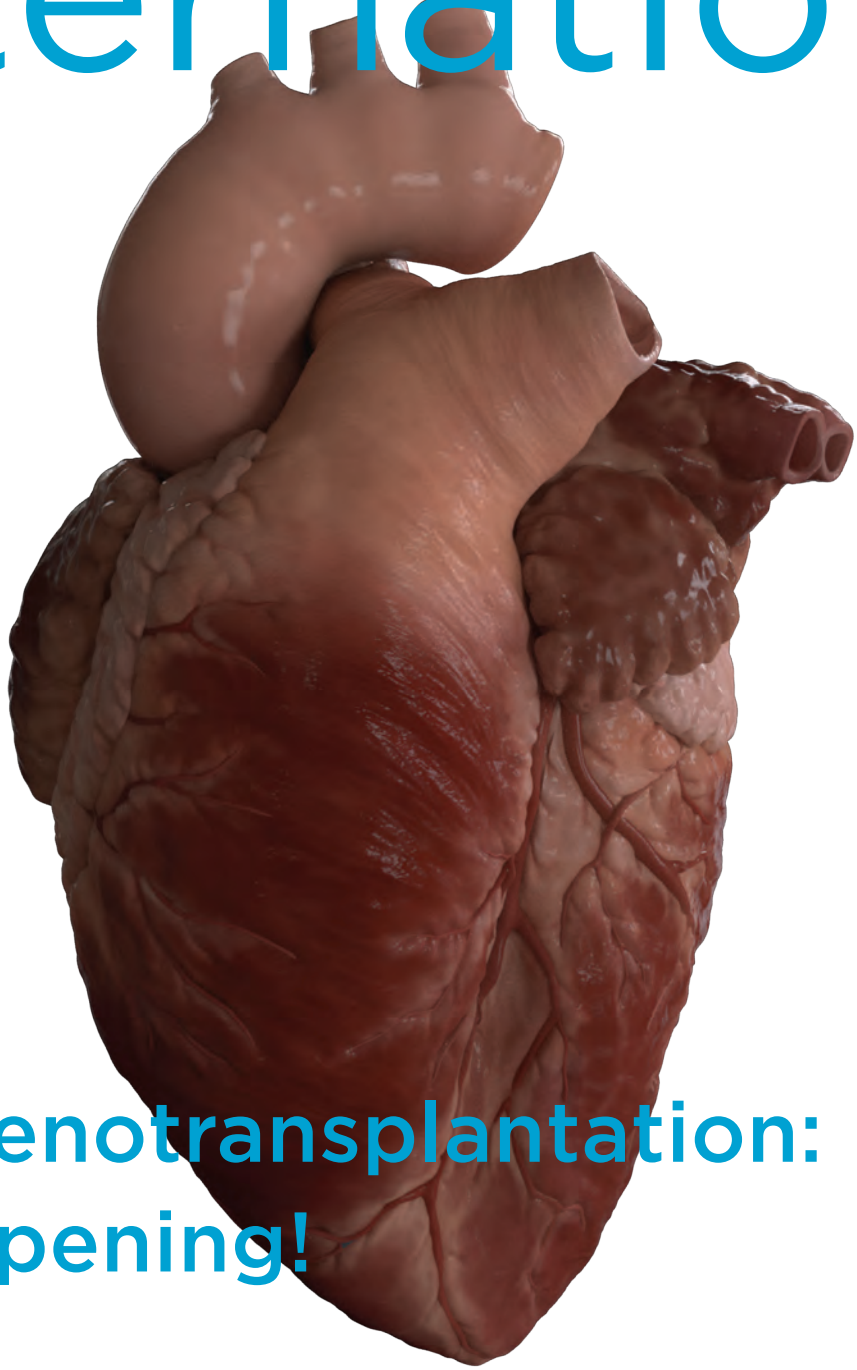
Volume 35 | Issue 3
March 2022

Transplant International



Heart xenotransplantation: It's happening!

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Transplant International

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ESOT

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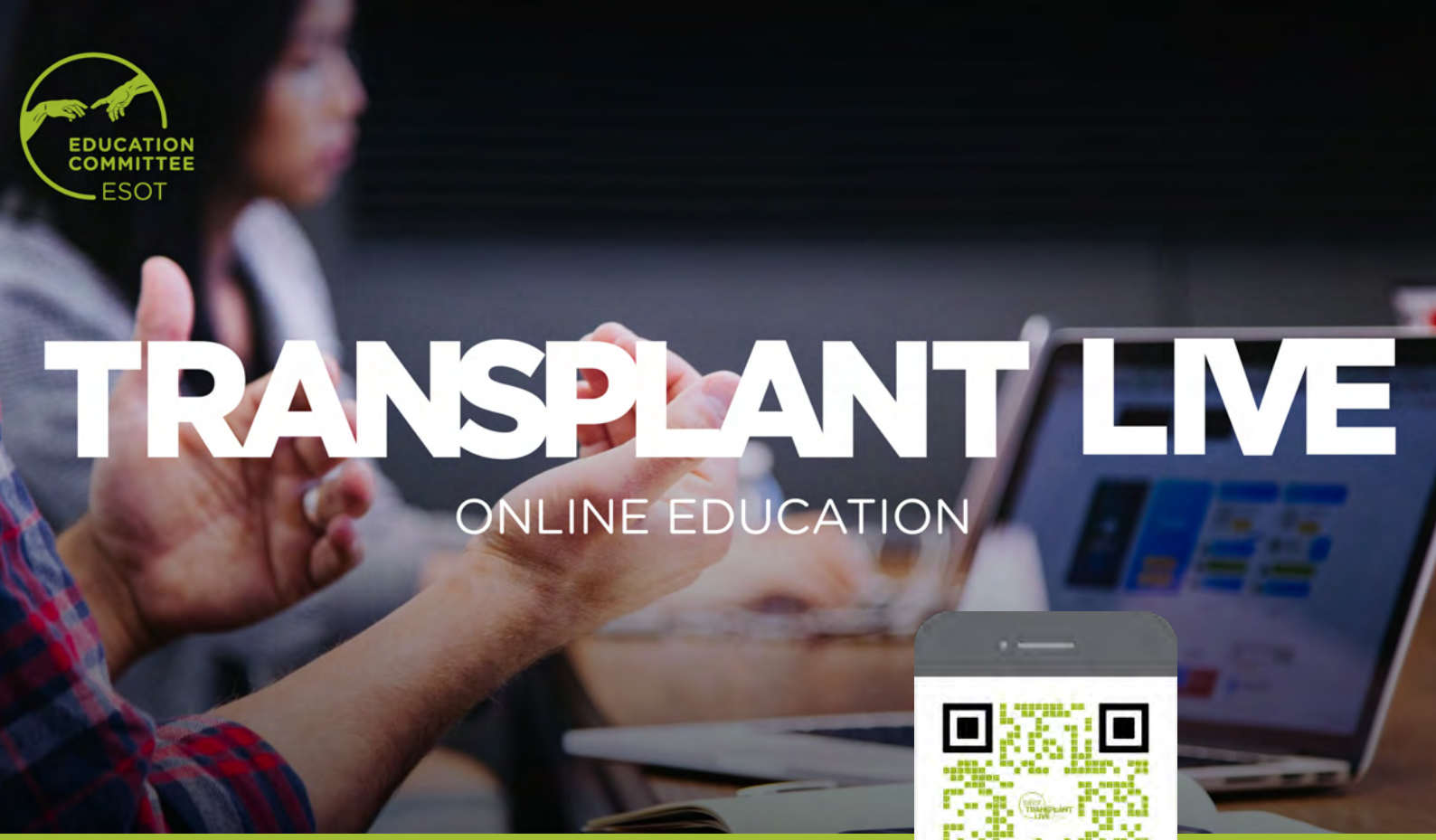

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- Stem cells
- organoids
- machine perfusion
- regeneration

Learning Objectives:

- Hear the latest developments in clinical regeneration
- Get updated on immunomodulatory cell therapy in transplantation
- Be informed about the introduction of cell therapy in machine perfusion
- Learn about novel developments in organoid research

Target Group:
Researchers and clinicians from the transplant field interested in regenerative medicine

TRANSPLANT TRIAL WATCH
published: 28 March 2022
doi: 10.3389/ti.2022.10365

Transplant Trial Watch

John O'Callaghan[1,2]*

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Keywords: kidney transplantation, Vaccination, COVID-19 vaccine, randomised controlled trial, Belatacept conversion

Randomised Controlled Trial 1

Randomized Trial of a Third Dose of mRNA-1273 Vaccine in Transplant Recipients
by Hall, V. G., et al. New England Journal of Medicine 2021; 385 (13): 1244–1246.

Randomised Controlled Trial 2

Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial
by Budde, K., et al. Journal of the American Society of Nephrology 2021 [record in progress].

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

RANDOMISED CONTROLLED TRIAL 1

Randomized Trial of a Third Dose of mRNA-1273 Vaccine in Transplant Recipients
by Hall, V. G., et al. New England Journal of Medicine 2021; 385 (13): 1244–1246.

OPEN ACCESS

***Correspondence:**
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Received: *17 January 2022*
Accepted: *04 February 2022*
Published: *28 March 2022*

Citation:
O'Callaghan J (2022) Transplant Trial Watch. Transpl Int 35:10365. doi: 10.3389/ti.2022.10365

Aims
This study aimed to investigate the effect of a third dose of the mRNA1273 (Moderna) vaccine in solid organ transplant (SOT) recipients.

Interventions
Participants were randomised to receive either a third dose of mRNA1273 vaccine or saline placebo.

Participants
120 adult solid organ transplant recipients.

Outcomes
The primary endpoint was a serologic response. Secondary endpoints included the percent neutralization, and the polyfunctional T-cell response.

Follow-up
4 months.

CET Conclusion
Published as a correspondence letter in the NEJM, this study randomised transplant recipients to a third coronavirus vaccine dose (moderna) or to placebo. 120 transplant recipients were included, with median age 66 years and median time after transplant to third vaccine dose of 3 years. Making use of the supplemental appendix and protocol, we can see that this study was conducted in an adequately blinded and randomised fashion, with allocation concealment. Four months after the third injection, 55% in the study group and 18% in the placebo group had antibody levels over the threshold of 100 U/ml for anti-receptor-binding domain antibodies. There was also a significant improvement in virus neutralisation in the study group (71% vs 13%). The third moderna vaccine dose showed a significantly higher immunogenicity than placebo in this patient group, but the authors acknowledge that the study was not powered to, nor had long enough follow up, to assess clinical outcomes.

Jadad Score
5.

Data Analysis
Per protocol analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov–NCT04885907.

Funding Source
Non-industry funded.

RANDOMISED CONTROLLED TRIAL 2

Conversion from Calcineurin Inhibitor to Belatacept-based Maintenance Immunosuppression in Renal Transplant Recipients: a Randomized Phase 3b Trial

by Budde, K., et al. Journal of the American Society of Nephrology 2021 [record in progress].

Aims
Participants were randomised to either the belatacept conversion group or the CNI continuation group.

Interventions
Participants were randomised into two groups: the intervention group, in which the patients participated in a personalised exercise rehabilitation program in addition to standard care, or the control group where the patients received standard care alone.

Participants
446 stable adult kidney transplant recipients.

Outcomes
The primary outcome was the percentage of patients surviving with a functioning graft. Secondary outcome included patient survival, graft survival, incidence and severity of biopsy-proven acute rejection (BPAR), renal function, mean changes in systolic and diastolic blood pressure, proportion of patients with preexisting donor-specific antibodies, and adverse events.

Follow-Up
24 months.

CET Conclusion
This large multicentre phase 3b study randomised renal transplant recipients 6–60 months post-transplant to continue CNI, or to switch to Belatacept-based immunosuppression. The primary endpoint (survival with a functioning graft at 24 months) did not differ between groups. There was, however, a clinically significant superior GFR in the Belatacept arm with lower rate of *de novo* DSA, tempered by numerically higher acute rejection rates. It should be noted that the population recruited is relatively low risk, with no recent acute rejection, stable function and EBV seropositive due to risk of PTLD. In reality, the study is underpowered to demonstrate non-inferiority for the primary endpoint, although outcomes in both arms in this respect were excellent. Longer-term follow-up will be interesting to see, as it is quite possible that the improvements in graft function and reduction in dnDSA seen will translate to better long-term graft survival.

Jadad Score
3.

Data Analysis
Strict intention-to-treat analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov–NCT01820572.

Funding Source
Industry funded.

CLINICAL IMPACT SUMMARY

This is a large, well-conducted RCT that took place across multiple centres in several countries. The included patients were adult recipients of both live and deceased donor kidney transplants who were stable 6–60 months after surgery. Patients were also excluded if they had experienced: antibody-mediated rejection at any time; any form of rejection within 3 months of the study start; recurrent acute rejection; greater than or equal to Banff grade-IIA acute rejection in the current allograft; previous graft loss due to BPAR; or had a positive T cell crossmatch prior to the current transplant. Randomisation was adequate and included only one form of stratification; by GFR so that an equal distribution of low functioning kidneys was entered into each study arm. Renal biopsies were mandated in any suspected acute rejection episode, surveillance biopsies were not done.

The primary endpoint was graft survival at 24 months and this was assessed in an intention to treat analysis to preserve randomisation. The withdrawals, dropouts and cross-overs are described and in any event were at tolerable levels for a study of this size. Due to the lack of prior data on graft function on which to base power calculations, the study was powered to exclude significant differences in graft survival instead.

The results show that there was a similar patient and graft survival at 24 months after randomisation and there was no significant difference in acute rejection rates or overall adverse events. However, there was a significant and evolving improvement in eGFR in patients in the belatacept group from baseline, compared to a decline in eGFR in the CNI group. The paper demonstrates a lower proportion of patients that developed *de novo* DSA in the belatacept group than the CNI group, but there is no statistical analysis presented for this outcome.

This study shows that low-risk, stable renal transplant recipients can be converted to belatacept from CNI-based immune suppression with comparable graft survival at 2 years. Whilst there were some post-conversion rejection events, they did not lead to any graft loss in this study and were successfully treated with steroids. Conversion to belatacept in this low-risk population is associated with an improvement renal function and provides a safe option in patients who are intolerant of CNI. If the follow up for the study could be extended, then this improvement in function might also be associated with improved graft survival.

AUTHOR CONTRIBUTIONS

JO'C wrote the clinical impact summary.

CONFLICT OF INTEREST

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

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NEWS AND VIEWS
published: 30 March 2022
doi: 10.3389/ti.2022.10439

Xenotransplantion: Defeating the "Shumway Curse" An Interview With Drs. Bartley Griffith, Jayme Locke, Robert Montgomery, and Bruno Reichart

Thierry Berney, Maarten Naesens and Stefan Schneeberger*

Transplant International

Dr. Robert Montgomery, is a Professor and Chair of Surgery, and Director of the Transplant Institute at New York University (NYU) Langone Medical Center, United States.

On Sep. 25, 2021, Dr. Montgomery transplanted a kidney from a genetically modified pig into a deceased human body donor, an experiment he successfully reproduced a few weeks later. Urine production could be observed for up to 3 days.

Dr. Jayme Locke, is a Professor of Surgery and Director of the Comprehensive Transplant Institute and Division of Transplantation at the University of Alabama at Birmingham (UAB), United States.

On Sep. 30, 2021, Dr. Locke transplanted two genetically modified pig kidneys inside the abdomen of a brain-dead human after removing the recipient's native kidneys. Urine production for over 72 hours was also observed (1).

Dr. Bartley Griffith, is a Professor of Surgery and Director of the Cardiac and Lung Transplant Programs at the University of Maryland School of Medicine, Baltimore, United States.

On Jan 7, 2022, Dr. Griffith performed the first successful xenogeneic heart transplant from a genetically modified pig to a human.

Dr. Bruno Reichart, is an Emeritus Professor of Surgery and project leader at the Walter Brendel Center for Experimental Medicine, Ludwig-Maximilian University (LMU), Munich, Germany.

Dr. Reichart performed Germany's first successful heart transplant in 1981, and has been a leading scientist and a spokesman for experimental xenotransplantation since over 20 years.

It is commonplace to quote Dr. Norman Shumway as saying that "xenotransplantation is the future and always will be." You and your teams seem to have successfully challenged this prediction. You have achieved and reported, in a super rapid sequence, what we feel is one of the most exciting breakthroughs in transplantation medicine since the turn of the century.

Dr. Bartley Griffith (BG).

The key to these successes seems to have been the availability of pigs genetically engineered to evade xenogeneic rejection mechanisms (1).

BG: D. Craig Miller (note: cardio-thoracic surgery pioneer from Stanford) wrote to me after our news broke. He congratulated us. He went on to chuckle over this well-traveled quote of his mentor Norman Shumway. He felt that if Norm had lived to see cloning of mammals and CRISPR gene editing, he might have softened his negativity. Further, he told me that Norm would have appreciated the lengthy study period and substantial animal trials that told of the group's preparedness. Today we are POD#24, so it is way early to conclude on long-term success, but the heart is doing quite well (note: the interview was

News & Views

OPEN ACCESS

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Received: *16 February 2022*
Accepted: *17 February 2022*
Published: *30 March 2022*

Citation:
Berney T, Naesens M and Schneeberger S (2022) Xenotransplantion: Defeating the "Shumway Curse" An Interview With Drs. Bartley Griffith, Jayme Locke, Robert Montgomery, and Bruno Reichart. Transpl Int 35:10439. doi: 10.3389/ti.2022.10439

conducted on 1 February 2022; since then, the death of the recipient, apparently for reasons unrelated to rejection, was communicated by the Maryland team).

Q: Can you tell us what are the molecules/pathways that had to be targeted to produce organs that would have a reasonable chance of escaping the immunologic hurdles of xenotransplantation? How many genes had to be inserted/knocked-out? Have you used the same porcine "strains" and are the genes to target different for kidney or heart?

JL, BG: At University of Alabama and University of Maryland, we transplanted kidneys from a pig donor with 10 gene edits (10 GE). Four genes were knocked out: three related to carbohydrate antigens known to cause hyperacute rejection (α 1-3 Gal, β 1-4 Gal, CMAH) and one involved the deletion of the pig growth hormone receptor (GHR). There were also six human transgene insertions. These edits were designed to further modulate the human immune system to help decrease inflammation (hCD47, hHO-1), and regulate complement (hCD46, hDAF) and coagulation (human thrombomodulin, human endothelial protein C receptor) (2).

BG: The goal of Revivicor/United Therapeutics (note: the company producing the genetically modified porcine donors for the Maryland transplant and the Birmingham experiment) is to have a single commercial pig for all organs if possible.

RM: We have taken the approach that "less is more." α 1-3 Gal has always been the clear barrier to xenotransplantation with up to 1% of total human immunoglobulin targeting this epitope. We have crossmatched a large number of patients against α 1-3 Gal KO pigs and most have very reasonable crossmatches (note: α 1-3 Gal KO (GalSafe) pigs were recently developed and approved by the FDA, primarily as a source of allergy safe meat). A lot of the transgenes that are being dropped into the pig genome have variable expression and targets that can be regulated by drugs that are approved for human use.


Dr Robert Montgomery (RM).

I do think different organs are going to have different genetic engineering requirements. The challenge will be designing trials that can test individual "knock outs" and "knock ins" so we don't just accumulate complex constructs with unproven individual components.

BR: For pig-to-baboon models, knocking out α 1-3 Gal is sufficient. This is enough to prevent hyperacute rejection, but when moving to the human, you need to target additional genes, as explained by Drs. Locke and Griffith. One major concern is that pigs grow to sizes much larger than humans, and the size of an organ in an animal is genetically determined by the size of the adult body. This made it necessary to knock-out the human GHR to prevent the donor organ from outgrowing its new human host. One problem with GHR-KO animals is that their reproduction is affected and they are therefore very difficult to breed. Because of the breeding difficulties, among other reasons, the UAB and Maryland groups have utilized cloned, as opposed to reproduced, animals.

I agree with Dr. Montgomery that it may be preferable to change as little as possible in the genes of the porcine donors and utilize pharmacological agents (e.g., inhibitors, monoclonals,. . .) rather than extensive genetic modifications. As for the size issue, instead of knocking-off GHR, the Munich group is looking for smaller breeds of pigs that only grow to 70–80 kg.

Q: How long and how did you prepare yourselves and your teams before taking the final step? How much of a leap of faith has it been?

