.

In conclusion, CMV exposure (cumulative, recent or at high level) was not independently associated with an apparent increase in the subsequent risk of non-CMV infection in this cohort of KT recipients. Taken together, our findings do not clearly support the hypothesis that the immunomodulatory effects driven by CMV replication exert an impact on the overall risk of infection and would rather point to its role as a surrogate marker of immunosuppression, particularly during the first months after transplantation and in KT recipients under preemptive therapy. Further studies are needed to unravel the complex interplay between CMV and the susceptibility to post-transplant infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee for Research with medicinal products Hospital Universitario "12 de Octubre." The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IR-G participated in the study concept and design, data collection, and manuscript writing, drafting and reviewing; MR-R, FL-M, HT, EG, NP, EG, RS, TR-M, PP, and AA participated in the clinical management of patients, data collection, and manuscript editing and reviewing; LC participated in data collection and manuscript editing and reviewing; MF participated in the laboratory procedures, and manuscript editing and reviewing; AA participated in the study concept and design, clinical management of patients, data collection, and manuscript writing, drafting and reviewing; JA participated in the study concept and design, and manuscript editing and reviewing; MF-R participated in the study concept and design, data analysis and interpretation, and manuscript writing, editing and reviewing. All authors read and approved the submitted manuscript.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10273/full#supplementary-material

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LETTER TO THE EDITOR
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Pre-Kidney Transplant Screening for Coronary Artery Disease: Current Practice in the United Kingdom

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Keywords: kidney transplantation, screening, cardiovascular disease, survey, risk factors

Dear Editors,

Randomised control trial (RCT) evidence is not available to guide screening for asymptomatic coronary artery disease before kidney transplantation [1]. United Kingdom observational data show no clear benefit from screening [2]. To gain data representative of current practice in the United Kingdom, we invited a lead transplant nephrologist from each kidney transplant centre to complete a survey examining cardiac screening practice, work-up pathways, and appetite for a national RCT in June 2021. Ethical approval was not required.

Responses were received from all 23 (100%) centres, of which 22 had a protocol for cardiac assessment prior to listing. In three centres, asymptomatic individuals were not required to undergo cardiac investigation beyond an ECG or echocardiogram prior to transplantation. The remainder followed a risk-stratified approach; no centres performed universal screening.

In centres adopting risk-stratified screening, factors used to screen patients included a history of ischaemic heart disease (100% of centres), diabetes (100%), peripheral vascular disease (50%), smoking (50%), stroke (35%), limited exercise capacity (35%), hyper/hypotension (15%), or an abnormal echocardiogram (95%) or ECG (70%). Two centres stratified using the Newcastle Risk Index [3]. Thirteen centres had a specific age threshold (mostly 50 or 60 years), whilst others included age in combination with additional risk factors or Newcastle Risk Index scores.

The most frequent screening investigation was a myocardial perfusion scan (55%) followed by stress echocardiogram (20%). Coronary angiography and cardiopulmonary exercise testing were the initial investigation in one centre each. Other indications for coronary angiography included an abnormal initial screening test (39%) or on cardiology advice (35%). In one third of centres, the waiting time for investigations was over 10 weeks.

Nine centres had cardio-renal multidisciplinary meetings, whilst 14 had a designated cardiologist providing transplant candidate assessments. In 16 centres cardiology review was only needed for patients with abnormal screening tests, whilst in three cardiologists reviewed all screened patients.

Of 23 centres, 10 had updated their screening protocol within the past 2 years and three were in the process of an update. Whilst 19 centres reported experience of patient declines from listing based on an abnormal screening test, this amounted to one patient per month or less in 11 centres.

Respondents commented on the challenges of outdated evidence, reliance on observational data, and differences between real-world cohorts and RCT study populations when assessing the evidence for cardiac screening. The importance of cardio-renal meetings was noted in units not adopting

Abbreviations: ECG, Electrocardiogram; RCT, Randomised controlled trial.