BG: Dr. Muhammad Mohiuddin has been studying xenoheart for nearly 3 decades. He developed the immunosuppression protocol in pig-to-baboon in an intra-abdominal implant model. His 3-year success was a major factor in his relocation from the NIH to the University of Maryland 5 years ago. Our goal has been to translate his work to a model that would directly translate to humans. We focused on orthotopic xenoheart transplantation in 18–30 kg baboons. We refined our processes and approach. We have consistent survivals of 3 months and beyond with minimal evidence of rejection. Our longest animal was going strong at 9 months, when he died of respiratory failure from a presumed virus that hit other animals in the post-operative care facility.

Prior to making an incision in our patient the team took a quiet moment of reflection to give thanks and a hope for help in our quest. We were confident the time was right to try.

JL: We launched the University of Alabama Xenotransplant Program in 2016. We invested heavily in building out the necessary infrastructure in order to make xenotransplantation a reality for the many patients in need. This included the development and implementation of a pathogen-free facility in which the donor source animal (pig) can be bred and raised in an environment that decreases/eliminates the risk of viral or other disease transmission to human recipients from these pig organs. We also simultaneously continued to study the 10 GE pig kidney transplant in non-human primates (NHP). However, it became very clear that the NHP model was not immunologically similar enough to humans to answer key safety questions: would hyperacute rejection be avoided in the setting of standard immunosuppression used in allo-human-to-human transplantation? would viral transmission or chimerism develop in a human host? would the pig vasculature withstand adult human arterial pressure? and importantly the NHP model was insufficient to validate our novel flow crossmatch designed to predict tissue compatibility a priori (e.g., prior to transplant as is required by federal regulators for human-to-human transplantation).


Dr Jayme Locke (JL).

We therefore sought a novel pre-clinical human model—human brain death. The implementation of this model began at UAB 2 years ago. We initiated an external ethics review and engaged our Institutional Review Board (IRB). Although by definition the model is not human subject research and therefore not under the purview of an IRB, we felt given the magnitude of the study and our goals of recapitulating every step in the process

that would be necessary to move this into living people, we elected to perform the study under IRB approval. As with all science, there is some element of a "leap of faith," but in reality the UAB study was hypothesis-driven to answer early endpoints that simply could not be answered in a NHP model, and were absolutely critical prior to risking the life of a living person—e.g., demonstration that we could assess tissue compatibility a priori, demonstrate no hyperacute rejection in the setting of conventional immunosuppression, and ensure no porcine endogenous retroviruses (PERV) were transmitted.

RM. We have been working on setting up the studies in the recently deceased for 5 years. It required a lot of vetting and regulatory work. Honestly, when we did the first transplant on 25 September 2021, I said to the team just before we removed the vascular clamps, "I don't know what is going to happen here but we will learn something very important today."

Q: These reports have almost taken the transplant community by surprise. Do you see these as a quantum leap or as the natural progression of xenotransplantation research over the past decades? How much of the success is due to the discovery CRISPR/Cas9 genome editing system of Nobel prize fame?

BG: The coming together in cases is likely a response to the availability of edited pigs. Certainly, gene editing is a huge accelerant.

JL: We believe these advances represent the natural progression of xenotransplantation research, which has most definitely benefited from the introduction of CRISPR/Cas9 genome editing allowing for greater precision and rapidity.

RM: The α-Gal KO in the pig we used was created by homologous recombination. I think the big leap was made possible by the concept of whole-body donation for the purpose of high stakes studies like xenotransplantation as an intermediate step between animal and phase I trials. This could be done without the risk of harm to the patient since they were brain dead. I think once we did that the genie was out of the bottle.

Q: Is transmission of porcine retroviruses (PERV) still a problem or has it ever been? How, if at all, have you tackled it?

JL: PERVs have long been a concern in the field, but these concerns have evolved over time as well. Regulators now acknowledge that PERV A and B are endemic in pig herds and that it is PERV C that is known to cause disease in humans. The donor source pigs at UAB are housed in a pathogen free facility and undergo routine biosurveillance. They are negative for PERV C. Importantly, our deceased human recipient was negative for PERV A, B, and C post-transplant suggesting that NO disease transmission occurred. In addition, a study of pig islets into humans out of New Zealand has demonstrated no PERV transmission with 7 years of post-transplant follow-up.

BG: This remains an uncertain but theoretically real risk. Our animals are not PERV free. We have an opt out ability for intimate contacts (very few did so), a major surveillance program, and contact precaution in force.

RM: There has never been a transmission of PERV to a human despite more than 200 patients having received living porcine cells and tissue. Close surveillance has become an acceptable framework for zoonotic management.


Dr. Bruno Reichart (BR).

BR: When the field was starting, PERVs were considered as a major issue. It has only been possible to infect human cells *in vitro* with PERV, and under very specific conditions. In the few human trials of xenogeneic islet transplantation, no PERV transmission could ever be documented. Interestingly, anti-PERV antibodies could not be found in a serological study of slaughterhouse butchers, who literally "bathe" in porcine blood! (3) PERV A and B exist in low copies and are not considered as dangerous to the humans, so only PERV C should be controlled. I believe that eGenesis (note: a US company developing gene-edited pigs for xenotransplantation) has the technology to target PERV genes but does not consider it necessary to knock off these genes because it may lead to off-target effects.

One infectious consideration regards transmission of porcine CMV. This virus from the herpes family is very different from human CMV and no drug is effective against it. Porcine CMV transmission has been associated with high lethality in pig-to-primate experiments. Fortunately, it is very easy to breed and maintain porcine CMV-free animals.

Q: Dr. Locke, Dr. Montgomery: While both of you have reported urinary output, in the Alabama case this was not associated with creatinine clearance. Was it the same in the NY cases? What would be an explanation? Was it just a matter of timing? Could you prolong the experiment beyond 3 days (from regulatory and ethical standpoints)?

JL: Kidney function was not a primary outcome for our study at UAB. Our recipient had already been brain dead for 5 days prior to enrollment in our study and was undergoing the pathophysiologic derangements associated with brain death. It is unlikely that extending the time frame in a preclinical human model of brain death will yield more information on kidney function as brain death physiology worsens with time. It is also important to place kidney function in this model in the context of what is known in human-to-human allotransplantation. Specifically, human recipients of kidneys from human brain-dead donors often experience delayed graft function after transplant, which is characterized by no to minimal urine output or renal clearance necessitating dialysis in the first post-transplant week. Thus, lack of urine output and renal clearance are common early after human-to-human deceased donor kidney transplantation.

RM: After implanting our two kidney xenografts we saw more than a doubling of eGFR but we did not remove the native kidneys.

Q: Dr. Montgomery, Dr. Locke: what boxes still have to be ticked before you move to actual kidney xenotransplantation? Are you preparing to perform a first case?

JL: At UAB, we are in conversation with the FDA regarding an IND (investigational new drug) approval for the 10 GE pig

kidney. Once we have an IND, it will approve us for a phase I clinical trial. We have 10 GE pigs at our pathogen free facility that will be of size between March and June of this year. Our goal is to start our phase I clinical trial during that time frame.

RM: I think through the Maryland heart transplant eIND (emergency IND) process we were able to get a clearer picture of what the FDA is looking for in terms of milestones for them to grant permission for phase I trials. We are working towards these goals.

Q: Dr. Griffith: what follow-up will you require on your first patient before you decide the time has come for a second case?

BG: We are approved for 1 case by the FDA. Revivicor/United Therapeutics plans a formal multi-institutional IND as soon as possible, but expect it may take up to 2 years for FDA approval. We have already had an INTERACT (note: informal non-binding consultation with the Center for Biologics Evaluation and Research at the FDA) pre-IND meeting. I believe several months should pass before we know whether an additional few cases might be indicated. That said, we have already learned a great deal about xenoheart care. Surely, should additional expanded access cases be deemed reasonable, they will add to the knowledge necessary for the best formal IND study.

Q: How would you judge the risk of late rejections, antibody-mediated rejection (AMR) and chronic inflammation in xenotransplantation of the heart and the kidney?

BG: I am comfortable we can deal with AMR but must prevent it rather than have to treat it. The pigs are edited for reduced inflammatory response. Unlike our animals we will be using endomyocardial biopsies, cell-free DNA testing, and allo-mapping, frequent stress echocardiography, and advanced immunosuppressant drug monitoring. We will learn.

JL: These are great questions that will be difficult to answer without moving into living persons. This should be done in the context of a clinical trial.

RM: In addition to the α 1-3 Gal KO, we also used a "thymokidney," of which the Columbia University team has published promising results demonstrating tolerogenic effects of autologous pig thymic transplantation under the capsule of the xenograft. We believe that this innovation will help further protect the pig kidney from late rejections and chronic AMR.

Q: Dr. Reichart: How would you comment on the fact that a clinical xenogeneic heart transplant could be performed, while for the kidney decedent recipient models still had to be used?

BR: I strongly believe that the heart is an easier organ than the kidney for xenotransplantation. There have been consistent observations of prolonged heart transplant survival in pig-to-primate models, including in Munich. This has not been the case for the kidney, although I am admirative of Dr. J. Markmann's recent reports with kidney xenotransplants (4). The reason is probably that the kidney is a much more complex organ than the heart from a purely functional standpoint. David Cooper would disagree with me and is a strong advocate for going to kidney first, notably because of the much lower technical complexity of the procedure (no heart-lung machine necessary). At the end of the day, the reality is that the heart was first!

Q: Dr. Reichart: What made it possible to do this first clinical xenoheart transplant in the United States?

BR: I think the major reason is that the institutional commitment was extremely strong. I think that there was a strong will to be first to do it. This is one reason why they opted for a cloned rather than farmed animal. This has produced a single 10 GE animal, but much faster. This strategy can be applied for a small number of initial cases but is not sustainable. There is a very emotional mentality in the United States, that is not really seen in Europe. Obviously, the money was there, but financial considerations were not the major issue, in contrast to the perspective of a groundbreaking achievement. From a regulatory standpoint, the FDA has been very responsive. The transplant was done on a compassionate protocol, which made it possible without the very stringent rules applied by the FDA for clinical trials.

It is very encouraging to see that worldwide, the reporting by the lay press has been overall very favorable and that the public has received the news with enthusiasm and admiration. There has been very little opposition in the society. In Germany, journalists form major newspapers wrote articles in support of the ethical acceptability of the procedure.

Q: Dr. Reichart: What is your appraisal of the xenokidney experiments and their results?

BR: I wonder how relevant these experiments have been for advancing xenotransplantation of kidneys. In my opinion, it is necessary to first demonstrate consistently predictable long-term success in preclinical studies in non-human primate models before moving to the clinic. The major achievement of these experiments is that a line was crossed with unquestionable acceptance by the society. The ethics of utilizing organs from animal origin should not be underestimated, and earlier attempts at utilizing baboon hearts were not accepted at the time. The population widely accepts to use of pigs, which are obviously an important source of food, as xenogeneic organ donors.

Q: What will happen next?

RM: More focused primate work with the exact pig construct and immunosuppression that the group wants to move forward to phase, I trials and more studies on the recently deceased.

BR: The Maryland case is a great landmark in the history of transplantation, and I have personally congratulated Dr. Griffith for his, and his team's achievement. The move to subsequent cases is potentially tricky. Several heart transplant programs will want to perform porcine heart transplantation, but this will have

to be done with extreme caution. In the year following the Barnard's first heart transplant in 1967, many programs opened throughout the world, with dismal survival results. This led to a near-stopping of this activity that lasted for about a decade.

Regarding xenotransplantation in general, upscaling the procedure will require to move from cloned animals to dedicated breeding farms. This will be extremely costly, since the breeding of these genetically modified pigs will require pathogen-free facilities with high standards defined by regulatory agencies: microbiological filters, showers, masks and clean room gowning, autoclaved food,. . . The number of facilities will be naturally limited. I envision that there will be no more than 1–2 facilities per continental region (North America, Europe,. . .), working in close interaction with their respective regulatory agencies (FDA, EMA,. . .).

AUTHOR CONTRIBUTIONS

The interview was conducted by Thierry Berney, editor-in-chief, and Maarten Naesens, and Stefan Schneeberger, deputy editors-in-chief, on behalf of Transplant International editorial board.

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FORUM
published: 17 March 2022
doi: 10.3389/ti.2022.10262

Reply To—Gender Distribution Among Transplant Journal Editorial Members

Deborah Verran[1]*, *Annemarie Weissenbacher*[2], *David Paredes-Zapata*[3] and *Fernanda Ortiz*[4]

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Keywords: gender, equity, transplant, journals, editors, data

A Forum discussing:

Gender Distribution Among Transplant Journal Editorial Members: A Call to Empower Women in Academic Medicine
by Lim, WH, Quek, J, Tay, PWL, Ng, CH, Vathsala, A, and Muthiah, MD. (2021) Transpl Int 34(11): 2897–8. doi:10.1111/TRI.14117

Dear Editors,

We read with interest the recent publication in Transplant International by Lim et al. (1) on the gender ratios of professionals on the editorial boards of transplant scientific journals. The authors provide summary data for the ratios of men versus women for the editorial boards plus associate editors positions of 29 journals sourced via Scimago. They demonstrated that there is a disparity in the gender ratios for these particular positions. No other specific information is provided about these particular journals bar one.

This is an important issue because it has become evident that there is a degree of gender disparity for all of the listed editorial-type positions across a range of top medical and surgical journals published around the world (2–4).

We wonder whether Lim et al may have analysed website information for both cellular and solid organ transplantation journals (1). By examining the websites of the 45 "transplant" journals currently listed via Scimago, we discovered that 10 were no longer being published, 6 were not published in English, 4 had a focus on cellular therapies only, 2 were directed at a non-medical audience and 1 had the Chief Editor only listed. This left us with 22 potential solid organ transplant journals in comparison to the 29 found by Lim et al. This raises the question of whether there has been some inadvertent introduction of additional variance into their results due to a lack of uniformity of the journals that they selected.

It is now known that there is a reasonable amount of variation between medical and surgical journals as to how many females are either associate editors or chief editors, which ranges in reports from 0 to 82% (2–6), with concern being expressed over the lack of gender equity for the top tier positions. With the summary data as reported by Lim et al being broken down into quartiles this does not allow for any further understanding to be gained by the reader as to where the variance exactly lies between all of the transplant journals for the full range of listed editorial positions.

A preliminary analysis of the Chief Editors of the 22 journals we located revealed that 4/22 (18%) are female compared to 32.3% of the associate editors of the 29 journals obtained by Lim et al. There is also a range of second tier editorial positions listed for transplant journals including Deputy editors, associate editors, editors and scientific editors, which are potentially the pool of individuals

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Received: *24 November 2021*
Accepted: *18 January 2022*
Published: *17 March 2022*

Citation:
Verran D, Weissenbacher A, Paredes-Zapata D and Ortiz F (2022) Reply To—Gender Distribution Among Transplant Journal Editorial Members. Transpl Int 35:10262. doi: 10.3389/ti.2022.10262

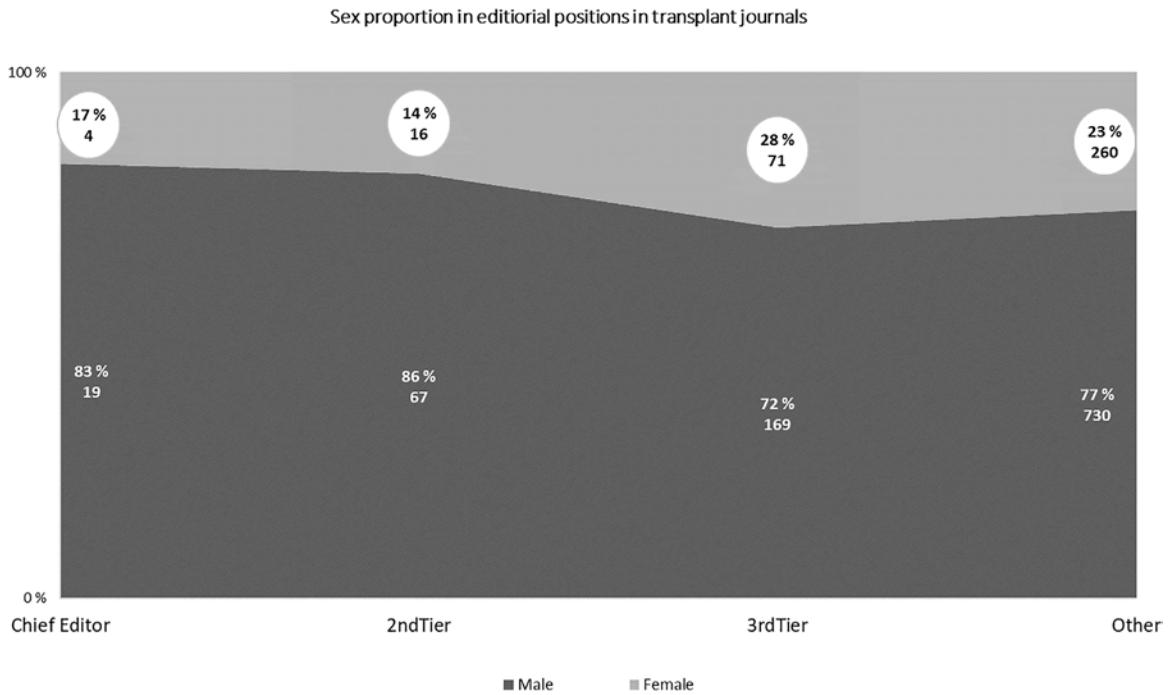

FIGURE 1 | Number and percentage of female representation in 22 transplant journals (Transplantation, Am J Transpl, Neph Dial Transpl, Liver Transplantation, JHLT, Trans Proc, Transpl Int, Clinical Transpl, Paediatric Transpl, Transpl Infect Disease, Transpl Immunology, Xenotransplantation, Current Opin Organ Transpl, Transplantation Reviews, Annals of Transpl, Saudi J Kid Disease Transpl, Experimental Clin Transpl, Transpl Direct, Transpl Research Risk Management, Turkish Nephrology Dialysis Transpl J, Transplantation Reports, Cell and organ Transplantology).

all designated as associate editors by Lim et al. The discrimination of the gender distribution in a full range of editorial positions we identified is shown in **Figure 1**. Noteworthy, 7 out of 22 journals (32%) do not have women in the top tier positions along with the 1 journal with all-men in the editorial board.

Nevertheless, we agree with Lim et al. that there are discrepancies in the gender ratios which have implications for corrective actions (7), noting that some transplant journals are already adopting specific measures (8).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DV conceived the response and undertook the initial data analysis followed by assisting with drafting of the manuscipt. AW assisted with interpreting the data and drafting of the manuscript. DP-Z provided critical insights with respect to the drafting of the manuscript. FO assisted with interpreting the data, assisted with data visualization as well as drafting 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.

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FORUM
published: 17 March 2022
doi: 10.3389/ti.2022.10299

Classifying Polyomavirus Nephropathy: The "Banff" Initiative

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Keywords: polyomavirus, PyVN, banff, validation, classification, biopsy, renal transplantation, outcome

A Forum discussing:

Assessment of the Banff Working Group classification of definitive BK polyomavirus nephropathy
by Kowalewska J, El Moudden I, Perkowska-Ptasinska A, Kapp ME, Fogo AB, Lin MY, et al. (2021) Transpl Int 34(11):2286–96. doi:10.1111/tri.14003

Dear Editors,

All classification systems, especially if newly designed such as the Banff classification of polyomavirus nephropathy (PyVN) (1–3), have to be further validated. In this context, we read, with great interest, the article by Kowalewska et al. "*Assessment of the Banff Working Group classification of definitive BK polyomavirus nephropathy*" in the November 2021 issue of Transplant International (4). We are encouraged to learn about their findings confirming aspects of the Banff 3-tier polyomavirus nephropathy classification system. We are also not too surprised to read about some differences.

Based on statistical analysis, the Banff working group on polyomavirus nephropathy (here referred to as "Banff") has identified two histologic variables, the ci and pvl scores as predictors of renal function (2, 3); those are used in the Banff system to define polyomavirus nephropathy disease classes (1). Kowalewska et al. reported similar observations (4). They also noted a significantly earlier diagnosis of PyVN in classes 1 and 2 compared to class 3. Post diagnosis all studies observed progressive deterioration of renal function in all PyVN classes, most pronounced in disease class 3. Both Banff studies (2, 3) and Kowalewska's report (4) showed patients in disease class 3 with protracted viral resolution. Vice versa PyVN patients with disease resolution were more often found in disease classes 1 and 2. Interestingly, "Banff" (2) reported that early disease resolution indicated improved overall graft function and survival with most pronounced effects seen in class 2. Early clearance in class 2 (seen in 35% of cases) resulted in good outcome like class 1 and vice versa no clearance (in 65%) in inferior outcome like class 3 (2). Since Kowalewska et al. presumably were only able to collect a single serum creatinine data point post index biopsy at the 24-month mark, in contrast to the "Banff" reports with data collection at 1, 3, 6, 12, and 24 months, study results may not be fully comparable. However, there is general agreement among the studies that the detection of a lower PyVN class, often diagnosed early after transplantation, predicts good allograft function. In addition, early/efficient viral clearance and disease resolution are factors preserving graft integrity and stable S-Cr levels.

In order to assess the impact of a PyVN diagnosis on allograft function at time of the initial index biopsy, "Banff" compared the lowest S-Cr level before diagnosis (= best preceding baseline S-Cr) with the highest one at time of index biopsy/diagnosis, i.e. the maximum delta-change. Using this approach, "Banff" noted significant differences in function at time of diagnosis that were most pronounced in class 3.

Forum

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Received: *12 December 2021*
Accepted: *07 January 2022*
Published: *17 March 2022*

Citation:
Nickeleit V, Singh HK, Davis VG and Seshan SV (2022) Classifying Polyomavirus Nephropathy: The "Banff" Initiative. Transpl Int 35:10299. doi: 10.3389/ti.2022.10299

Kowalewka's study design appears to have been less rigorous, presumably explaining the reported differences.

Differences between the studies were also seen in the graft failure rates that may most easily be explained by the applied definitions of "failure." An additional aspect to consider in this context is the improved graft survival rate in PyVN observed over the last decade. In a PyVN patient cohort transplanted between 1996 and 2008 the overall graft failure rate within 24 months was 30% (3), compared to only 8% in a cohort transplanted post 2008 (2). A more favorable graft survival rate was also noted by Kowalewska et al. in their cohort of more recent kidney transplants with PyVN. Thus, in contrast to original studies presumably more reflective of the natural PyVN disease course (3), adaptations in patient management, such as regular screening of BK-DNAemia by PCR and early pre-emptive lowering of baseline immunosuppression (5, 6) have resulted in improved graft survival. Consequently, and not surprisingly the predictive power of PyVN disease classes to mark graft loss in current patient (2) compared to historic patient cohorts (3) is limited. "Not much failure can be predicted if loss is minor." Very similar observations can be made with other disease entities, such as Banff type I rejection, where changes in patient management have resulted over time in improved clinical presentation and outcome.

We are surprised to learn about Kowalewska's findings on BK-DNAemia in the PyVN classes. In both "Banff" studies different histologic viral load levels in class 1 (pvl-score: 1) versus class 3 (pvl-score: 3) resulted not surprisingly in significant differences in BK-DNAemia levels. Spearman's rho, correlating histologic intra renal viral load levels, i.e. Banff pvl-scores, and BK-DNAemia is between 0.35 and 0.48 (7, 8). Thus, differences in BK-DNAemia between PyVN classes 1 and 3 are expected. Why Kowalewska et al. found very similar PCR reads in those PyVN classes in their study is undetermined; possibly differences in PCR test methodologies among centers are a reason (9, 10).

Any validation study faces challenges. Concurrent renal diseases, with rejection being one example, variations in inter observer lesion scoring, differences in PCR methodologies, or differences in study design can all influence data analysis and interpretation. We assume that Kowalewska et al., similar to "Banff," exclusively used the time of the initial/first PyVN biopsy diagnosis as the primary reference point. We also assume that all cases of active and chronic rejection were excluded (although descriptions in their paragraph "characteristics of PyVAN classes" with "v," "g," and "cg" lesion scores render this assumption less clear). We also assume that the Banff ci-score/degree of interstitial fibrosis was evaluated in trichrome stains.

Kowalewska et al. conclude that PyVN ". . ..classes do not correlate with the previously identified prognostic indicators such as interstitial inflammation or viral load." Indeed, the "Banff" studies were not designed to confirm previous reports, but rather to propose a statistically based histologic PyVN classification system. By explicitly excluding cases of concurrent rejection and graft injury unrelated to PyVN, in depth statistical analyses did not reveal a significant association between interstitial inflammation and outcome. This "Banff" approach excluding confounding diseases differs significantly from other reports (11, 12). BK-DNAemia levels assessed by PCR allow for (diagnostic) risk stratification, i.e. low risk/high risk/presumptive PyVN. However, PCR test methodologies and results vary considerably, and the prognostic predictive value of BK-DNAemia levels is very limited (7, 8, 13). This notion was also confirmed in statistical analyses by "Banff" (see supplemental data (3)).

PyVN is a complication post kidney transplantation with major effects on allograft function. The "Banff" disease classes provide prognostic information. As Kowalewska et al. pointed out, their study approach mimicking ". . .the day-to-day practice" of pathology, is a very valuable contribution confirming some key findings of "Banff." This day-to-day approach also illustrates that certain disease specific aspects are only uncovered in more rigorous studies. Thus, we interpret Kowalewka's paper (4) as complementary to the Banff working group studies and report (1–3).

AUTHOR CONTRIBUTIONS

All authors (VN, HS, VD, SS) contributed equally to the preparation and editing of this letter to the editors. The final version was unanimously approved by all authors, There are no conflicts of interest to report.

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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POINT OF VIEW
published: 21 March 2022
doi: 10.3389/ti.2022.10131

Donor Autonomy and Self-Sacrifice in Living Organ Donation: An Ethical Legal and Psychological Aspects of Transplantation (ELPAT) View

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Clinical teams understandably wish to minimise risks to living kidney donors undergoing surgery, but are often faced with uncertainty about the extent of risk, or donors who wish to proceed despite those risks. Here we explore how these difficult decisions may be approached and consider the conflicts between autonomy and paternalism, the place of self-sacrifice and consideration of risks and benefits. Donor autonomy should be considered as in the context of the depth and strength of feeling, understanding risk and competing influences. Discussion of risks could be improved by using absolute risk, supra-regional MDMs and including the risks to the clinical team as well as the donor. The psychological effects on the donor of poor outcomes for the untransplanted recipient should also be taken into account. There is a lack of detailed data on the risks to the donor who has significant co-morbidities.

Keywords: risk, kidney, transplantation, living donation, autonomy 2

Point of View

OPEN ACCESS

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Received: *21 October 2021*
Accepted: *02 February 2022*
Published: *21 March 2022*

Citation:
Mamode N, Van Assche K, Burnapp L, Courtney A, van Dellen D, Houthoff M, Maple H, Moorlock G, Dor FJF and Lennerling A (2022) Donor Autonomy and Self-Sacrifice in Living Organ Donation: An Ethical Legal and Psychological Aspects of Transplantation (ELPAT) View. Transpl Int 35:10131. doi: 10.3389/ti.2022.10131

INTRODUCTION

The donation of a solid organ for transplantation by a person who is alive at the time represents a unique event in healthcare, since the donor will gain no physical benefit from undergoing major surgery, which has a low but nevertheless significant rate of major complications and death (1, 2). Living donors are usually highly motivated individuals, whose appetite for risk differs substantially from that of the healthcare team (3). This may lead to conflicts between the clinical team and potential donors-some examples are given in **Figure 1**. Were the decisions of the clinical teams correct? This article explores the issues raised by these cases and others, and considers the principles

Abbreviations: ELPAT, ethical legal and psychological aspects of transplantation; MDM, multidisciplinary meeting; ESRD, end stage renal disease; LDN, living donor nephrectomy.

Case 1: A 71-year-old man wished to donate a kidney to a young boy with whom he had no emotional or genetic connection. He had the same surname as the child, and having become aware of his need for a transplant felt that this similarity was an indication to donate. His prior history of heart disease, along with his age, led the clinical team to decline him as a donor.	Case 2: A 40-year-old man wished to donate a kidney to his wife, and is found to have Type II diabetes during his work-up. On this basis he was declined as a donor. 14 months later, he re-presents, having lost 10kg and followed a recommended diet. His glycaemic control is excellent (HbA1c is 5.8%), and he is adamant, despite attempts to persuade him otherwise, that he wishes to donate as he cannot bear to see his wife suffering. His family support him, the hospital ethics committee find his decision to be freely made, and psychological evaluation finds a significant psychological benefit from donation. He is permitted to donate.
Case 3 A 75-year-old male retired Professor of Statistics, was declined in two other centres, due to an incidental 5.5cm aortic aneurysm, and wished to donate to his wife. He was adamant about donation, and calculated his absolute versus relative risk, in the context of an expectation to have a couple of good years in good health with his wife off dialysis even if their lifespan would be shortened. He proceeded to donate.	

FIGURE 1 | Examples of potentially difficult decisions regarding living donor candidates.

which might help to guide decision-making. It is an overview aimed at healthcare professionals, and is not intended to be an in-depth ethical review. Suggestions for further reading are given in **Figure 2**.

AUTONOMY VERSUS PATERNALISM

Although not universally adopted, principlism remains the dominant approach to medical ethics (4), particularly amongst the clinically-orientated. Under a principlist approach, four principles are considered in the determination of whether an intervention is ethically appropriate: autonomy, beneficence, non-maleficence, justice (5). Beauchamp and Childress suggest that each principle should be afforded equal weight, but nonetheless autonomy is often regarded as "first amongst equals" (6). In living kidney donation, beneficence is difficult to both specify and quantify accurately. There is likely to be some psychological benefit (7, 8) but there is clearly no physical benefit of donation itself. Whilst non-maleficence, or more specifically the minimisation of harm is a concomitant aim of donation surgery, some harm is unavoidable, such as the physical harm routinely associated with surgery, and sometimes unanticipated complications occur. Although teams attempt to assess the risk to the donor independently, the benefit to the recipient also plays a part (9), since without this the donation would not be justified (**Figure 3**). Some have argued for a "donor-centred" approach, where the importance of the emotional benefits to the donor is expanded when considering risks (10).

The clinical team are also agents here and ultimately responsible for decisions to offer donation as an option to an individual: an on-table death of a donor would certainly affect them profoundly, and potentially their programme and others, and hence other patients. But this could perhaps be overcome by having centralisation of high risk cases in dedicated centres or by having surgeons for "high risk" cases in centres, where everyone understood that the risks were higher and appropriate protections were in place, including transparent audit, support for staff, and avoidance of punitive actions in the event of below average outcomes.

It is quite common for clinical teams to adopt a degree of paternalism (11), whereby autonomy is infringed upon to some extent in order to serve a patient's best interests. Consider, for example, the postoperative patient who would rather not get out of bed, but is essentially cajoled into doing so. In this scenario, it might be considered that the patient's wish to stay in bed is not strongly held, and that it is heavily in their best interests to mobilise, so beneficence overrules respecting the rather weak autonomous wishes of the patient. It might then seem logical that there is a gradation of potential benefits or harms, which could be weighed against a scale of autonomous desires of increasing strength, rather than simple binary outputs for these potentially competing interests. Considering that there may be effectively different levels of autonomy, related to a degree of understanding and strength of feeling, may help here. Similarly, it might be considered that there is a scale of paternalism, ranging from "weak to strong (12)" or "soft to hard (13)." In practical terms, such an interpretation is necessarily a matter of subjective judgement, but a potentially paternalistic approach might include consideration of the following: how strongly do you feel about donating, and

Williams, NJ. On harm thresholds and living organ donation: must the living donor benefit, on balance, from his donation?', Medicine, Health Care and Philosophy 2018 21(1). 11-22.	Discusses whether it is a requirement of ethically acceptable living donation that the donor themselves should receive benefit and argues that it should not be.
Spital, A. Donor benefit is the key to justified living organ donation. Cambridge Quarterly of Healthcare Ethics 2004, *13*(1): 105-109.	Spital is someone who is notable for holding the opposite view to the above: that donors should benefit overall from donation in order for it to be permissible.
Bailey, P & Huxtable, R. When Opportunity Knocks Twice: Dual Living Kidney Donation, Autonomy and the Public Interest. Bioethics 2016 30(2):119-128.	Argues that someone should be permitted to donate both of their kidneys in some situations.
Draper, H, & Moorlock, G. "A Challenge to the Duty to 'First Do No Harm'". In: Hansen SL and Schicktanz S, editors. Ethical Challenges of Organ Transplantation: Current Debates and International Perspectives. Bielefeld: Transcript Verlag (2021) p. 151-166.	Discusses how the notions of harms and benefits have been expanded in living organ donation to include: i) The abstract moral benefit of doing something good ii) The harms of frustrating the wishes of an autonomous individual
Biller-Andorno, N, Agich, GJ, Doepkens, K and Schauenburg, H. Who shall be allowed to give? Living organ donors and the concept of autonomy. Theoretical medicine and bioethics, 2001 *22*(4): 351-368.	Explores the relationship between donor autonomy and broader contextual factors when determining suitability of a living donor.

FIGURE 2 | Suggested further reading.

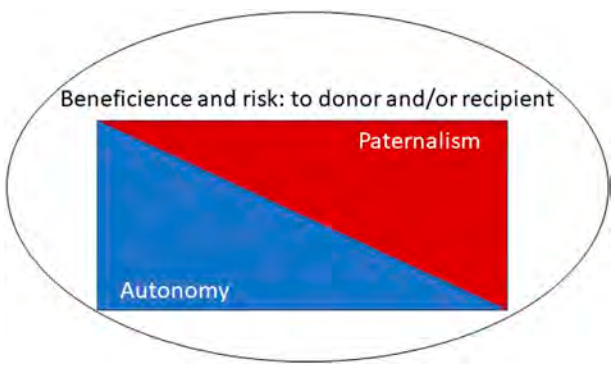

FIGURE 3 | The interplay of potentially conflicting ethical principles.

why? Do you have a reasonable understanding of the risks? How likely are you to regret this later? Despite the difficulty in answering these questions, it might be a first step in resolving the conflicts described above.

A key problem in considering the importance of autonomy in medical decision making is the difficulty in the determination of the value that should be accorded to a particular autonomous wish. That is, at what point does an apparently autonomous decision carry sufficient weight to outweigh other considerations (9). This is a key issue when considering decision making in children, who may not yet be considered independent and adults who are incompetent to make any decision, but whose wishes are nevertheless taken into account. Indeed, children not infrequently express a wish to donate to siblings, but in most jurisdictions this would be refused (14, 15). Perhaps a useful ethical approach

would be to balance the clinical team's view of the potential benefits and harms, with the depth and strength of conviction of the individual concerned. One might consider a central aspect of autonomy to be the ability to use relevant information to reason in certain ways and adopt a considered approach (5). Thus, it might be, for example, that an experienced transplant surgeon with non-insulin dependent diabetes who felt strongly that they wished to donate to their spouse could have a reasonable understanding of the risks, and should be allowed to proceed. In clinical practice, a clear understanding of the risks is often given greater validity in terms of decision making; however, it could be argued that neither depth nor strength of conviction are valid reasons for assessing the degree of autonomy. Furthermore, freedom from external pressures beyond the clinical team, for example from family members, is an important consideration in determination of the extent to which a patient's wishes are truly autonomous.

RISK BENEFIT BALANCE

The risks of donor nephrectomy are mortality 1 in 3,000 and major complications 2–5% (1, 2), while for a living liver donation the mortality rate is 1 in 200 (16). This could mean that a "high risk" kidney donor might still be exposed to less risk than a low risk liver donor. It could be argued that the difference here is the combination of lack of availability of other options and need for urgent surgery in the recipient, since a liver patient might not survive for long without a transplant, while most kidney recipients would have a dialysis option. However, in considering the risk/benefit balance for the donor, the implication must be that the difference is only a psychological one, and not physical-that is, the liver donor has the higher psychological risk of seeing a loved one die, which justifies the higher risk of donation. There can't be any other moral imperative to expose the donor to higher risks because the stakes are higher for the recipient. The logical extension of this argument suggests, however, that outcomes other than death might have a profound psychological detrimental effect on the potential donor-for example, parental donation to a child who is not thriving on dialysis, or spousal donation where the life of the donor is severely impacted by having an unwell partner (17).

One of the common errors in considering the risks of donation is to focus on relative, rather than absolute, risk. The use of absolute risk has been recommended specifically for living donors (18). A mortality rate of 1 in 1,500 is twice the normal risk but still very low, and lower than for the liver donor. Furthermore, we do not have good data on what the actual risks are in those with co-morbidities, in part because they are usually refused surgery (19). For example, previous myocardial infarction is often an exclusion criterion for kidney donors, yet if successful rehabilitation has taken place, risk factors addressed and cardiac tests are adequate, then it probably does not confer a high absolute risk (20, 21). An alternative approach might be to consider what is an acceptable upper mortality rate, and to permit donation if this threshold is not reached, even if the relative risk is doubled. Clearly challenges would remain in determining this rate, and in assessing individual donors who are below this threshold. There is certainly a need to determine more accurately and objectively the risks to both donor and recipient, in order to make the appropriate decision-just as we may not be aware of the real perioperative risk to a donor conferred by a co-morbidity, data on the risk to the recipient of not proceeding with a living donor transplant at that time is often lacking.

It is also important to consider long term as well as perioperative risk. There is even less data here. For example, the lifetime risk of ESRD after LDN in a 70-year-old man is 0.15% (95% CI 0.05, 0.28), and the relative risk for ESRD from non-insulin dependent diabetes is 3.01 (1.91, 4.74)- the absolute risk would appear to be low, but we have no data on the effect of donation on subsequent ESRD in this scenario (22).

Risk aversion may sometimes vary with specialty; surgeons and nephrologists sometimes have differing appetites for risk. Whilst the multidisciplinary meeting (MDM) or protocols and guidelines may mitigate some of these differences, an exploration of how these operate in practice, and the underlying thought processes could help in smoothing decisions. An emerging literature on cognitive biases and loss aversion, where the fear of a low probability but high loss outcome tends to outweigh potential gains, in decision making indicates an interesting start (23, 24).

Finally, risks apply not only to the potential donor, but to the operating surgeon, the clinical team, and to a national programme, since donor deaths have typically impacted on all of these. One way to mitigate this might be to take national decisions on high-risk cases, in a sense as a supra-regional MDM, which would in part shift some of the risk away for the local team in the same way that local MDM advice shares the risk beyond the operating surgeon. Equity of access is an important principle to consider, since widely differing views may pertain in different centres (18). It is also important to consider the risk to the recipient-a donor who suffers severe complications may lead to considerable distress for the recipient.

SELF-SACRIFICE AND HEROISM

We applaud self-sacrifice in many walks of life-firefighters, military, even sport, such as Formula 1, mountaineering, round the world sailing. Those who take risks to save others, or for glory or money, are often considered heroes. Why is someone who takes a risk as a donor different?

It might be argued that the difference is that they need a clinical team to facilitate their operation- but then many of the others listed above need support from teams. Arguably in these cases there is oversight of risk by another group. For example, a military unit might be ordered to retreat if the risk is too high, or the race director may stop a Grand Prix if rain makes it unsafe. It could be considered that the MDM in each unit provides a similar oversight, but given the potential risks to individual clinicians, and to programmes, of poor

Case1	Case 2
A 45-year-old man suffering from multiple sclerosis wished to end his life, due to unbearable physical suffering. He approached a clinical team asking to donate his organs as part of a procedure which would result in euthanasia. He was declined on the basis that this form of euthanasia is not permitted.	A 40-year-old woman with severe motor neurone disease, who had campaigned for euthanasia and the right to end her own life made an enquiry to NHS Blood and Transplant to request living kidney donation prior to referring herself to DIGNITAS. Her husband was supportive. She had a permanent urinary catheter (supra-pubic) and was immobile in a wheelchair, and was deemed to have full mental capacity. The case was discussed in the multidisciplinary meeting There were surgical reservations due to her immobility, anaesthetic risk, positioning on the table, risk of venous thrombosis during surgery, immediate and recovery at home More significant reservations were about her decision-making related to her perception of risk as a living donor i.e. if her plan was to end her life in any case, she would not have the usual 'stops' in her decision-making in relation to risk of death or complications from the surgery. (Equally, life-long risk would be less of a consideration for her or the team). The final decision was to not accept her self-referral for living kidney donation.
Case 3 A 45-year-old man with Huntington's disease underwent unspecified donation of a kidney. Later in life he became more unwell, and underwent euthanasia followed by retrieval of the remaining kidney as well as other organs [20]	

FIGURE 4 | Examples of living donor candidates in the context of euthanasia.

outcomes as mentioned above it might be that we are not independent enough. The wide variability in assessment criteria illustrates the difficulty here (19, 25). Nevertheless, if the local clinical team is reluctant to proceed, there is an argument for a second opinion, or for national or regional bodies to make these assessments.