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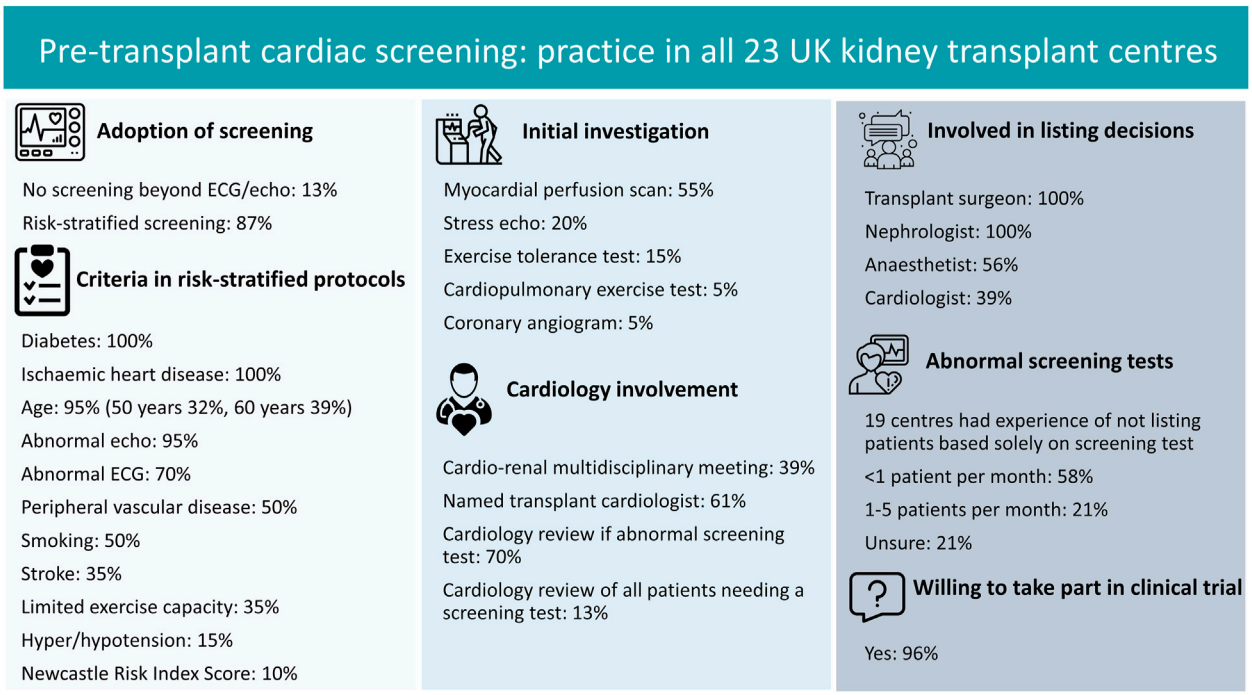

FIGURE 1 | Summary of findings from survey of pre-transplant cardiac screening.

screening. Of 23 centres, 22 expressed interest to participate in an RCT to examine the utility of screening, 12 of whom supported recruiting the highest cardiac risk candidates.

Our survey highlights variation in screening practice across the United Kingdom (**Figure 1**). Similar heterogenous practice has been shown in the United States [4], although our survey was undertaken following publication of ISCHEMIA-CKD [5]. Whilst no centres perform universal screening and many have recently updated their protocols, which may represent a trend away from routine screening, responses highlight nephrologists' concerns over the evidence upon which practice is based. Capturing views of other transplant professionals and patients is essential, but this survey suggests support for an RCT to evidence utility of screening.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors contributed to study design and writing of the survey questions. AN performed the analyses, produced the figure and wrote the letter under the supervision of RR and DT. All authors contributed to manuscript preparation.

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
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Comparison of mRNA-1273 and BNT162b2 SARS-CoV-2 mRNA Vaccine Immunogenicity in Kidney Transplant Recipients

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Keywords: Covid-19, Sars-CoV-2, kidney transplantation, vaccination, mRNA vaccine

Dear Editors,

Kidney transplant recipients are at high risk for severe COVID-19 disease or death in case of SARS-CoV-2 infection (1). There is growing evidence suggesting that anti-SARS-Cov2-antibody response is markedly blunted in kidney transplant patients after vaccination (2). Severe COVID- 19 and COVID-19-related death has been recently reported in kidney transplant recipients despite prior complete (two dose) vaccination with SARS-CoV-2 mRNA vaccines (3). (4).