EXTREME RISKS

Some potential donors might have a limited life expectancy, for example Huntington's chorea, or a reduced capacity due to illness, for example, early dementia, but still wish to donate. In these cases, it might be argued that if the organ is unaffected by the underlying medical condition, donation does not hasten death, and there is sufficient capacity to make the decision, it would be reasonable to proceed (25). However other donors might wish to take more extreme risks-for example, donating their heart and thus ending their life (26–28). Similarly, there are those who are undergoing euthanasia (28), and wish to donate as part of that process, as detailed in **Figure 4**. In this case, the acceptance of such a donor would potentially help a number of recipients to have a better quality and quantity of life. However, apart from the fact that it is not permitted, such a procedure might have very negative consequences on wider donation rates, as the perception could be that life may be ended specifically to provide organs-a concern that has been expressed in general by some who are reluctant to agree to deceased donation. The principle that individuals are entitled to decide how and when they will die has been established in some countries (Switzerland), but some may struggle with the idea that doctors should participate in organ donation which might either precipitate death or be part of the final interventions.

CONCLUSION

Decision making in the case of living donation remains difficult. There is a lack of detailed objective data regarding the risks in donors with co-morbidities, and the impact on the recipient of not proceeding. There are a number of potentially competing interests, including donor autonomy, the effect on the clinical team and wider societal effects on donation rates. One solution would be

to introduce oversight removed from the clinical centre, or to designate some centres as those for "high risk" donors. Consideration of the understanding of risk by the donor may also help guide decisions. This manuscript provides an overview of the relevant issues for a clinical audience, and does not attempt a detailed ethical analysis, which is available in the bioethical literature; we have suggested further reading in **Figure 2**.

AUTHOR CONTRIBUTIONS

NM wrote the manuscript. AL and FD coordinated the group and supervised. All other authors contributed to the discussions and writing 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.

ACKNOWLEDGMENTS

We acknowledge the support of the European Society of Transplantation for ELPAT. The ELPAT working group on Living Organ Donation greatly acknowledge Maria Valentin for her contribution to the manuscript during the time she has been involved in the writing process.

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POINT OF VIEW
published: 24 March 2022
doi: 10.3389/ti.2022.10257

Hypothermic Machine Perfusion in Liver Transplantation—A Randomised Trial and Beyond

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Keywords: liver transplantation, machine perfusion, hypothermic perfusion, oxygen, DCD

The randomised controlled trial of oxygenated hypothermic machine perfusion of DCD livers, reported by van Rijn et al. in NEJM in February 2021 is of great importance, not only because of the novel technology under investigation, but also because of the trial design and methodology.

After several decades in which static cold storage (SCS) has been the organ preservation methodology of choice, there is now great interest in the development of machine perfusion systems of abdominal and cardiothoracic organs. Whereas previously this was the preserve of a number of relatively simple hypothermic machine perfusion (HMP) systems for the kidney, clinical units around the world are now using/testing, perfusion systems under both cold (hypothermic) and warm (normothermic) conditions. The relatively recent demonstration of the benefits of adding oxygen to the perfusate under hypothermic conditions (1, 2) has increased the interest and application in this approach.

The publication of van Rijn et al., from the Groningen group of Robert Porte (3), addresses a vitally important question in liver transplantation: whether machine perfusion technology is of benefit with respect to the biliary complications seen in DCD transplantation. Donation after circulatory death is increasing in many countries and is now a major source of donor organs (e.g., up to 40% of all deceased donors in the United Kingdom), but utilisation of livers from DCD donors (the proportion of offered organs that result in a transplanted liver) is of the order of 25% both in Europe and the US. For this reason, the impact of DCD transplantation has been much lower in liver transplantation than in kidney transplantation. The reason for this low level of uptake is not hard to understand: not only do DCD liver transplants have a much higher rate of primary non-function and Early Allograft Dysfunction, but also there is a greatly increased risk of non-anastomotic biliary strictures (NAS), also referred to as ischaemic-type biliary lesions, and as ischaemic cholangiopathy (4). Although this complication is reported in DBD liver transplants, it is so much more commonly seen in DCD grafts as to be pathognomonic. This is the primary reason for the inferior outcomes (and higher costs) associated with DCD liver transplantation.

Within the modern era, hypothermic machine perfusion of the liver was first shown to be feasible and safe by Guarrera et al. (5), adopting relatively simple HMP technology as used in kidney transplantation. The addition of oxygenation of the circuit, pioneered by the groups at Zurich (1) and Groningen (6), later provided evidence that dissolved oxygen in an HMP circuit, even when applied for only a short period after SCS, may be associated with reduced levels of ischaemia-reperfusion injury. More is known now about the potential mechanism of benefit, much centred on the role of oxygen in maintaining aerobic mitochondrial metabolism, thereby avoiding the accumulation of succinate and subsequent release of reactive oxygen species (even at low temperature) (7). The preliminary evidence generated by the trials of hypothermic oxygenated machine perfusion (HOPE) and dual hypothermic oxygenated machine perfusion (D-HOPE) have suggested that this mitigation of ischaemia-reperfusion would translate into

Point of View

OPEN ACCESS

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Received: *21 November 2021*
Accepted: *27 January 2022*
Published: *24 March 2022*

Citation:
Friend P and Pollok J-M (2022) Hypothermic Machine Perfusion in Liver Transplantation—A Randomised Trial and Beyond. Transpl Int 35:10257. doi: 10.3389/ti.2022.10257

reduced levels of graft injury and, in particular, a reduction in the all-important syndrome of NAS. It is this hypothesis that van Rijn et al. set out to test.

The primary endpoint of this trial was the incidence of symptomatic NAS. Much has been written in recent years about endpoints for trials in organ preservation, because trials have relied upon surrogate endpoints (e.g., EAD, or peak postoperative transaminase) rather than measures of direct clinical relevance (e.g., graft survival). This is in order to design clinical trials of feasible proportion: indeed, to illustrate this, it has been pointed out that a preservation trial based upon liver graft survival would typically require in excess of 4,000 patients (8). However, the relatively high incidence of NAS in DCD liver transplants renders this complication a suitable endpoint within a trial of manageable proportions. van Rijn et al. measured, therefore, a directly clinically-relevant metric: the use of NAS as the primary endpoint in this trial is an enlightened choice.

In this trial, livers were randomised once deemed transplantable by the implanting surgical team: the trial was not, therefore, designed to test any effect on organ utilisation (a separate key issue in liver transplantation). Indeed, there was a very low discard rate of organs: only three livers were discarded as unsuitable for transplantation, these due to steatosis ($n = 2$) and retrieval damage ($n = 1$).

Similarly, the investigators did not set out to test the effect of HMP at (or beyond) the limits of current DCD practice: donors were relatively young (median age 52 years and 49 years in HMP and SCS respectively), non-obese (BMI 25 kg/m^2 in both groups) and the warm ischaemia time were relatively short (median 11 min in both groups). Also the recipients were low-risk (MELD 14 and 16 in HMP and SCS groups respectively). Donor and patient selection was, therefore, well within the accepted range for DCD liver transplantation, and the groups were well-matched.

Diagnosis of the primary endpoint was clinical, based on the development of jaundice or cholestatic liver function tests. The diagnosis was confirmed in all cases by later MRCP. Indeed all patients underwent MRCP at 6 months postoperatively, as part of the trial protocol. This allowed not only corroboration of the clinical findings, but also objective assessment of the effect of the intervention (oxygenated HMP) with respect to biliary stricture formation. All scans were reviewed by two independent radiologists who were unaware of the treatment allocation, with a third radiologist providing the casting vote in the event of discordant opinions. The study was powered on the basis of a reduction of the NAS rate from 29% (a notably high rate in comparison to published rates) to 11%: the results of the trial showed a reduction from 18% to 6%.

The results of the protocol MRCP investigation are of some interest, because this is potentially an important endpoint in trials of future interventions in DCD liver transplantation. Here, the evidence is less clear-cut: not only did all the symptomatic patients have radiological evidence of NAS (as expected), but 70% of all MRCPs were positive, including 65% of asymptomatic patients. Including all scans graded as showing mild, moderate or severe strictures, there were no differences in the incidence or severity of radiological cholangiopathy between the two arms of the trial. As noted by the authors, this dissonance between clinical and radiological manifestations of biliary pathology is unexplained and requires more research.

Protocol MRCP assessment at 6 months postoperatively was also included in the previously-published normothermic machine perfusion trial, carried out by the Consortium for Organ Preservation in Europe (COPE): 70% of 222 transplanted patients underwent protocol MRCP. In this randomised controlled trial (9), patients receiving DCD organs comprised only a minority of the recruitment in a study with wider enrolment criteria: the incidence of radiologically-determined biliary strictures in DCD recipients was 11.1% in NMP livers (3 out of 27), compared to 26.3% in SCS livers (5 out of 19). Notably (and in line with the findings of the van Rijn paper), only two patients (one in each arm of the trial) underwent retransplantation as a result of NAS within 1 year of the initial transplant.

Other benefits were shown in the van Rijn study: these include clinically important reductions in the rate of post-reperfusion syndrome (12% vs. 27%), EAD (26% vs. 40%), the requirement for biliary interventions (5 vs. 22), and the need for readmission (6 vs. 17). These findings are all indicative of an improved preservation technology that has had the effect of reducing the severity of ischaemia-reperfusion injury. There is no doubt that such benefits are needed, especially in the context of DCD liver transplantation, in which the risk/fear of complications is responsible for organ utilisation rates of the order of 25%. However, NAS is not the only driver of poor utilisation: there are other facets of organ preservation that need improvement if optimum utilisation of the critical resource of donor organs is to be achieved.

There is little doubt that the utilisation of marginal donor organs (both DCD and DBD) is improved by the ability to assess the functional viability of the organ before deciding whether to subject a patient to the risk of transplantation. This can be achieved at normothermic temperature, potentially allowing organs that would otherwise be discarded to be transplanted: indeed a proof-of-principle study has already tested the clinical implementation of this and been published (10). Normothermic machine perfusion is intrinsically superior as a means of testing the donor organ, compared to hypothermic perfusion. As noted by van Rijn et al., there is current interest in the measurement of mitochondrial flavin mononucleotide (as a mitochondrial injury marker) during HMP, but it is not yet clear to what extent this predicts longer-term outcome (11). However, functional assessment by this or other means was not part of the study as conducted.

A further and hitherto unmet need is that of extended preservation. No real progress has been made in static cold preservation since the introduction of University of Wisconsin solution three decades ago: indeed, with more transplants of higher risk organs, average preservation times are shorter now

than in the past. There is no published evidence of the utility of extending the period of safe preservation using hypothermic machine perfusion of liver grafts. Investigation of this is much needed in order to assess whether this technology is a potential solution to the very real logistic challenges of running a liver transplant programme, in which offers of donor organs may come in rapid succession, but where only one transplant can be undertaken at a time. Normothermic preservation has been shown to enable prolonged preservation times, not only allowing sequential transplantation, but also offering the real prospect of scheduling liver transplants during the day (12).

Another machine perfusion technology which is showing great promise in the context of DCD liver transplantation is that of normothermic regional perfusion (NRP)—the re-institution of oxygenated blood flow to the abdominal organs *in-situ* following the declaration of death. Although this has not been subjected to the level of randomised clinical trial analysis conducted by van Rijn et al, nonetheless accumulating evidence suggests a substantial benefit from this peri-retrieval intervention. In a publication from the United Kingdom (13), 43 livers were transplanted after NRP with no occurrence of NAS, compared with 27% in 187 DCD livers transplanted contemporaneously without the use of NRP. Notably, however, the logistics of NRP are complex, requiring additional technology and skilled personnel at the donor site, this contrasting with the much simpler logistic demands of HMP. A trial comparing HMP, NMP, and NRP in the management of DCD livers is much needed.

After several decades of relative stagnation, the field of transplant organ preservation is undergoing a renaissance, with the implementation of machine perfusion systems. Although it is easy to characterise the current state of the art as a debate about hypothermic vs. normothermic perfusion, it is likely that future implementations will exploit temperature not as a binary but as a continuous variable parameter with temperature transition being seen as a key issue. Also, not only will organs be thereby preserved in better condition and for longer, but specific targeted interventions will be applied to repair and modify organs to the benefit of post-transplant outcomes. The delivery of oxygen at cold temperatures is just a first step into this exciting future.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

PF is co-founder and Chief Medical Officer of OrganOx Ltd, a company established to develop machine perfusion technology in Transplantation.

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

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ESOT | Transplant International

POINT OF VIEW
published: 28 March 2022
doi: 10.3389/ti.2022.10302

Impact of COVID-19 on Global Kidney Transplantation Service Delivery: Interim Report

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This article gives a personal, historical, account of the impact of the COVID-19 pandemic on transplantation services. The content is based on discussions held at two webinars in November 2020, at which kidney transplantation experts from prestigious institutions in Europe and the United States reflected on how the pandemic affected working practices. The group discussed adaptations to clinical care (i.e., ceasing, maintaining and re-starting kidney transplantations, and cytomegalovirus infection management) across the early course of the pandemic. Discussants were re-contacted in October 2021 and asked to comment on how transplantation services had evolved, given the widespread access to COVID-19 testing and the roll-out of vaccination and booster programs. By October 2021, near-normal life and service delivery was resuming, despite substantial ongoing cases of COVID-19 infection. However, transplant recipients remained at heightened risk of COVID-19 infection despite vaccination, given their limited response to mRNA vaccines and booster dosing: further risk-reduction strategies required exploration. This article provides a contemporaneous account of these different phases of the pandemic from the transplant clinician's perspective, and provides constructive suggestions for clinical practice and research.

Keywords: kidney transplantation, COVID-19 pandemic, cytomegalovirus management, service delivery, historical record, 2020

Point of View

OPEN ACCESS

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***Received:** 13 December 2021*
***Accepted:** 23 February 2022*
***Published:** 28 March 2022*

Citation:
Papalois V, Kotton CN, Budde K, Torre-Cisneros J, Croce D, Halleck F, Roze S and Grossi P (2022) Impact of COVID-19 on Global Kidney Transplantation Service Delivery: Interim Report. Transpl Int 35:10302. doi: 10.3389/ti.2022.10302

INTRODUCTION

All aspects of health care are affected by the COVID-19 pandemic (1–3). In November 2020, kidney transplantation experts from Europe and the US attended two webinars to describe the challenges of COVID-19 and measures taken to maintain kidney transplantations during the pandemic. Discussants were re-contacted in late 2021, to reflect on the continuing situation. By then, vaccination, booster and antibody testing programs were widespread, despite substantial ongoing cases of COVID-19 infection.

Abbreviations: CMV, cytomegalovirus; HDU, high dependency unit; ICU, intensive care unit.

TABLE 1 | Kidney transplantation rates in the US and Europe, 2014–2021 (4, 5).

	2021	2020	2019	2018	2017	2016	2015	2014
Deceased Donor, United States	13,214	11,925	11,152	9,867	9,401	9,116	8,250	7,763
Living Donor, United States	5,970	5,235	6,866	6,443	5,811	5,629	5,628	5,538
Deceased Donor, Eurotransplant[a]	2,933	2,831	3,161	3,480	3,093	3,278	3,424	3,348
Living Donor, Eurotransplant[a]	1,069	942	1,183	1,328	1,294	1,338	1,323	1,348

[a]*Eurotransplant countries: Austria, Belgium, Croatia, Germany, Hungary, Luxembourg, Netherlands, Slovenia.*

KIDNEY TRANSPLANTATION RATES

In November 2020, kidney transplantation rates were generally near normal [**Table 1** (4, 5)] after many institutions had reduced–or suspended–procedures (1, 2). Discussants estimated that, globally, kidney transplantation rates would be ~20% lower in 2020 than expected, but individuals held varying views.

At Charité (the largest transplant center in Germany), living-donor programs were suspended briefly during the first wave of the pandemic. Although the deceased-donor program continued, 'high-risk' transplantations (i.e., extended-criteria donors, recipients with comorbidities, or donor–recipient partnerships exhibiting immunological incompatibilities) were not performed. In Italy, 2020 transplantation rates were predicted to match previous years, but with fewer procedures performed in the north (where the pandemic had greatest impact) and more in the south. In the US, living-donor programs paused (some for several months): kidney transplant volume was at 97.5% in 2020 compared with 2019, with an increase in deceased donors but fewer living donors (1, 2, 6). In the UK, all five London centers stopped kidney transplantations for months, although some regional centers continued operating (7). European participants reported no changes in recipient-selection protocols.

By November 2020, although all transplantations were resuming, there were backlogs of non-urgent cases, with many patients presenting late or with complex needs. To improve efficiencies, innovative care-delivery practices were being trialled, including new enhanced recovery pathways (reducing the hospital length of stay post-transplantation to ~3 days, with remotely delivered aftercare (8). Discussants commented that such practices could become standard of care, if safety, financial efficiencies, and positive patient experiences were objectively demonstrated.

TELEMEDICINE

Early phases of the pandemic saw strong uptake (and acceptance) of telemedicine (9–11). Some discussants spoke of immediate efficiencies when consultations moved to virtual platforms, including reduced travel burden for staff and patients. Despite rapid implementation, telemedicine was well received, perhaps unexpectedly. However, virtual consultations could be technologically challenging, and therefore time consuming. Disparities in access to care were also evident: some patients did not own suitable devices or could not easily express themselves. Difficulties in maintaining patient confidentiality were mentioned, because clinicians could not influence patients' locations for virtual appointments. Such consultations were therefore considered useful by the group, but were not expected to be adopted permanently. Finally, several discussants had difficulties obtaining reimbursement for telemedicine services.

Nevertheless, the value of telemedicine in the work-up and monitoring of kidney transplant recipients was noted. For example, at Charité, if recently discharged patients did not input daily blood pressure and temperature data they were automatically contacted; the clinical team could intervene, if necessary (9).

Although the pandemic created uncertainty for transplant patients, general advice about COVID-19 exposure, shielding, or procedural delays could be provided efficiently online [e.g., Massachusetts General Hospital (United States), videos: https://www.youtube.com/watch?v=hbrFLzbVFTA; https://www.youtube.com/watch?v=RP7clFVsYsk) (12)].

HOSPITAL CAPACITY

Even countries modestly affected by COVID-19 in 2020 had to consolidate health care. The pandemic overwhelmed intensive care unit (ICU)/high dependency unit (HDU) services in some regions such that other urgent-care capacity reduced by 50%. In the UK and elsewhere, centers that stopped transplantations redeployed clinical staff to other acute services. Discussants hoped that complete shutdowns would be avoided in future because reactivating departments (and associated research) was extremely challenging.

The pandemic disrupted the close collaboration that often develops between specialist transplant centers and individual patients. To comply with stay-in-place directives, many patients could only receive in-person care from local 'general' hospitals or community clinics. Although this had potential drawbacks in terms of continuity of care, discussants noted that it created new bonds between specialist and generalist centers and is a model to explore further.

TRANSPLANT CANDIDATE SELECTION

Discussants considered ethical aspects of performing kidney transplantations (particularly high-risk procedures) when ICU/HDU capacity is limited. While they agreed was appropriate to

focus on low-risk transplantations when pressure on resources was greatest, the clinical dilemmas that this created should be acknowledged.

Of note, CMV-positive donor kidneys remained acceptable for CMV-negative recipients during the pandemic, but with greater emphasis on patient counselling (about transplantation risks and the importance of undergoing all scheduled post-procedural assessments).

CMV RISK MANAGEMENT

Saving the graft remained the main aspect of CMV risk management. CMV prophylaxis, using agents with low side-effect profiles (avoiding leukopenia and neutropenia, in particular) was vital, to limit unplanned hospitalizations and associated risks.

Some centers did not adjust CMV prevention protocols in 2020; others switched from pre-emptive therapy to antiviral prophylaxis, and some increased the duration of prophylaxis. Indeed, comprehensive antimicrobial prophylaxis for the first 6–12 months post-transplantation and more frequent monitoring of CMV viral load (with increased use of local testing) were instrumental in mitigating overall risk of CMV infection/reactivation. No group members treated CMV without knowing the patient's viral load.

No biomarker is commonly used to assess/adjust CMV prophylaxis, predict risk of neutropenia or leukopenia: decisions are based on clinical judgment. Tests for cellular-mediated immunity can predict CMV risk (especially R+ transplantatioins) but are rarely implemented. In addition, the absolute lymphocyte count can indicate risk for CMV infection at the end of treatment. Discussants suggested that an algorithm should be developed, to indicate specific prophylaxis regimens for specific patient types.

Although CMV reactivation was anticipated in kidney transplant recipients with concomitant COVID-19 because of immunomodulator use, only low-level reactivations were observed in 2020. Discussants were unaware of any research to investigate this further.

PREVENTING OTHER VIRAL INFECTIONS

Annual influenza vaccination remains strongly recommended for transplant recipients. However, research is needed to establish the number of hospitalizations and pneumonia cases prevented by vaccination, and to characterize efficacy and safety profiles of both influenza and COVID-19 vaccinations post-transplantation. In November 2020, before COVID-19 vaccinations were licensed, discussants felt it would be unlikely there would be an absolute requirement for COVID-19 vaccination before kidney transplantation (note that this has evolved, and many programs mandate pre-transplant vaccination).

In the context of unvaccinated kidney transplant patients, the unmet need for effective antiviral treatments (for COVID-19 and influenza in particular) remained. Treatments (especially oral agents) given during the initial biologically driven phase of COVID-19 might help to reduce the risk of poor outcomes associated with the immune-driven phase of infection.

COVID-19 RISK IN KIDNEY TRANSPLANT POPULATIONS

In November 2020 discussants commented that fewer kidney transplant candidates or recipients had contracted COVID-19 compared with general-population rates, possibly because the transplant community was well practiced in infection control and social distancing. Timing, duration and nature of stay-at-home directives differed internationally, therefore no conclusions could be drawn on their effectiveness, particularly among kidney transplant recipients.

Regarding COVID-19 screening, PCR testing (nasopharyngeal swabs) was recommended. Although reperfusion technology has extended the time between kidney retrieval and successful transplantation (up to 40 h), rapid diagnostics needed to be a 24/7 service, given that transplantations often occur outside standard hours.

TREATING COVID-19 INFECTION IN KIDNEY TRANSPLANT RECIPIENTS

In Spain in 2020, the incidence of COVID-19 was reportedly higher in people receiving kidney dialysis in hospital than in transplant recipients, possibly because they had limited ability to self isolate; this was demonstrated in UK research (13). The clinical course of COVID-19 infection was similar in transplant and dialysis patients, and worse than in the general population (14–18). Some discussants said that COVID-19 infections were rare within 6 months post-transplantation, probably because of antimicrobial prophylaxis.

Kidney transplant recipients were at a higher risk of death from COVID-19-related complications compared with the general population (19). However, data from an Italian center, collected during the early phase of the pandemic, indicated that all kidney transplant recipients with concomitant COVID-19 survived when immunosuppression was maintained (or switched from mycophenolate mofetil to high-dose steroids) (20). Centers that withdrew immunosuppression reported high rates of rejection and 30% mortality. Similar findings were described by discussants from Spain and the US. COVID-19 mortality rates were lower for patients who were several years post-kidney transplant if they were hospitalized at centers that were also specialist transplantation centers (21).

Opportunistic infections were not of concern in ICU patients with COVID-19, even if they were kidney transplant recipients: those who died were generally many years post-transplantation, elderly and had comorbidities, the group commented.

IMPACT OF COVID-19 ON HEALTH POLICY

In November 2020, the picture was similar across Europe and the US: treatment delays for non-COVID-19-related conditions created a backlog that discussants felt would stretch resources for years (22, 23).

Patients were presenting with later stages of non-COVID-19 diseases than would be expected (24, 25): some were reluctant to attend hospital because they feared nosocomial COVID-19 (26). Across Europe, a substantial increase in use of anti-depressants and sleeping tablets (often self-medicated) was also noted.

For end-stage kidney disease, the impact of maintaining patients on dialysis (because of lack of transplantation services) is considerable. Maintenance dialysis costs ~€50–90 000 per year; kidney transplantation in the first year costs ~€30–86 000 while the annual ongoing management of a functioning graft reduces substantially, to ~€5–20 000 (27, 28).

BUDGET IMPACT

COVID-19-related healthcare costs in 2020 anticipated to reach billions of Euros in Europe) were funded separately; no changes to standard budgets were anticipated for 2021. However, given the backlog of non-COVID-19 cases, discussants wanted to identify and implement more efficient practices.

They commented that some initiatives (e.g., shorter in-patient stays) might be less valuable than initially anticipated: for example, even if patients are discharged early, fewer procedures were being performed because of reduced hospital capacity, social distancing, staff absences, etc. After the pandemic, analyzing big data might help to identify which improvements provided genuine benefits for specific services, including transplantation. Such analyses have been successful in HIV, hepatitis C management and oncology. Big data might also determine the number of excess deaths caused by the pandemic more accurately.

DRUG DEVELOPMENT

Health Authority drug evaluations generally continued during 2020: potential treatments for COVID-19 were fast-tracked but processes continued with equal rigor, suggesting that efficiencies could be retained, especially for urgent medical needs. However, other new-drug evaluations were de-prioritized, and slower development pathways for non-COVID-related treatments may create access delays at a time when efficient methods to reduce backlogs are urgently needed.

HEALTH-RELATED QUALITY OF LIFE FOR KIDNEY RECIPIENTS

Discussants agreed that a universal instrument to measure health-related quality of life in kidney transplant recipients would be valuable. Standard questionnaires may not capture what is important for patients, particularly in extraordinary times.

POST SCRIPT: OCTOBER 2021 REFLECTIONS FROM THE GROUP

Table 2 compares discussants' views in 2020 and late 2021. Discussants did not expect the pandemic to have long-lasting impact, with many countries experiencing restrictions through October 2021 despite widespread vaccination campaigns. In November 2020, no one anticipated that the pandemic had yet to peak. The largest wave hit Germany in early 2021, severely stretching its health system for the first time: capacity was halved in university hospitals and staff were redeployed to COVID-19 wards. Of note, deceased-donor transplantations generally continued, but living-donor procedures were greatly reduced (in part, because of patients' concerns about nosocomial COVID-19) (2, 29).

Factors affecting decisions to suspend, redeploy or continue transplantation services require full evaluation (2); data on this remain extremely limited (30). Some publications have emphasized the need to provide autonomy to transplantation centers, even if there are stay-at-home directives, given the impact on life years lost for waitlisted candidates (2, 29).

In October 2021, a population-based study investigated effects of the pandemic on transplantations across 22 countries (2). The study corroborated many points and predictions made at the November 2020 webinars, cautioning that other factors (including natural disasters) also affected service delivery (2). However, the study showed a 19% decline in kidney transplantations in 2020 compared with 2019, which was the highest reduction for any solid organ (2). Rates of deceased donor kidney transplantations fell by 12%, and living-donor transplantations by 40%. For those waitlisted for a kidney in 2020, the pandemic was associated with ~37,664 patient life-years lost (2). Difficulties undertaking living-donor transplantations and paired kidney exchange during the pandemic, given the many societal restrictions, were inevitable. It is to be applauded that so many transplant centers managed to reduce their backlogs and return to near-normal service in 2021.

In terms of CMV management, patients receiving pre-emptive therapy risked losing virological control mechanisms because of reduced access to care (31, 32). Some discussants reported that severe disease in D+/R–transplantations negatively affected morbidity and mortality in patients with concomitant COVID-19; data corroborate this (33). In addition, clinical-trial participants lost protocolized visits, which implied they became protocol deviations. It remains unclear how this might affect data reporting (34, 35).

Discussants said that patients remained reluctant to attend in-person appointments, even when essential, although telemedicine remained well received (11, 36–39). Nevertheless, data on the impact of telemedicine in kidney transplant populations specifically are lacking, and the group spoke of continuing inequalities/access barriers (38, 39). Reimbursement for telemedicine remains unresolved in many

TABLE 2 | Key reflections from the 2020 webinars and 2021 discussions

Aspect of care	Views, November 2020	Views, October 2021
Transplant activity	• Decisions to continue/reduce transplantations, and redeploy clinical team differed by region • Transplantation capacity reduced (focus on DCD transplantations) • Complete cessation of transplant services to be avoided (difficult to restart)	• No transplant centers closed, but living donor procedures paused when COVID-19 admissions were high • Transplantations generally at near-normal level • Most centers had a backlog of cases and increase in patients with complex needs
Process adaptations	• Telemedicine and shared care (with local hospitals) were successful, but not expected to become permanent • Information provision via social media was efficient • Technology enabled remote patient monitoring (and rapid hospital discharge) • Technology poverty and poor skills created care disparities • Reimbursement issues for telemedicine apparent	• All forms of telemedicine remain widely accepted (new normal) • Patients reluctant to attend hospital (fear of infection) • Technology poverty remains of concern • Reimbursement issues unresolved in some countries
Candidate selection	• Focus on low-risk transplantations was necessary, but ethically and clinically challenging	• Autonomy needed for transplantation centers, given the life-years lost for waitlisted candidates
CMV risk management	• D+/R– transplantations continued but with greater emphasis on risk-management and pre-transplant counseling • Some centers switched from pre-emptive therapy to antiviral prophylaxis (or from 6 to 12 months' prophylaxis), with frequent viral load monitoring • CMV reactivation: not a concern for kidney recipients with concomitant COVID-19	• Virological control at risk because of reduced access to care and/or poor adherence • Severe CMV-related disease in D+/R– transplants increased morbidity/mortality with concomitant COVID-19
Infection prevention in transplant recipients	• Influenza vaccination mandatory • No COVID-19 vaccine licensed • Mixed views on whether COVID-19 vaccination would be mandatory for transplant candidates	• Transplant recipients reluctant to attend in-person appointments, even when essential • Most centers require transplant candidates to be fully vaccinated (including COVID-19, influenza); some extend this to immediate family • Initial mRNA vaccinations less effective in kidney recipients than in general population: numerous additional boosters required
COVID-19 risk/outcomes for infected kidney transplant recipients	• Lower incidence of infection in recipients than in general public (pre-COVID-19 social distancing/infection control habits may have been beneficial) • Outcomes worse in recipients than general public. Risk factors: older age, comorbidities	• Recipients at higher risk of death from infection than general population. Risk factors: older age, comorbidities, many years post-transplantation
Unmet needs	• Big data analysis: How many people contracted COVID-19 (excess mortality)? • Understand safety/efficacy of practice modifications, especially telemedicine, shortened hospital stay, and community–hospital collaboration • Develop health-related quality of life tool, specific for kidney transplant recipients • Algorithm to individualize CMV prophylaxis would be beneficial	• How to adjust studies for lost protocol visits? • Efficacy and safety of vaccinations (influenza and COVID-19) in kidney transplant populations • How to reduce the immense, ongoing pressure on all members of the healthcare team

countries, although for successful implementation see Duettman et al. (11).

Hospital–community and hospital–diagnostic partnerships continue, but initiatives have not been objectively evaluated. Nevertheless, discussants felt that COVID 19-related challenges were well managed by kidney transplantation communities. A tremendous amount of patient/family education was distributed. One participant commented, "Transplant patients looked to their clinical specialists for guidance. A lot of what we went through reinforced the strong relationship between transplant recipients and their clinicians." The group remain concerned, however, about patients living with long-term illness and isolation, lacking access to appropriate clinical or respite care.

Discussants also described the unprecedented levels of mental and physical stress affecting healthcare professionals, given the unrelenting workloads. They were particularly concerned about the negative impact of the pandemic on morale among nurses, and on undergraduate medical education, especially the lack of access to patients. Such issues have been explored outside transplantation (40).

On reflection, if the pandemic continues, to reduce impact on health services some discussants would call for earlier stay-at-home directives, citing that a German lockdown in late 2020 may have helped hospital services in early 2021. Discussants agreed that reallocation of transplantation resources is inevitable folowing any rise in infections and hospitalizations, but

stricter adherence to public-health measures could reduce infection rates, and therefore reduce the need to divert resources.

COVID-19 vaccination programs have so far been extremely successful, rapidly reducing the rates of serious infection and death in 201. Primary and booster vaccination remains the highest priority globally, as well as among transplant patients and their families. COVID-19 vaccination is generally (not universally) mandatory before organ transplantation and the requirement may even extend to close family members. The burden placed on transplantation teams to inform, counsel, vaccinate, determine immune responses and administer boosters to patients is considerable.

Research is required to ascertain short- and long-term levels of COVID-19 immunity among transplant recipients (41–46), and how best to treat those with severe infections. Administering a monoclonal antibody has been suggested (21). Without robust clinical evidence, prevention remains key: kidney transplant recipients' antibody responses are monitored frequently (44) and repeated mRNA vaccine boosters are administered (44–47), given that immunity often appears to be short lived in these patients (48).

In 2021, COVID-19 infections were managed similarly in kidney transplant recipients and in the general population (49, 50), although transplant recipients had heightened risk of poor outcomes (51). Given the risks inherent in commenting on any single publication, the authors encourage regular review of guidance from the US National Institutes of Health (47) and the European Medicines Agency (52).

To conclude, discussants were relieved that many nearl-normal services resumed in 2021, despite ongoing challenges of COVID-19. They await a time when activities settle within a new-normal, where there are opportunities to meet, reflect and educate in person, so that more can be learned from this important time in our history.

HUMAN COST OF THE PANDEMIC: POST-SCRIPT ACKNOWLEDGMENT

Maintaining transplantation services with the additional infection-control measures required for COVID-19 has been extremely challenging for healthcare professionals. The pandemic lowered staff morale, and increased levels of fatigue. In addition, many healthcare professionals have been severely infected with COVID-19: some have lost colleagues or loved ones, or been debilitated by the infection and its aftermath. Most transplant clinicians have seen a substantial number of transplant recipients succumb to COVID-19 infection. Discussants agreed that this has been a very emotional and challenging time for all.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

VP, PG, CNK, KB, JT-C, DC, FH, and SR made substantial contributions to the conception of the report developed from the webinars, drafting/revising the work critically for important intellectual content, providing final approval of the version to be published and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

The authors declare that the webinars in 2020 received funding from Merck Sharp & Dohme. The funder was not involved in the writing of this article or the decision to submit it for publication.

CONFLICT OF INTEREST

CNK received honoraria and/or research funding from Merck, Takeda, Roche Diagnostics, Beigene, and Regeneron. KB received honoraria and/or research funding from Alexion, Astellas, Bristol Myers Squibb, Chiesi, Fresenius, Hansa, Hexal, Merck, Novartis, Otsuka, Pfizer, Roche, Sandoz, Siemens, and Veloxis. FH received honoraria and/or research funding from Astellas, Bristol Myers Squibb, Hexal, Merck, and Novartis. SR is the CEO and sole shareholder of Vyoo Agency. PG has served as a consultant for Allovir, Angelini, BD, Biotest, Gilead, MSD, Nordic, Paratek, and Takeda; and on speaker's bureaus for Atara, BD, Gilead, Biotest, MSD, Pfizer, Takeda, and Vertex.

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

ACKNOWLEDGMENTS

Medical writing support was provided by Linda Edmondson and Rebecca Mant, independent medical writers, funded by the European Society for Organ Transplantation.

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ESOT | Transplant International

REVIEW
published: 23 March 2022
doi: 10.3389/ti.2022.10171

Cardiac Xenotransplantation: Progress in Preclinical Models and Prospects for Clinical Translation

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Survival of pig cardiac xenografts in a non-human primate (NHP) model has improved significantly over the last 4 years with the introduction of costimulation blockade based immunosuppression (IS) and genetically engineered (GE) pig donors. The longest survival of a cardiac xenograft in the heterotopic (HHTx) position was almost 3 years and only rejected when IS was stopped. Recent reports of cardiac xenograft survival in a life-sustaining orthotopic (OHTx) position for 6 months is a significant step forward. Despite these achievements, there are still several barriers to the clinical success of xenotransplantation (XTx). This includes the possible transmission of porcine pathogens with pig donors and continued xenograft growth after XTx. Both these concerns, and issues with additional incompatibilities, have been addressed recently with the genetic modification of pigs. This review discusses the spectrum of issues related to cardiac xenotransplantation, recent progress in preclinical models, and its feasibility for clinical translation.

Keywords: xenotransplantation, cardiac transplantation, transplantation, pre clinical model of xenotransplantation, heart transplantation

OPEN ACCESS

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Received: *01 November 2021*
Accepted: *11 January 2022*
Published: *23 March 2022*

Citation:
Singh AK, Goerlich CE, Shah AM, Zhang T, Tatarov I, Ayares D, Horvath KA and Mohiuddin MM (2022) Cardiac Xenotransplantation: Progress in Preclinical Models and Prospects for Clinical Translation. Transpl Int 35:10171. doi: 10.3389/ti.2022.10171

INTRODUCTION

Xenotransplantation (XTx) is an alternative source of a human organ for patients with end-stage organ failure. Many of these patients will die waiting for a human organ, as the current availability of donor organs falls short of its demand. In the past few years, substantial progress has been made in the xenotransplantation field. With the discovery and use of novel molecular biology techniques, genetically engineered (GE) porcine organ donors have been created to overcome numerous XTx barriers. The first transgenic pig for XTx was produced expressing human complement regulatory protein (hCRP) decay acceleration factor (hDAF). Organs from these pigs were transplanted in non-human primate (NHP), but hyperacute rejection (HAR) was only partially avoided (1, 2), and antibody-mediated immune response induced to terminal galactose sugar molecules (α1-3 Galactose

Abbreviations: B4GALNT2, beta-1,4-N-acetyl-galactosaminyltransferase; CC, consumptive coagulopathy; CMAH, CMP-N-acetylneuraminic acid hydroxylase; CRISPR, clustered regularly interspaced short palindromic repeats; CSA, cyclosporin; GHRKO, growth hormone receptor knockout; GTKO, 1-3 alpha Galactosyltransferase knockout; hCRP, human Complement Regulatory Protein; hDAF, human Decay Acceleration Factor; HHTx, Heterotopic Heart xenotransplantation; Neu5Gc, N-glycolylneuraminic acid; OHTx, Orthotopic Heart xenotransplantation; SPF, specified pathogen-free; TBM, thrombomodulin; TKO, triple knockout; TPC, alpha-Gal Polyethylene glycol polymer.

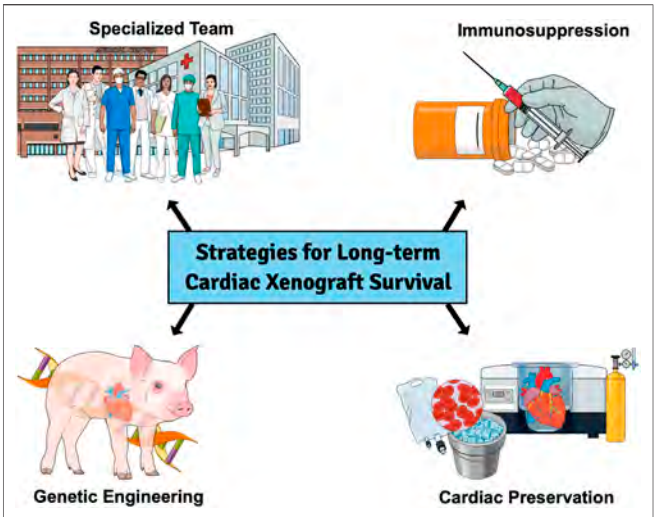

FIGURE 1 | Strategies for achieving success for long term cardiac xenograft survival.

or Gal) expressed on graft vascular endothelial cells continued to cause HAR. By using gene-editing techniques, Gal antigen was knocked out in pigs, and organs from these pigs were protected from HAR (3–5).

Other combinations of antigen knockout and human transgene expressing GE pigs were produced, and xenograft survival was extended further (6–13). We (HHTx Heart) and others (Kidney, Liver) have also reported long-term xenograft survival in NHP from genetically modified pigs (10, 12, 14–17). Recently, Langin et al. reported consistent survival in an experimental life-supporting (OHTx) in NHP (18). Strategies which have helped to achieve this success have also been summarized in **Figure 1**. In this review, we discuss the challenges faced in cardiac xenotransplantation and solutions that have culminated from the last several decades of work and speculate on the next steps required to make cardiac XTx a clinical reality (19, 20).

CHALLENGES FOR CARDIAC XENOTRANSPLANTATION

Immunological

Preformed Natural and Elicited Antibodies

The presence of natural preformed antibodies (nAbs) against pig antigens in recipients is a primary and significant hurdle for the success of cardiac XTx. These antibodies trigger immune responses and causes hyperacute (HAR) and acute humoral xenograft rejection (AHXR) (21). These nAbs against donor antigens (xenoantigens) trigger the activation of complement proteins, which further cause activation and damage to endothelial cells, leading to platelet aggregation and microvascular thrombosis. This ischemic injury leads to the destruction of cardiomyocytes, interstitial hemorrhage, and eventually fibrosis. Most of nAbs are against porcine carbohydrate antigens not found in humans and NHP. The most predominant of these is Galactose-α1-3 galactose, due to the acquired mutation of α1-3 galactosyltransferase (GT), an enzyme responsible for synthesizing this carbohydrate antigen. Others include SDa, and N-glycolylneuraminic acid (Neu5Gc). While preformed antibody responses dominate Gal antigens, it has been shown that elicited Abs responses can occur in cardiac XTx also towards these other antigens (i.e., non-Gal antigens) (22, 23, 24–30). Elicited Abs also play a major role in posttransplant thrombotic microangiopathy (TM), consumptive coagulopathy (CC), and AHXR (10, 31–34).