In this retrospective cohort study involving 320 prevalent kidney transplant recipients from a single transplant center (Ordensklinikum Linz—Elisabethinen hospital), anti- Spike (S) protein IgG antibody titers were measured 3–6 weeks [BNT162b2: median 28 days (IQR: 6 days), mRNA1273: median 28 days (IQR: 8 days)] after administration of the second dose of either mRNA-1273 or BNT162b2 SARS-CoV-2 vaccine. Vaccinations took place between January 15th and June 8th, 2021 according to the Austrian national SARS-CoV-2 vaccination program. Vaccine doses were allocated by the Austrian government depending on availability. Patients were vaccinated ranked by age beginning with the oldest as soon as vaccines were available. Allocation to a certain vaccine (BNT162b2 or mRNA-1273) was therefore determined by the vaccination progress in our kidney transplant cohort and vaccine availability at that time. A two-dose vaccination regimen was applied, with 3-weeks (BNT162b2) and 4-weeks (mRNA-1273) intervals between the first and second vaccination, regardless of a history of prior infection with SARS-CoV-2.

Anti-SARS-CoV-2-antibodies directed against the receptor binding domain (RBD) of the S1 subunit of the Spike (S) protein were measured with the SARS-CoV-2 IgG II Quant assay (Abbott Ireland Diagnostics Division, Finisklin Business Park, Sligo, Ireland), which was reported to have high sensitivity and specificity for detection of anti-SARS-CoV-2 S-protein antibodies (5) and high correlations with anti-SARS-CoV-2 neutralizing antibodies (6). Results were reported in BAU/ml (binding antibody units). Differences between vaccine groups (mRNA-1273 vs BNT162b2) in S-antibody-positivity were tested for statistical significance using the Chi^2-Test. To further investigate the impact of vaccine type on S-antibody-positivity, we computed a multivariate logistic regression model taking potential confounding factors of seroconversion after SARS-COV2 vaccination into account. Results are reported as odds ratios (OR) and 95% confidence intervals (95% CI). We performed a complete case analysis as covariate information was missing in one patient only for the multivariate model. The study was approved by the Ethics Committee of the Johannes Kepler University Linz (ID: 1100/2021). Patients provided written informed consent. Patient demographics and additional analyses are shown in the **Supplementary Material**.

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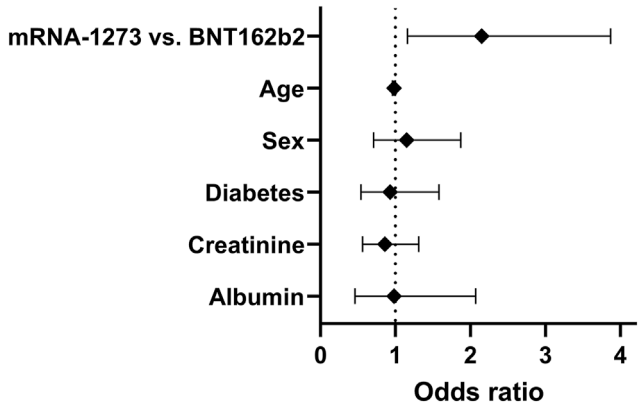

FIGURE 1 | Odds ratios and 95% confidence intervals of a multivariate logistic regression analysis for anti-S-antibody seroconversion after 2 doses of SARS-CoV-2 mRNA vaccine in 320 prevalent kidney transplant patients irrespective of prior history of SARS-CoV-2 infection.

Anti-S-antibody positivity was detected in 51% of the patients in our study cohort. A higher proportion of mRNA-1273 vaccinated patients achieved antibody-positivity compared to those vaccinated with BNT162b2 (61.6 vs 47.7%, $p = 0.037$, Chi^2-test). After correction for age, diabetes status, sex, serum albumin and serum creatinine, the odds ratio for anti-S- antibody seroconversion was significantly higher for mRNA-1273 vaccinated patients compared to BNT162b2 in a multivariate regression analysis (odds ratio: 2.12, 95% confidence interval: 1.16 to 3.87, $p = 0.013$, **Figure 1**). After exclusion of patients with a history of prior SARS-CoV-2-infection [$N = 21$; 17 patients with BNT162b2, four patients with mRNA-1273; six patients with IgG antibodies directed against the nucleocapsid (N) protein, 15 Patients with positive SARS-CoV-2 polymerase chain reaction (PCR) test], results remained similar. In patients without prior SARS-CoV-2 infection ($N = 299$) a higher proportion of patients vaccinated with mRNA-1273 achieved seropositivity compared to patients vaccinated with BNT162b2 (59.4 vs 44.3%, $p = 0.027$, Chi^2-test). The odds ratio for seroconversion was higher in mRNA-1273-vaccinated patients compared to BNT162b2-vaccinated patients in multivariate analysis (OR: 2.2, 95% CI: 1.19 to 4.08, $p = 0.011$).