Cellular Xenograft Rejection

Besides HAR and AHXR, acute cellular rejection of cardiac xenografts can be mediated by innate (i.e., macrophages, neutrophils, dendritic cells, and NK cells) and adaptive (i.e., T and B cells) immune responses (35–37). However, acute CXR has not been reported frequently in xenotransplantation (34, 38). Innate immune cells, like macrophages and NK cells, have been found in pig organs perfused with human blood *ex vivo* and in pig-to-NHP xenografts, which may trigger CXR (34). Macrophages may also be activated by xenoreactive T cells and release proinflammatory cytokines (e.g., tumor necrosis factor-alpha (TNF-α, IL-1, and IL-6), which can further stimulate T cells. Both macrophages and NK cells can also be activated by direct interaction between donor endothelial antigens and their surface receptors, which may trigger CXR by direct NK cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC) (39, 40).

T cells can be activated through both direct and indirect pathways after xenotransplantation. However, the responses against xenoantigens, especially indirect responses, are more robust than seen in allotransplantation (41). T cell activation requires interaction between TCR and MHC peptide complex from the antigen-presenting cells (APC) and a costimulatory signal (e.g., CD40–CD154 and CD28–CD80/86 pathway interactions) (42, 43).

Coagulation Dysfunction

Coagulation dysregulation is also another major impediment to the success of xenotransplantation. The most extreme manifestations of it are systemic consumptive coagulopathy, characterized by thrombocytopenia and bleeding, which ultimately leads to graft loss due to ischemia from thrombotic microangiopathy (TM). Coagulation is a complex pathway that involves interactions of inflammation, vascular injury, heightened innate, humoral, and cellular immune responses. Incompatibilities between primate and pig coagulation/anti-coagulation factors can alter their function, contributing to coagulation dysfunction (44, 46). Notable proteins with cross-species incompatibilities include tissue factor pathway inhibitor (TFPI), thrombin, thrombomodulin (TBM), endothelial protein C receptor (EPCR) and CD39 (45–47).

Complement is also able to activate the clotting cascade, as it can be activated by the binding of complement fixing antibodies onto endothelium. As an example, activated product of complement C5a has been reported to induce tissue factor (TF) activity in endothelial cells (48) and has been reported to modifying the balance between pro- and anti-coagulation (49).

Preformed and elicited antibodies promote coagulation by activating porcine endothelial cells and platelets and contribute to graft loss due to TM (50–52). Systemic inflammatory responses and proinflammatory cytokines (notably IL-6) also upregulate or recruit recipient tissue factors (TF) on platelets and monocytes by interacting with porcine vascular endothelial cells which can lead to coagulation through thrombin production (54, 55).

Viral Transmission

A potential problem for cardiac xenotransplantation is a zoonotic viral transmission from swine. Most notable of which is a porcine endogenous retrovirus (PERV). There is no report yet for *in-vivo* pig-to-human PERVs transmission so the true risk in the context of xenotransplantation is not known (56). But, *in-vitro* studies have shown that PERVs could be transmitted from pig cells to human cells (57). Provirus DNAs of PERVs can be genetically transferred to offspring and cannot be eliminated by specified pathogen-free (SPF) breeding. Like other retrovirus, PERV theoretically predispose to the risks of tumors, leukemia, and neurodegeneration (58). However, studies have shown complete elimination of all copies of PERV in donor pigs (57).)

Porcine circovirus (PCV) from the Circoviridae family is also highly distributed among pigs and wild boars. Previously, two types of PCV1 and PCV2 have been characterized (59). PCV1, which is isolated from pig kidney cell culture (PK15 cells), and recently, Liu et al. have demonstrated that PCV2 can infect human cells *in vitro* with a reduced infection efficiency compared to pig PK-15 cells. Kruger et al. were unable to identify PCV1 and PCV2 in GE pigs. However, two other subtypes PCV3a and PCV3b, were found in the spleen, liver, lung kidney, and explanted heart of recipient baboons of GE cardiac xenografts after OHTx (60). The presence of PCV3 in the OHTx recipient baboon was higher among long-term survivors. However, the significance of PCV in causing clinical disease is unknown.

Xenograft Growth

Although there are several anatomical and physiological similarities between pigs and humans (or NHPs), their organs' growth rate is significantly different (61). Therefore, the use of minipigs has been suggested as their mature growth rate is 1/3 that of wild type Yorkshire (domestic) pigs (62). However, mostly domestic pigs have been used even for genetic modifications, but organs from these GE pigs continue to grow too large (61). Therefore, juvenile GE pigs are being preferred, but even still, continued organ growth after transplantation has been reported (8, 62, 63). Längin et al. have also reported left ventricular hypertrophy after pig OHTx in NHPs, but it is unclear its origin and whether this is from rejection, physiologic mismatch or natural growth (i.e., intrinsic or extrinsic causes or a combination of both) (18). In contrast, others have not seen pig heart growth after HHTx until the xenograft underwent delayed xenograft rejection (14, 15). In these experiments heart graft size was maintained until the co stimulation pathway blockade was reversed by stopping the anti-CD40 antibody.

While the growth of other organs such as the kidney can be accommodated within the abdomen, the growth of a heart xenograft could be problematic due to its position in the non-compliant chest and must be addressed before clinical translation.

OVERCOMING THE CHALLENGES FOR SUCCESSFUL CARDIAC XENOTRANSPLANTATION

Generation of Genetically Modified Donors

Several genetic strategies have been developed to prevent early graft failure from preformed antibodies and coagulation dysfunction resulting in generation of GE pigs. Genome editing using zinc-finger nucleases, transcription activator-like effector nucleases, or CRISPR-Cas9 is being used to delete multiple genes with high precision to produce GE pigs. Several pig genes are knocked out (e.g., α1-3 galactosyltransferase, B4GALNT2 and CMAH) and human genes are overexpressed (e.g., hCD46, hTBM, hEPCR, hTFPI, hCD39, etc.) in these GE pigs (**Table 1**).

The genetic constructs listed in **Table 1**, and the GE pigs produced, have been tested to various degrees. Kuwaki et al. reported the longest (179 days) heterotopic cardiac xenograft survival of GTKO hearts in NHP (6). Chen et al. also found an advantage in using GTKO pig kidneys over previously used transgenic kidneys (5). Recently, GTKO pigs along with other transgene have significantly improved the cardiac xenograft survivals in NHP to months in OHTx and years in HHTx models (14, 15, 69, 70).

CRISPR technology has now come into vogue as it affords complex genetic constructs to be employed with the highest fidelity compared to other techniques. Two carbohydrate antigen-expressing genes (e.g., GT and CMAH) have been deleted, and "double knockouts" (GTKO.CMAHKO) have been constructed (71, 72). Burlak et al. reported a reduced binding of human antibodies to cells from these GTKO. CMAH KO pigs (67). Later, Tector's group has produced three carbohydrate antigen knockout (TKO) pigs (i.e., GTKO.CMAHKO.B4GALNT2KO), which included deletion of B4GALNT2 responsible for SDa antigen along with GT and CMAH genes (26, 31, 73). They demonstrated that the binding of human IgG and IgM antibodies to peripheral blood mononuclear cells and red blood cells from triple knockout pigs was significantly reduced. Niu et al. inactivated all known porcine endogenous retrovirus (PERVs) within pig xenograft donors (74). A combination of various genetic constructs is being developed by other groups as well, a testament to the technology's ability to move the field forward quickly. "Multi-gene" expressing cardiac xenografts' effect on overall graft function and survival in HHTx and OHTx is currently a topic of investigation in our lab and others.

Immunosuppression

To achieve long-term xenograft survival, various immunosuppressive (IS) drug regimens have been used along with GE pigs. Earlier conventional corticosteroids and calcineurin

TABLE 1 | The "genetic toolbox" central to our strategies to minimize or abolish hyper-acute and delayed humoral rejection.

Genetic modification	Mechanisms	Properties
Alpha-Gal KO (GTKO) B4GalNT2 KO CMAH KO	Deletion of immunogenic Gal antigen expression Deletion of B4Gal Deletion of Neu5Gc	Anti-Immunogenic
hHO-1	Decreases oxidative products	Anti-Apoptotic
hHLA-E hCD46 hCD55 (DAF)	Protects the graft against human killer cells Suppresses human complement activity Suppress human complement activity	Anti-Inflammatory
hEPCR hTFPI hvWF hTBM	Activates Protein C Inhibits Factor Xa Reduces platelet sequestration and activation Binds human thrombin, and activates Protein C via activated thrombin	Anti-Coagulation
Multi-Genetic Modified Pigs		
• GTKO.hCD46		
• GTKO.CD55(DAF) (64, 65)		
• GTKO.hCD46.CD55(DAF) (14)		
• GTKO.hCD46.hTBM (15, 18, 63)		
• GTKO.hCD46.CD55.EPCR.TFPI.CD47 (63)		
• GTKO.hCD46.hTBM.CD47.EPCR.HO1		
• GTKO. B4GalNT2KO (66)		
• GTKO. B4GalNT2KO.hCD46.hHLAE		
• GTKO.B4KO.hCD46.hTBM.hEPCR. hCD47.hHO1.hVWF		
• GTKO.CMAHKO (67)		
• GTKO. B4GalNT2KO CMAHKO (68)		
• GTKO.CMAHKO.hCD46.hCD47. hTFPI		
• GTKO.CMAHKO.hCD46.hEPCR. hDAF		
• GTKO.CMAHKO.hCD46.hEPCR. hDAF.hTBM. hHO1		
• GTKO.CMAHKO.B4GalNT2KO.hCD46.hDAF		
• GTKO.B4GalNT2KO.GHRKO. hCD46.hTBM.hEPCR.hCD47 (69)		
• GTKO.B4GalNT2KO.CMAHKO.GHRKO. hCD46.hTBM.hEPCR.DAF.hCD47.HO1 (69)		

CMAH, cytidine monophospho-N-acetylneuraminic acid hydroxylase; EPCR, Endothelial Protein C Receptor; HO-1: Heme Oxygenase -1; TFPI, tissue factor pathway inhibitor; HLA, human leukocyte antigen; h, human; vWF, von Willebrand Factor; TBM, thrombomodulin.

based (CSA) immunosuppression (IS) was used in NHP recipients, which prevented acute rejection, but failed to prolong cardiac xenograft survival (75–77). The longest reported cardiac xenograft survival using a CSA-based IS regimen was 32 days from a wild type (WT) pig (75) but was extended up to 99 days (median 26 days) using hDAF transgenic hearts (78). Various other IS regimens were used which include splenectomy or total body irradiation, non-antigenic alpha-Gal polyethylene glycol polymer (TPC) alone or in combination (9, 23, 79). Effect of these immunosuppression regimen on cardiac xenograft survival has been summarized in **Table 2**. Later, anti-thymocyte globulin (ATG), rituximab, mycophenolate mofetil, tacrolimus, and sirolimus were also used in various combinations as alternative regimens (10, 80–82). For complement inhibition, either cobra venom factor (CVF) or overexpression of complement regulatory protein gene expression for a donor organ or both were used (10, 15, 81). By using these IS drugs, McGregor et al. 2005 reported consistent graft survival (median 96 days; range, 15–137 days) in an HHTx model, but xenograft rejection was associated with a rise in non-Gal antibody titers. They did not observe a significant difference in graft survival when GTKO or GTKO.hCRP donors were used (35, 64).

Significant progress in cardiac XTx occurred when newer agents were used that block the co-stimulation, which aids in T cell activation upon antigen exposure (10, 11, 15, 92). In 2000, Buhler et al. demonstrated that the blocking of the CD40/CD154 pathway by anti-CD154 antibody prevents an induced anti-pig humoral response (99). Kuwaki et al. also reported the longest cardiac xenograft survivals for 179 days (median 78 days) (6) in HHTx with anti-CD154 antibody treatment. We have also reported more than 8-month survival of GTKO.CD46 cardiac xenograft in HHTx with continuous co-stimulation blockade by

TABLE 2 | Progress in Cardiac Xenograft Survival (Heterotopic and Life Supporting Orthotopic) and Immunosuppression Regimen used.

Type of graft	Broad immunosuppression category	GE cardiac xenograft survival (Days)	References
Heterotopic		<1	(10)
	Without Immunosuppression		
	With Immunosuppression		
	• Without Corticosteroids[a]	3–62	
	• Total body irradiation[a]	8–15	(9)
	• Immunoadsorption[a]	9–39	(32, 83)
	• Thymic irradiation[a]	8–15	(84)
	• Splenectomy[a]	0–139	(84–87)
	• Immunosuppressive Reagents e.g., Cyclosporine, MMF 15-Desocyspergualin TPC, Gas914,Tacrolimus, Rapamycin[a]	0–139	(64, 76, 78, 82, 84, 91)
	• CVF[a]	16–179	(10, 14, 15, 81, 92, 93)
	• ATG[a]	5–236	(10, 14, 15, 86)
	• Anti-CD20[a]	0–236	(10, 14, 15, 86)
	• Costimulation blockade (Anti CD154 and anti CD40 Antibody)[a]	8–945	(10, 14,15, 81, 92, 93)
Orthotopic			
	With Immunosuppression		
	• Immunoadsorption, TBI, CsA, Methotrexate[a]	18–19	(79)
	• Immunosuppressive reagents, e.g., Cyclosporine, Cyclophosphamide, MMF, Tacrolimus, Rapamycin[a]	1–25	(75, 94–96)
	• CVF, ATG, Anti CD20, Anti-CD40 antibody, Non-ischemic preservation technique[a]	51–264	(18, 23, 69, 97)

[a]*Introduction of new agents along with other immunosuppressive drugs.*

anti-CD154 antibody (25 mg/kg; clone 5C8) and B cell depletion with Rituxan at the time of transplantation (10). Although the use of anti-CD154 antibody has improved survival, it has been reported that anti-CD154 antibody is associated with bleeding and thrombotic complications such as consumptive thrombocytopenia and venous and arterial thrombi (10, 81, 99). As a result, replacement with an anti-CD40 (25 mg/kg; clone 2C10) monoclonal antibody (mAb), which targets the same interaction, has been the focus of the active investigation. When we used this antibody, there was no significant difference found in median 70 vs. 75 days) compared to anti-CD154 blockade.

However, we demonstrated that cardiac xenograft (GTKO.CD46. TBM) survival in HHTx was significantly prolonged (median 298 days) when the anti-CD40 antibody was used at a higher dose (50 mg/kg) (15, 100). Iwase et al. also demonstrated anti-CD40 mAb combined with belatacept proved effective in preventing a T cell response (14). The anti-CD40 mAb used in these studies is a mouse/rhesus chimeric IgG4 antibody, which may not be suitable for use in humans. Still, several other humanized anti-CD40 blocking antibodies under development can be used for human use if approved as an immunosuppression adjunct in cardiac XTx (101).

Prevention of Viral Transmission

The risk of PERV transmission can be minimized by selecting PERV negative porcine donors. Thorough screening of PERV can be done by serology, western blot, ELISA, immunofluorescence, scanning electron microscopy, and PCR. Recently, Yang et al. have inactivated all PERV proviruses (62 copies of PERV's gene *pol*, leading to a 1,000 times reduction in the virus's ability to infect human cells) in the pig genome the CRISPR/Cas technique (102). The use of PERV inactivated pigs may provide tissue, organs that may address the safety issue from a porcine virus in pig-to-human xenotransplantation. However, the impact of PERV inactivation and gene editing on PERV-inactivated pigs and the necessity of these complex constructs is not known.

Prevention of Xenograft Growth

In one approach, xenograft growth is controlled by using drugs such as rapamycin (8, 14, 18). Inhibition of mTOR protein kinase has been shown to control cell growth and proliferation to treat cancers in the clinical setting (103). Längin et al have used mTOR inhibitor and anti hypertensive drugs to control the blood pressures to prevent overgrowth cardiac xenograft in OHTx (18). Recently, Hinrichs et al. have produced GHRKO pigs in order to address intrinsic organ growth. They demonstrate that GHRKO pigs have slow or reduced growth, including their organs' growth, compared to normal wild-type pigs (61, 104–107). Recently, Goerlich, et al. have examined intrinsic and extrinsic causes of graft growth after transplantation in an OHTx model using "multi-gene" pigs growth hormone receptor knockout pigs (GHRKO) (69). Post-transplantation xenograft growth was measured by echocardiography longitudinally after transplantation between multi-gene cardiac xenografts with and without GHRKO. Extrinsic causes of graft growth, namely blood pressure and heart rate, were left without treatment. GHRKO grafts demonstrated a 50.4% increase in LV mass up to 9 months (264 days) after OHTx compared to 140.1% in xenografts with a limited survival of less than 3 months. Terminal histology demonstrated fibrosis, interstitial edema and hemorrhage as the cause of this growth and not classical hypertrophy. Moreover, blood pressures and heart rates were significantly elevated after transplantation regardless of GHRKO status, suggesting physiologic mismatch occurs after transplantation. Altogether, these data suggest that post-

transplantation xenograft growth in OHTx is multifactorial; largely driven by intrinsic growth with some extrinsic component not related to physiologic mismatch. Terminal histology would suggest this extrinsic component could be rejection related.

PROGRESS IN CARDIAC XENOTRANSPLANTATION TOWARD CLINICAL TRANSLATION

The progress of cardiac xenotransplantation has been immense (**Figure 2**) but the transition from HHTx to OHTx (i.e., to the life-supporting function of xenografts) has been fraught with its own challenges as the recipient's native heart is replaced entirely by the xenograft (108–112). Thus, any perturbations in the graft (arrhythmias, ventricular function, or rejection) can have devastating consequences to the recipient. Peri operative cardiac xenograft dysfunction (PCXD) has been observed in 40%–60% of OHTx which has also made the transition difficult (23). However, there has been a success in the OHTx with GTKO.hCD46.hTBM (3-GE) graft survival up to 6 months, despite these hurdles with the aid of non-ischemic continuous xenograft preservation (70, 112). This has been observed by others as well, but the underlying mechanism in cardiac preservation preventing primary graft dysfunction in this setting is poorly understood (113).

The advancement in donor genetic engineering capabilities has also resulted in xenografts with additional transgenes and knockouts for successful long-term OHTx survival. While multi-gene xenografts have certainly fallen into favor, there has been a recent increase in interest for "triple knock out (TKO)" xenografts, which lacks three carbohydrate antigens. In addition to Gal antigens, knockout for additional non-Gal antigens addresses other preformed antibodies that can contribute to humoral rejection. However, like our HHTx experience, we have also seen that hTBM is important in increased survival in xenografts, but specifically, we have seen that TKO grafts exhibit accelerated antibody-mediated rejection and increased incidence of thrombotic complications (16). This could be because of the lack of human transgenes in these TKO xenografts or because TKO xenografts create *de novo* synthesis, a novel xenoantigen on their surface due to CMAH knockout in the TKO pig that baboon recipients see as foreign (114).

However, multi-gene pigs with double and triple carbohydrate knockouts have been developed for cardiac xenotransplantation and are currently being tested in OHTx and HHTx models. Recently, we have achieved up to 264 day survival of a multi-gene cardiac xenograft with additional human transgene and knockouts (69). Notable modifications in these pigs were are carbohydrate enzyme KO (GTKO and β4GalNT2), growth hormone receptor knockout (GHRKO) and the addition of human transgenes (hCD46, hTBM, hEPCR and hCD47). We are testing cardiac xenograft survival which have over expression of other human genes (8–10 GE) in addition to these from pigs in OHTx with mixed success. These studies, along with others, will soon shed light on the advantages and disadvantages of iterative genetic modifications and pave the way for pre-clinical efficacy required for human clinical trials.

Conclusion

We are now entering an exciting time in xenotransplantation with the progression of survival in preclinical models of pig cardiac xenotransplantation. With the understanding now that a multi-pronged approach toward these recipients' immunosuppression increases graft survival, most critical of which to date is co-stimulation blockade, attempts to reduce the burden of immunosuppression has placed genetic engineering of cardiac xenografts in the forefront. Increasing the immunocompatibility of xenografts from genetically engineered pigs are a noble approach utilizing technology that has progressed the field further. However,

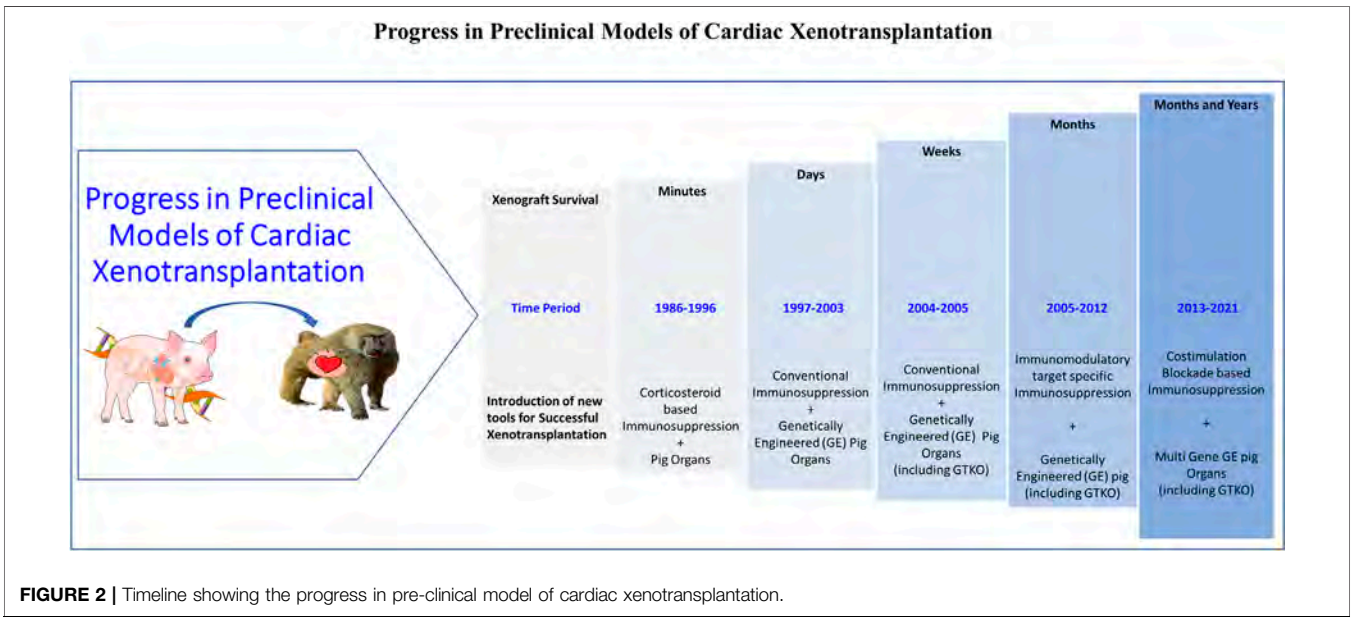

FIGURE 2 | Timeline showing the progress in pre-clinical model of cardiac xenotransplantation.

genetic engineering should proceed with caution, utilizing *in vitro* evidence for every iterative improvement in the genetically engineered cardiac xenograft. Given the field's current progression and demonstration of success, it is our opinion that multi-gene xenografts which include iterative addition of human transgenes or knockouts of pig genes along with targeted immunosuppression will pave the wave for clinical translation a reality.

AUTHOR CONTRIBUTIONS

AS, CG, AS, TZ, and IT wrote the paper, DA, KH, and MM reviewed, critiqued revised, and approved the paper.

FUNDING

Funding is generously provided by public funding-NIH U19 AI090959 "Genetically-engineered Pig Organ Transplantation into Non-Human Primates" and private funding by United Therapeutics. Additional funding was provided by public funding-NIH T32 5T32HL007698-26. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

CONFLICT OF INTEREST

Author DA was employed by the company Revivicor Inc.

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

ACKNOWLEDGMENTS

We would like to thank all the authors whose work is cited here and sincerely apologize to significant contributors to xenotransplantation whose work is not reported due to this review's limited scope.

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REVIEW
published: 21 March 2022
doi: 10.3389/ti.2022.10258

Machine Perfusion for Human Heart Preservation: A Systematic Review

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Currently, static cold storage (SCS) of hearts from donations after brainstem death remains the standard clinically. However, machine perfusion (MP) is considered an approach for donor organ management to extend the donor pool and/or increase the utilization rate. This review summarizes and critically assesses the available clinical data on MP in heart transplantation. We searched Medline (PubMed), Cochrane, Embase, and clinicaltrials.gov, along with reference lists of the included publications and identified 40 publications, including 18 articles, 17 conference abstracts, and five ongoing clinical trials. Two types of MP were used: hypothermic MP (HMP) and normothermic MP (NMP). Three studies evaluated HMP, and 32 evaluated NMP. Independent of the system, MP resulted in clinical outcomes comparable to traditional SCS. However, NMP seemed especially beneficial for high-risk cases and donation after circulatory death (DCD) hearts. Based on currently available data, MP is non-inferior to standard SCS. Additionally, single-centre studies suggest that NMP could preserve the hearts from donors outside standard acceptability criteria and DCD hearts with comparable results to SCS. Finally, HMP is theoretically safer and simpler to use than NMP. If a machine malfunction or user error occurs, NMP, which perfuses a beating heart, would have a narrower margin of safety. However, further well-designed studies need to be conducted to draw clear conclusions.

Keywords: review, heart transplantation, machine perfusion, heart preservation, donor

INTRODUCTION

Heart transplantation is the most effective method used to treat end-stage heart disease. Currently, static cold storage (SCS) of hearts from donations after brainstem death (DBD) remains the standard practice. SCS combines cardioplegia and hypothermia, which can significantly reduce the energy demand of the donor heart (1). However, despite decades of effort, the cold ischemia time has been limited to 4–6 h. Prolonged cold ischemia and ischemia-reperfusion injury (IRI) have been recognized as significant causes of post-transplant graft failure. According to the International Society for Heart and Lung Transplantation registration, the survival rate decreases as the ischemic time increases (2). The continuous shortage of donor hearts has always been a major limiting factor for heart transplantation (3).

Machine perfusion (MP) is considered an ideal approach for donor organ management to extend the donor pool and/or increase the utilization rate. Perfusion can supply the metabolic need of the myocardium, thus minimizing irreversible ischemic cell injury and death. Several heart perfusion systems, which are either hypothermic MP (HMP) or normothermic MP (NMP), have successfully preserved animal and/or human hearts (4). The longest reported successful human heart preservation time was 16 h with NMP (5). Currently, there is only one commercially available

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Received: *22 November 2021*
Accepted: *27 January 2022*
Published: *21 March 2022*

Citation:
Qin G, Jernryd V, Sjöberg T, Steen S and Nilsson J (2022) Machine Perfusion for Human Heart Preservation: A Systematic Review. Transpl Int 35:10258. doi: 10.3389/ti.2022.10258

perfusion system for clinical use, the organ care system (OCS), and one recently tested system, the non-ischemic heart preservation system (NIHP) (6, 7). Another approach to extend the donor pool is to utilize organs from donation after circulatory death (DCD) (4, 8). For these donor hearts, MP can provide a platform to resuscitate, preserve, assess and even possibly recondition the cardiac function prior to planned transplantation.

Well-designed machine perfusion can theoretically expand the donor pool in different ways. A prolonged safe preservation time allows to utilize remote donor hearts and functional assessment allows to utilize some of the DCD and high-risk donor hearts. Pediatric heart transplantation may have an extra benefit since pediatric donor shortage is even worse, and long transport time occurs more frequently.

Despite the growing number of human donor hearts preserved with MP, it remains controversial whether MP is superior to SCS. In this systematic review, we summarize and critically assess all available clinical data on MP of adult donor hearts, highlighting its therapeutic potential as well as the current limitations and shortcomings.

METHODS

Search Strategy and Data Sources

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines. The literature search consisted of two parts: searching for published studies and searching for ongoing clinical trials (inception to 27 June 2020). Published studies were searched in the Medline (PubMed), Cochrane, and Embase databases. The following searching terms were used in combination with AND or OR: heart transplantation, organ perfusion, *ex vivo* perfusion, *ex vivo* reperfusion, heart perfusion, cardiac perfusion, non-ischemic heart preservation, perfusion preservation, antegrade perfusion, and machine perfusion. Ongoing clinical trials were searched in clinicaltrials.gov using the term of heart transplantation for condition or disease in combination with preservation or perfusion for other terms. Only original publications in English were considered. All questions regarding the literature search and article selection were resolved by discussion between two independent reviewers. All references listed in the selected articles were screened for any further publications that were not identified in the initial search.

Inclusion and Exclusion Criteria

Articles reporting the outcome of MP in donor hearts during primary adult heart transplantation were included. Reports that met any of the following criteria were excluded: 1) irrelevant topics, 2) duplicated data, 3) non-English language, 4) not transplanted, 5) not human, 6) pediatric, or 7) reviews, editorials, and letters to the editor.

RESULTS

The initial search yielded 3,446 potentially relevant records. **Figure 1** shows a flowchart of the study selection process. Screening resulted in 39 relevant studies. One additional study was identified from the screening of reference lists in the included publications. Ultimately, 40 studies were included in this review: 18 papers (6, 7, 9-24), 17 conference abstracts (5, 25–40), and five ongoing clinical trials (41-45). Three studies reported multicenter data (7, 25, 40), and three were randomized controlled studies (7, 12, 13).

In clinical practice, two types of MP have been used to preserve donor hearts: HMP and NMP. The system temperature was controlled below 10°C during HMP, in contrast to 34°C during NMP. We identified three non-randomized, single-centre studies that used in-house designed HMP systems (**Table 1**) (6, 9, 11). Wicomb et al. demonstrated the first system for HMP of the human heart (9). In this study, four hearts were perfused with an oxygen- and carbon dioxide-bubbled crystalloid cardioplegic solution at a pressure of 8–10 cm H_2O. All four hearts were transplanted after a total preservation time of 6, 7, 12, or 15 h. Only one patient survived after 16 months with normal heart function (9). Hill et al. reported successful heart transplantation with HMP using a colloid cardioplegic solution to perfuse eight hearts with a low flow rate (17 ml per 100 g per hour) for 221 min. For comparison, 13 hearts were preserved with cardiosol (185 min) and 50 hearts with modified St. Thomas solution (187 min). The 7-year survival rate was 70% in the St. Thomas solution group and 100% in the other two groups (11). In the third study, Nilsson et al. preserved six hearts using NIHP with a perfusion pressure of 20 mm Hg at 8°C. The perfusate comprised a hyperoncotic cardioplegic nutrition solution supplemented with hormones and erythrocytes. These six NIHP transplantations were compared with 25 SCS transplantations during the same period. The median total preservation time was longer for the NIHP group (223 min; IQR, 202–263) than for the SCS group (194 min; IQR, 164–223). The primary outcome showed a 100% event-free 6-month survival rate for NIHP recipients, compared to 72% for SCS recipients. Furthermore, creatine kinase-muscle/brain, assessed 6 h after ending perfusion, was 76 ng/ml for NIHP compared with 138 ng/ml for the SCS recipients (non-significant), indicating less myocardial damage when using the NIHP method (6).

The only NMP system for clinical heart transplantation is currently the OCS. With the OCS, oxygenated donor blood is used to perfuse coronary arteries at a temperature of 34°C with a perfusion pressure of 60–90 mmHg. Lactate concentration is monitored to verify that adequate perfusion is achieved and if it is above 5 mmol/L, the heart is discarded (7). In the PROCEED II trial, five donor hearts were discarded, four because of rising lactate concentrations and one because of technical issues (7).

Twenty-one publications, including eight papers (7, 10, 12–16, 21) and 13 conference abstracts (5, 25–35, 40) presented results from using the OCS at transplantation of DBD hearts with or without a control group (**Tables 2**, **3**). Three of these studies were randomized (**Table 2**). The only randomized and multicenter study, PROCEED II, which recruited 130 patients from 10 heart transplant centres in the United States and Europe, showed no significant differences in the primary endpoint (30-day patient and graft survival) or secondary endpoints. However, the mean

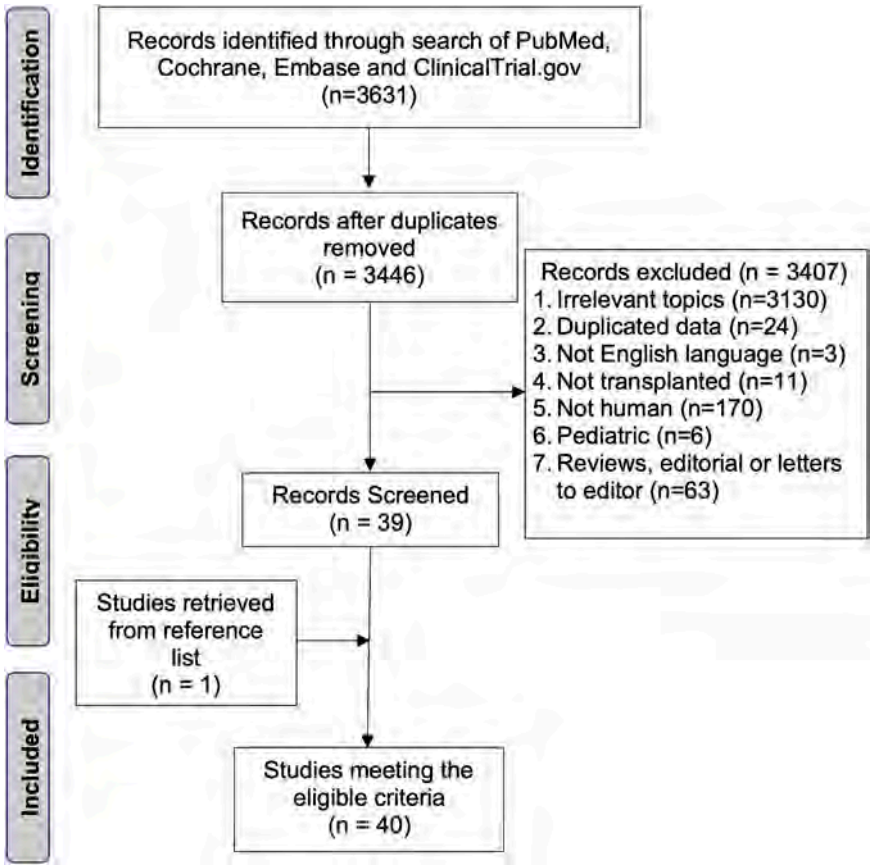

FIGURE 1 | Flowchart of the search strategy.

TABLE 1 | Hypothermic machine perfusion.

Study	Number of patients	Temperature (°C)	Perfusate	Outcome	Publication type
Wicomb et al., 1984 (9)	HMP = 4	4–10	Crystalloid cardioplegic solution	Total preservation time 12, 7, 15, and 6 h. One patient survived over 16 months	Single-center
Hill et al., 1997 (11)	HMP = 8, SCS = 12	Ice-cooling	Colloid cardioplegic solution	7-year survival rate 100% in both the HMP and the SCS groups	Single-center
Nilsson et al., 2020 (6)	HMP = 6, SCS = 25	8	Albumin-rich solution with erythrocytes	6-month event-free survival rate 100% in the HMP group and 72% in the SCS group	Single-center

HMP, hypothermic machine perfusion; SCS, static cold storage.

total out-of-body time was significantly longer in the OCS group than in the control group (324 vs. 195 min) (7). The other two randomized studies reported data from single institutional heart transplant candidates, previously enrolled in the PROCEED II study and subsequently followed for an additional one and 2 years (12, 13). There were no significant differences between the OCS and SCS groups regarding changes in intimal thickness for the left main and left anterior descending coronary arteries (13). Chan et al. followed the recipient for 2 years and did not find any significant differences in patient survival, freedom from non-fatal major cardiac events, or cardiac allograft vasculopathy (12).

Thirteen studies (5, 14, 16, 21, 25, 26, 29–33, 35, 40) used the OCS in high-risk cases. High risk was defined as an adverse donor/recipient profile, including an estimated ischemic time longer than 4 h, left ventricular ejection fraction less than 50%, left ventricular hypertrophy, donor cardiac arrest, alcohol/drug abuse, coronary artery disease, recipient mechanical circulatory support, and/or elevated pulmonary vascular resistance.

In nine publications, the OCS was compared with SCS (**Table 2**) (14, 15, 25–31). The results of three of these studies favored OCS perfusion (27, 29, 31), including two studies that used the OCS for high-risk cases (29, 31). The other six studies

TABLE 2 | Studies of normothermic machine perfusion for hearts from donation after brainstem death with static cold storage as the control group.

Study	Number of patients	Total preservation time (min)	Outcomes	Publication type	Risk case
Ardehali et al., 2015 (7)	OCS = 67, SCS = 63	OCS = 324, SCS = 195	No difference in 30-day survival rate and SAE between groups	Multi-center, randomized, article	No
Chan et al., 2017 (12)	OCS = 19, SCS = 19	OCS = 361, SCS = 207	2-year patient survival rate: 72.2% in OCS group, 81.6% in SCS group (p = 0.38)	Single-center, randomized, article	No
Sato et al., 2019 (13)	OCS = 5, SCS = 13	OCS = 362, SCS = 183	ΔMIT ≥0.5 mm with no significant difference between groups. From baseline to 1 year post-transplant, ΔMIT, maximal intimal area, and percent stenosis were similar between groups	Single-center, randomized, article	No
Botta et al., 2017 (26)	OCS = 7, SCS = 95	OCS = 296, SCS = 187	No significant difference in CK-MB post- transplant	Conference abstract	Yes
Falk et al., 2019 (27)	OCS = 16, SCS = 24	Not reported	OCS perfusion reduces IRI at the cytokine and endothelial level in recipient blood immediately after transplantation	Conference abstract	Not mentioned
Fujita et al., 2018 (28)	OCS = 29, SCS = 169	Not reported	Survival rate similar between groups	Conference abstract	Not mentioned
Garcia et al., 2015 (29)	OCS = 15, SCS = 15	OCS = 373, SCS = 204	30-day survival rate: 100% in OCS group and 73.3% in SCS group (p = 0.03)	Conference abstract	Yes
Jain et al., 2017 (14)	OCS = 1, SCS = 1	OCS = 495, SCS = 412	Total cost of OCS transplantation significantly less than SCS transplantation	Article	Yes
Koerner et al., 2014 (15)	OCS = 29, SCS = 130	OCS = 313, SCS: not reported	No significant difference in cumulative survival rates at 30 days, 1 year, and 2 years	Article	No
Rojas et al., 2020 (30)	OCS = 49, SCS = 48	OCS = 402, SCS = 225	No significant difference in 30-day, 1-year, and 2-year survival rate	Conference abstract	Yes
Sponga et al., 2019 (31)	OCS = 17, SCS = 70	Not reported	Improved 30-day, 1-year, and 5-year survival rate in the OCS group	Conference abstract	Yes
Sponga et al., 2020 (25)	OCS = 44, SCS = 21	OCS = 428, SCS = 223	No significant difference in 30-day mortality	Conference abstract	Yes

IRI, ischemia-reperfusion injury; MIT, maximal intimal thickness; NS, not significant; OCS, organ care system; SAE, serious adverse events; SCS, static cold storage.

TABLE 3 | Non-randomized studies of normothermic machine perfusion for hearts from donation after brainstem death, without control group.

Study	Number of patients	Total preservation time (min)	Outcomes	Publication type	Risk case
Ayan Mukash et al., 2019 (32)	47	Not reported	Kaplan-Meier survival estimates 91%, 85%, and 80% at 3 months, 6 months, and 1 year	Conference abstract	Yes
Garcia et al., 2016 (33)	60	Not reported	Survival rate similar between regular donor group (n = 24) and extended criteria donor group (n = 36)	Conference abstract	Yes
Garcia et al., 2014 (16)	26	371	Survival rate 100% at 1 month and 96% at follow-up of 257 days	Article	Yes
Kaliyev et al., 2019 (10)	43	344	30-day survival 100%	Article	Not mentioned
Koerner et al., 2012 (34)	13	Not reported	1- and 2-year survival rate 89%	Conference abstract	Not mentioned
Nurmykhametova et al., 2018 (5)	1	960	Total out-of-body time 16 h, longest out-body time to date	Conference abstract	Yes
Rojas et al., 2020 (40)	76	382	Survival rate 92.1% and 82.9% at 30 days and 1 year	Conference abstract	Yes
Stamp et al., 2015 (21)	1	611	Total out-of-body time 10 h	Article	Yes
Yeter et al., 2014 (35)	21	388	Freedom from cardiac-related death 95% at 30 days and 6 months, 87% at 1 and 4 years	Conference abstract	Yes

did not find any significant difference in the primary outcomes (14, 15, 25, 26, 28, 30). The total preservation time was reported in five studies, and it was significantly longer in the OCS groups (14, 25, 26, 29, 30).