Reasons for a higher rate of seroconversion after mRNA-1273 vaccination compared to BNT162b2 are currently uncertain. Possible explanations include differences in mRNA content per vaccine dose, differences in mRNA modification or differences in the lipid formulation between the vaccines, all of which may influence expression of spike (S)-proteins and therefore immunogenicity. A limitation of this study is the lack of data in cellular immune responses, which may underestimate the immunogenicity of the vaccines. Another limitation is the retrospective nature of this study. However, similar results were recently reported in another observational study on immunogenicity of the mRNA-1273 and BNT162b2 vaccines in patients on renal replacement therapy (7), corroborating our findings.

In conclusion, vaccination with mRNA-1273 is associated with higher odds of anti-S-antibody seroconversion compared to vaccination with BNT162b2 in prevalent kidney transplant recipients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Johannes Kepler University Linz, Austria. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Research idea and study design: MH, RK, PA, HK, and DC. Data acquisition: RK, SL, HK, CB, and DC. Data analysis/interpretation: MH, RK, SL, PA, HK, CB, and DC. Statistical analysis: MH and DC. Supervision or mentorship: DC. Each author contributed important intellectual content during manuscript drafting or revision and agrees to be personally accountable for the individual's own contributions and to ensure that questions pertaining to the accuracy or integrity of any portion of the work, even one in which the author was not directly involved, are appropriately investigated and resolved, including with documentation in the literature if appropriate.

CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10026/full#supplementary-material

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LETTER TO THE EDITOR
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Humoral Response in SARS-CoV-2 Convalescent Compared to Vaccinated Kidney Transplant Patients

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Keywords: SARS-CoV-2, kidney transplantation, vaccination, humoral response, SARS-CoV-2 spike protein

Dear Editors,

Solid organ transplant patients are at high risk for severe or fatal COVID-19 (1), even after two vaccinations (2). Recent studies show, that after a double vaccination course, the antibody response rate is as low as 48% (3). However, to our knowledge there is no data on differences in the natural or vaccine-induced SARS-CoV-2 humoral immunity evaluated in one and the same cohort of kidney transplant recipients (KTR). Here, we are reporting on and comparing the humoral response in 164 KTR (mean age 59.1 years (range 21–85 years), 61.6% male). The group included 142 patients who were vaccinated twice (72% Moderna mRNA-1273 vaccine; 27% Pfizer/BioNTech mRNA-BNT162b2 SARS-CoV-2 vaccine, 1% Oxford-AstraZeneca ChAdOx1-COVID-19 vaccine) and 22 patients after symptomatic and PCR-confirmed Covid-19. We assessed the humoral response on average (25th percentile, 75th percentile) 50 days (33.8, 62.0) after the second vaccine dose or 90 days (39.8, 143.0) after infection by quantifying anti-SARS-CoV-2 spike IgG antibodies. Most patients were treated with tacrolimus (74%), mycophenolic acid (71%) and prednisolone (57%). Eight percent were treated with belatacept. Convalescent KTR were significantly younger ($p = 0.009$), had lower eGFR ($p = 0.021$) and were more often treated with prednisolone ($p = 0.042$) as shown in **Table 1**. Seroconversion was defined as an anti-SARS-CoV-2 IgG antibody concentration above the respective cut-off value according to the manufacturer of the assay. Details about the assays in use and their cut-off values are given in the **Supplementary Material**.

The seroconversion rate in convalescent patients was 90.9 and 48.6% in vaccinated patients ($p < 0.001$). In the patients treated with belatacept, only one out of 12 (8.3%) vaccinated individuals had a seroconversion, whereas both naturally infected patients showed a response. In a multivariable logistic regression analysis infection compared to vaccination [odds ratio (OR) 18.98; 95% CI: 3.41, 105.58] and transplantation vintage (OR 1.01; 95% CI: 1.01, 1.02) were associated with a significantly higher likelihood of seroconversion. On the other hand, older age (OR 0.95; 95% CI: 0.93, 0.99) and belatacept treatment (OR 0.13; 95% CI: 0.02, 0.68) significantly decreased the likelihood of seroconversion. All model coefficients and odds can be found in the **Supplementary Table S1**.

Like our results in convalescent KTR, Magicova et al. recently found a preserved humoral response after SARS-CoV-2 infection comparable to immunocompetent persons in a large Czech cohort of 1,037 kidney transplant recipients with a seroprevalence of 6.8% during the second infection wave in fall 2020 (4). In line with our findings, recent data in dialysis patients also show a superior humoral immune response in convalescent compared to vaccinated patients (5). Natural infection seems to be a stronger and quantitatively higher antigenic challenge than vaccination. In contrast to intramuscular vaccination, natural infection stimulates the resident immune system of mucous membranes, especially designed to

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TABLE 1 | Characteristics of convalescent and vaccinated kidney transplant patients with and without seroconversion.

	Total (*n* = 164)	Infection (*n* = 22)			Vaccination (*n* = 142)			*p*-value[a]
		Seroconversion (*n* = 20)	No seroconversion (*n* = 2)	Total (*n* = 22)	Seroconversion (*n* = 69)	No seroconversion (*n* = 73)	Total (*n* = 142)	
Age (years), mean (SD)	59.1 (13.9)	51.7 (15.6)	55.0 (1.4)	52.0 (14.9)	59.0 (51.5, 65.0)	62.2 (13.4)	60.2 (13.4)	0.009
Gender (male), *n* (%)	101 (61.6%)	14 (70%)	2 (100%)	16 (72.7%)	44 (63.8%)	41 (56.2%)	85 (59.9%)	0.347
eGFR (ml/min/1.73 m^2), mean (SD)	52.8 (17.6)	45.0 (14.8)	42.5 (patient 1:49.0; patient 2: 36.0)	44.7 (14.2)	55.2 (16.8)	53.0 (18.8)	54.0 (17.8)	0.021
Transplantation vintage (months), median (25th percentile, 75th percentile)	104.5 (50.3, 174.5)	87.5 (34.0, 151.5)	207.0 (patient 1: 41.0; patient 2: 373.0)	87.5 (38.0, 161.3)	147.0 (93.5, 223.0)	70.0 (37.5, 120.5)	108.5 (53.0, 175.5)	0.361
Time between antigenic contact (infection or vaccination) and antibody assessment (days), median (25th percentile, 75th percentile)		88.0 (37.3, 118.3)	252.5 (patient 1: 230.0, patient 2: 275.0)	90.0 (39.8, 143,0)	51.0 (34.5, 64.0)	43.0 (31.5, 60.0)	50 (33.8, 62.0)	0.001
Immunosuppression, *n* (%)								
Prednisolone	94 (57.3%)	15 (75%)	2 (100%)	17 (77.3%)	30 (43.5%)	47 (64.4%)	77 (54.2%)	0.042
Tacrolimus	121 (73.8%)	17 (85%)	1 (50%)	18 (81.8%)	48 (69.6%)	55 (75.3%)	103 (72.5)	
Mycophenolic acid	116 (70.7%)	15 (75%)	0 (0%)	15 (68.2%)	38 (55.1%)	63 (86.3%)	101 (71.1%)	
Everolimus	1 (0.6%)	0 (0%)	0 (0%)	0	1 (1.4%)	0 (0%)	1 (0.7%)	
Sirolimus	5 (3%)	0 (0%)	1 (50%)	1 (4.5%)	3 (4.3%)	1 (1.4%)	4 (2.8%)	
Belatacept	14 (8.5%)	2 (10%)	0 (0%)	2 (9.1%)	1 (1.4%)	11 (15.1%)	12 (8.5%)	
Cyclosporin A	23 (14.0%)	2 (10%)	0 (0%)	2 (9.1%)	16 (23.2%)	5 (6.8%)	21 (14.8%)	
Azathioprine	16 (9.8%)	2 (10%)	1 (50%)	3 (13.6%)	11 (15.9%)	2 (2.7%)	13 (9.2%)	

[a]p-*value for group comparison infection* versus *vaccination.*

fight respiratory viral diseases. These considerations and first experience showing a 49–70% humoral response rate after a third vaccine dose in kidney transplant patients without seroconversion after two doses (6–8) support an early third vaccination to improve the seroconversion rate in this vulnerable population.

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

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

AUTHOR CONTRIBUTIONS

JS, HS-M, KL, and EZ designed the study, JS, HS-M, and TD collected data, JS and EZ analyzed data and wrote the first draft of the manuscript. All authors reviewed the manuscript approved the submitted version of the manuscript.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10060/full#supplementary-material

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