Botta et al. compared day-0/day-1 CK-MB levels between an OCS group and an SCS group and did not find any significant difference (26). Falk et al. compared IRI between the OCS and SCS groups by measuring interleukin (IL)-6, IL-8, IL-18, angiopoietin-2, and insulin-like growth factor-binding protein-1 immediately after and 24 h after heart transplant (27). The results showed that OCS preservation significantly reduced all these proteins. Seven studies compared short- and long-term patient survival rates and found no significant difference between the groups (14, 15, 25, 28-31).

One case report reported two long-distance heart transplantations, with or without the OCS. Although both patients remained well at 6 months with normal cardiac function, the patient who received the SCS-preserved heart

TABLE 4 | Studies of normothermic machine perfusion for hearts from donation after circulatory death.

Study	Number of patients	Outcomes	Publication type
Chew et al., 2017 (36)	DCD = 12, MBD = 12	All hearts retrieved with DPP, comparable survival rate between OCS-preserved DCD hearts and OCS-preserved MBD hearts	Conference abstract
Chew et al., 2019 (22)	DCD = 23, DBD = 94	All DCD hearts retrieved with DPP, comparable survival rate between OCS-preserved DCD hearts and SCS-preserved DBD hearts	Paper
Dhital et al., 2015 (23)	DCD = 3	All hearts retrieved with DPP, survival to date: 77, 91, and 176 days	Article
Garcia et al., 2016 (17)	DCD = 2	Both hearts retrieved with DPP, survival to date: 290 and 291 days	Article
Mehta et al., 2019 (18)	DCD = 7	All hearts retrieved with DPP, 90-day survival rate 86%	Article
Messer et al., 2016 (20)	DCD = 9	8 hearts retrieved with TA-NRP + OCS; all patients survived during follow-up (range, 48–297 days)	Article
Messer et al., 2017 (24)	DCD = 26, DBD = 26	DCD hearts retrieved with DPP or TA-NRP, comparable results of the OCS-preserved DCD hearts and the SCS-preserved DBD hearts	Article
Messer et al., 2019 (37)	DCD = 50, DBD = 50	DCD hearts retrieved with DPP or TA-NRP, comparable results in 30-day survival	Conference abstract
Mohite et al., 2019 (19)	DCD = 1	Heart retrieved with DPP, alive to date at 5 months	Article
Page et al., 2017 (38)	DCD = 20, DBD = not reported	Biopsies within first month after transplantation showed significantly lower positive C4d rate in OCS-preserved DCD hearts suggesting a lower IRI rate. During first year, acute cellular rejection (2R) was lower in DCD than DBD group	Conference abstract
Page et al., 2018 (39)	DCD = 31, DBD = 31	DCD hearts retrieved with DPP or TA-NRP, comparable results	Conference abstract

DBD, donation after brainstem death; DCD, donation after circulatory death; DPP, direct procurement and perfusion; IRI, ischemia reperfusion injury; MBD, marginal brain dead; TA-NRP, normothermic regional perfusion; OCS, organ care system; SCS, static cold storage.

had a longer hospital stay (50 vs. 12 days) and a higher cost (AU$ 234,160 vs. 56,658) compared with the OCS recipient (14). In nine publications, only the OCS was studied (**Table 3**) (5, 10, 16, 21, 32–35, 40). In general, the OCS preserved heart function well, resulting in a satisfactory postoperative survival rate for the recipients. Two case reports presented successful transplantations after 10 and 16 h preservation time (5, 21). In one study, hearts from both standard criteria donors and marginal donors (outside standard acceptability criteria) were preserved with the OCS, and no significant differences in 1-month, 1-year, and 2-year survival rates were found. However, there was an increased requirement for extracorporeal membrane oxygenation (ECMO) support in the standard criteria donor group (33% vs. 11%) (33).

The OCS was used for DCD hearts in 11 studies (**Table 4**) (17–20, 22–24, 36–39). In clinical practice, DCD hearts are retrieved with either direct procurement and perfusion (DPP) (17–19, 22–24, 36, 37, 39) or thoracoabdominal normothermic regional perfusion (TA-NRP) (20, 24, 37, 39). For DPP, after confirmation of death, a cardioplegic flush is applied. Thereafter, the heart is excised and transported in a beating state using an OCS. For TA-NRP, after confirmation of death, cardiac resuscitation is achieved with the help of an external pump. After weaning from the TA-NRP, cardiac functional assessment is performed using a pulmonary artery flotation catheter and transesophageal echocardiogram. Four studies reported comparable results between the OCS-preserved DCD hearts and the SCS-preserved DBD hearts (22, 24, 37, 39). However, two hearts were discarded after OCS preservation owing to machine failure (22). One study reported a 100% 3-month survival rate in both OCS-preserved DCD hearts and OCS-preserved marginal brain donor hearts (36). One study compared post-transplant biopsies for C4d and acute rejection episodes. The results suggested a lower IRI rate and similar patterns of cellular rejection for the OCS-preserved DCD hearts compared with the regular DBD transplantation (38). The other five publications presented successful DCD heart transplantations using OCS (17–20, 23). Messer et al. also compared the DPP plus OCS with TA-NRP plus OCS for DCD hearts and found no significant difference in 30- and 90-day survival rates (24, 37).

Five clinical trials are currently recruiting patients (**Table 5**) (41–45). Among these trials, three have a randomized design (42, 43, 45) and four are multicenter studies (41, 42, 44, 45). All ongoing clinical trials use patient/graft survival as the primary endpoint and patient/graft survival in a different time frame and/or graft function as secondary endpoints.

DISCUSSION

Despite encouraging results, considerable challenges still need to be overcome before sound conclusions can be drawn regarding MP for heart preservation. Existing literature in this field is limited. Most of the studies were non-randomized and retrospective, and half of the publications were conference abstracts. The total number of transplantations using MP was low, especially for HMP. A clear advantage of MP has not been observed in randomized controlled studies. Although NMP has shown its superiority in high-risk cases in non-randomized single-centre studies, high-quality clinical trials still need to be conducted.

TABLE 5 | Ongoing clinical trials.

NCT number	Institution	Study phase/ design	Starting date–estimated primary completion date	Estimated number of enrolled patients	Study arms	Outcome measures (time frame)
NCT03687723 (41)	Hannover Medical School, Hannover, Germany	Multicenter, observational	October 2016–December 2021	60	Clinical use of OCS	Primary outcome: patient survival (12 months); secondary outcomes: patient and graft survival (30 days)
NCT03991923 (42)	UZ Leuven, Leuven, Flemish Brabant, Belgium, etc., total eight centers in Europe	Multicenter, randomized	July 2020–July 2021	202	NIHP, STS	Primary outcome: mortality and graft dysfunction (30 days); secondary outcomes: mortality and graft dysfunction (time frame 12 months)
NCT04066127 (43)	Skane University Hospital Lund, Skane, Sweden	Randomized	June 2020–December 2022	66	NIHP, STS	Primary outcome: survival free of acute cellular rejection and re-transplantation (12 months); secondary outcomes: I/R-tissue injury, early allograft dysfunction, and health status
NCT03835754 (44)	Cedars-Sinai, Stanford University, Yale New Haven Hospital, etc., total 12 centers from United States	Multicenter	June 2019–November 2020	48	Clinical use of OCS, high risk donors	Primary outcome: patient survival (30 days), absence of severe PGD (24 h post heart transplant); secondary outcome: patient and graft survival (30 days), incidence of severe PGD and donor heart utilization rate (24 h post-transplant)
NCT03831048 (45)	Stanford University, Yale New Haven Hospital, Mayo Clinic, etc., total 16 centers from United States	Multicenter, randomized	December 2019–August 2021	212	DCD donors: OCS, SCS	Primary outcome: survival (6 months); secondary outcome: utilization rate (within 24 h post-transplant)

DCD, donation after circulatory death; NIHP, non-ischemic hypothermic preservation; OCS, organ care system; PGD, primary graft dysfunction; SCS, static cold storage.

Several publications have concluded that the effectiveness of the OCS seems to be more prominent in high-risk cases and for DCD hearts (5, 16, 46). One explanation could be that the OCS provided a platform for the functional assessment of donor hearts. During perfusion, perfusion parameters such as lactate production could be evaluated, and visual assessment could be performed. Only hearts that meet predefined criteria proceed to transplantation. However, as the only biomarker, serum lactate levels in the perfusate might not be reliable One study reported that five DCD hearts with a perfusate lactate concentration >5 mmol/L had been transplanted with a good outcome (22). As an alternative, TA-NRP can also assess DCD heart function *in situ* (24). During TA-NRP, donor hears can be assessed in a physiologic condition. With the help of a Swan-Ganz catheter and echocardiography, functional assessment can theoretically be better done during TA-NRP than OCS. In one study, two successful DCD heart transplantations were performed after TA-NRP and SCS preservation (37). However, whether the same result can be repeated for more significant number of candidates still needs to be confirmed.

MP may reduce acute graft rejection. A porcine heart study showed that NIHP could significantly reduce donor heart immunogenicity via loss of resident leukocytes, reducing recipient T cell recruitment up to 48 h following transplantation in the absence of immunosuppression (47). No clinical study has addressed on this topic so far. However, if this is confirmed clinically, all the transplantations can benefit from MP.

Ischemia is the main reason a donor heart can only be preserved within a few hours. The principle of the MP is to avoid ischemia. Both preclinical (46) and clinical (5, 21) studies have shown that successful transplantations after more than 10 h of MP preservation can be achieved. A prolonged preservation time would theoretically benefit the transplantation teams and reduce transplantation costs.

Literature on pediatric heart transplantation has been excluded in this review. As far as we know, no MP has been used for clinical pediatric heart transplantation so far. However, due to donor shortage, pediatric transplantations more often involve distant retrieval and complex operations. A MP system for pediatric donor hearts would be extra beneficial.

The perfusion technique and perfusate are the two keys to successful preservation. In Wicomb et al.'s study of HMP (9), only one of the four recipients survived over 16 months. Because the study was performed before 1982, many factors might have played roles in the low survival rate, such as the operative technique, perioperative care, etc. Among other factors, the combination of inadequate perfusion and lack of colloid in the perfusate might also have played a specific role. In pilot studies of porcine heart preserved using HMP, we observed that the albumin concentration in the perfusate was positively related to the myocardial water content (48, 49). The feasibility and effectiveness of this method have been shown in a clinical study (6). In contrast to this albumin-rich hyperoncotic and hyperkalemic solution supplemented with erythrocytes, the

OCS uses diluted whole blood. This can theoretically provide all the necessary nutrients for the heart. However, some donor blood components may have adverse effects, such as pharmacological substances, metabolites, and platelets.

MP could theoretically cause hemolysis, especially at higher pressures and extended preservation times. An animal study showed no hemolysis occurred after 24 h of porcine heart perfusion with the NIHP system (49). With a higher perfusion pressure and flow, the OCS has a higher risk of hemolysis. However, we have not seen any reports about this in clinical trials. Apart from hemolysis, prolonged MP time, especially with NMP, would also lead to metabolite accumulation in the perfusate. However, with post-transplant ECMO support, successful transplantations have been reported after 10 and 16 h of total preservation time with the OCS (5, 21).

In addition to better clinical outcomes, safety and simplicity are crucially important for MP. HMP is theoretically safer and simpler to use than NMP. If a machine malfunction or user error occurs, NMP, which perfuses a beating heart, would have a narrower margin of safety. It was reported that two hearts were discarded after using the OCS owing to machine failure in one DCD study (22). In PROCEED II, five donor hearts were discarded after OCS preservation, despite these hearts being appropriate for transplantation at harvest. However, whether the OCS caused this effect was unclear (7, 50).

Using MP leads to a longer preservation time (129 min longer in the OCS group and 29 min longer in the NIHP group than in the SCS group) (6, 7). Moreover, MP requires additional surgical and technical support, proprietary equipment, appropriate transport, and additional costs. However, it may reduce the length of stay in the intensive care unit or hospital, postoperative mechanical support, and need for reoperation. Therefore, the total cost and labor demand may be reduced (14).

A challenge emerged during literature collection because the same data on MP transplantation has been used repeatedly in different conference abstracts and papers. Such examples can be found in publications from the groups of Rojas S., et al, Nilsson J., et al, Yeter R., et al, Chew, H., et al and García Sáez, D., et al. When the same data have been used in a series of publications, we included only the latest the publications and when only part of the data has been used with different study design, we included all these publications to avoid missing data (16, 33). Consequently, this may jeopardize the objectiveness of this review. Fortunately, the conclusions of these publications have been consistent, and the impact is theoretically minimal.

In summary, the machine perfusion in the form of either HMP or NMP, has emerged a potentially beneficial method for heart preservation. Based on the currently available data, when preserving a regular human donor heart, MP seems to yield clinical outcomes comparable to traditional SCS. However, HMP seems especially beneficial for high-risk cases and DCD hearts. Compared to NMP, HMP seems to be less complex, which may make it more feasible and safer, and this is an excellent advantage for the transportation of donor hearts. In future studies, we believe it's important address the efficiency of MP for donor hearts with isolated risk factors, such as prolonged preservation time, hearts from higher age donors, or low ejection fraction. Additionally, it is also essential to develop an ideal perfusion medium for different types of MP and a system for pediatric transplantation considering the more significant donor shortage.

AUTHOR CONTRIBUTIONS

GQ: Study design; GQ and JN: Study conduction; GQ and JN: Data analysis; GQ, VJ, TS, SS, and JN: Paper writing; JN: Fund collection.

FUNDING

This work was funded by the Swedish Research Council (2019–00487), Vinnova (2017–04689), Swedish Heart-Lung Foundation (20190623), Government Grant for Clinical Research, Region Skåne Research Funds, Donation Funds of Skane University Hospital, Anna-Lisa and Sven Eric Lundgrens Foundation, and Hans-Gabriel and Alice Trolle-Wachmeister's Foundation for Medical Research.

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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REVIEW
published: 14 March 2022
doi: 10.3389/ti.2022.10312

Sense and Sensibilities of Organ Perfusion as a Kidney and Liver Viability Assessment Platform

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Predicting organ viability before transplantation remains one of the most challenging and ambitious objectives in transplant surgery. Waitlist mortality is high while transplantable organs are discarded. Currently, around 20% of deceased donor kidneys and livers are discarded because of "poor organ quality", Decisions to discard are still mainly a subjective judgement since there are only limited reliable tools predictive of outcome available. Organ perfusion technology has been posed as a platform for pre-transplant organ viability assessment. Markers of graft injury and function as well as perfusion parameters have been investigated as possible viability markers during *ex-situ* hypothermic and normothermic perfusion. We provide an overview of the available evidence for the use of kidney and liver perfusion as a tool to predict posttransplant outcomes. Although evidence shows post-transplant outcomes can be predicted by both injury markers and perfusion parameters during hypothermic kidney perfusion, the predictive accuracy is too low to warrant clinical decision making based upon these parameters alone. In liver, further evidence on the usefulness of hypothermic perfusion as a predictive tool is needed. Normothermic perfusion, during which the organ remains fully metabolically active, seems a more promising platform for true viability assessment. Although we do not yet fully understand "on-pump" organ behaviour at normothermia, initial data in kidney and liver are promising. Besides the need for well-designed (registry) studies to advance the field, the catch-22 of selection bias in clinical studies needs addressing.

OPEN ACCESS

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Received: *16 December 2021*
Accepted: *26 January 2022*
Published: *14 March 2022*

Citation:
Verstraeten L and Jochmans I (2022) Sense and Sensibilities of Organ Perfusion as a Kidney and Liver Viability Assessment Platform. Transpl Int 35:10312. doi: 10.3389/ti.2022.10312

Keywords: kidney transplantation, liver transplantation, hypothermic perfusion, machine perfusion, organ preservation, normothermic perfusion, organ viability assessment

INTRODUCTION

One of the underlying causes of the perpetuating organ shortage is the discarding of transplantable organs based on "poor organ quality". Currently, up to 20% of kidneys and 10% of livers that are recovered in the United states are not transplanted (1). Eurotransplant data show similar figures for kidney with considerably lower utilization rates for livers donated

Abbreviations: DCD, donation after circulatory death; DBD, donation after brain death; DGF, delayed graft function; PNF, primary non function; GST, glutathione S-transferase; H-FABP, heart-type fatty acid binding protein; NGAL, neutrophil gelatinase-associated lipocalin; FMN, flavin mononucleotide.

after circulatory death (DCD) compared to those donated after brain death (DBD) (2). A major contributor to organ discard is the fact that organ quality and viability remain difficult to predict accurately (1). With the increasing use of DCD kidneys and livers, the need for reliable pre-transplant viability assessment has become even more important. Indeed, DCD kidneys suffer from higher rates of delayed graft function (DGF) and primary non function (PNF) leading to a significant morbidity and mortality risk for the recipient (3, 4). DGF is associated with an increased risk of acute rejection, longer in hospital stay, higher cost and lower graft survival (5, 6). Higher-risk liver grafts, especially those from DCD donors, suffer higher incidences of PNF and intrahepatic cholangiopathy ultimately leading to higher graft failure rates compared to DBD livers (7, 8).

While with static cold storage, only limited options to assess organ function and viability are available, organ perfusion preservation has been posed as a platform for organ viability assessment (9). During organ perfusion, a perfusion solution is circulated through the vasculature, driven by a pump. The perfusion solution can be cooled or heated and, often with the help of a gas-exchanger, oxygenated. During hypothermic perfusion an acellular perfusion solution is used, in normothermic conditions an oxygen carrier is needed and this are often red blood cells. In this dynamic environment, the organ can be assessed real-time by evaluating perfusion parameters and injury markers (**Figure 1**). When the organ is metabolically active, markers of organ function can also be studied. As (patho)physiology involves a complex interplay of different cells, it is likely that true prediction of organ viability will need the assessment of more than a single parameter.

This review provides an overview of the available clinical evidence on the use of organ perfusion as a platform to predict kidney and liver viability before transplantation.

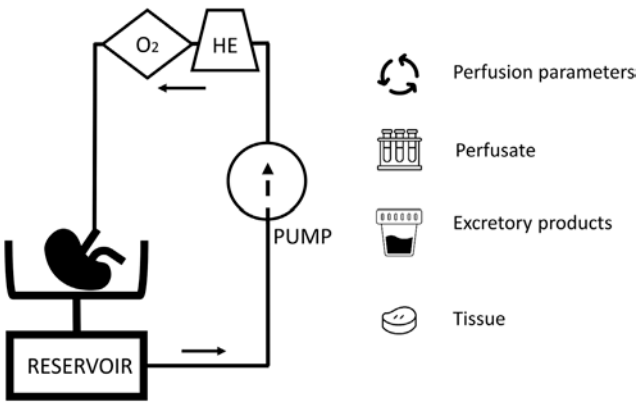

FIGURE 1 | A schematic overview of a kidney perfusion circuit: The presence of a heat exchanger (HE) and gas exchanger (O_2) depends on the perfusion mode. During perfusion, perfusion parameters, perfusate, excretory products (e.g., urine during normothermic perfusion), and tissue are available for viability assessment.

KIDNEY

Hypothermic kidney perfusion became a clinical reality after much preclinical work in the 1960s by pioneers like F.O. Belzer (10–12). Due to refinement of preservation solutions good results with the cheaper and simpler static cold storage were obtained and kidney perfusion disappeared to the background. Nevertheless, hypothermic kidney perfusion has been reintroduced in clinical settings after it was shown to reduce the risk of DGF compared to static cold storage (13). Normothermic perfusion is being investigated in research settings with a first randomised trial underway (14).

Pathophysiology of the Ischemic Injury

To assess kidney viability, understanding the pathophysiology of ischemia reperfusion injury is crucial. Every transplantation procedure is associated with ischemia reperfusion injury that impacts post-operative tissue injury and graft function. The biological pathways behind ischemia reperfusion injury describe functional and structural changes in the organ based on changes in cell metabolism (especially in the mitochondria). Various molecular mechanisms are active in ischemia reperfusion injury. There is the critical role of the anaerobic metabolism during ischemia, resulting in intracellular acidosis, ATP depletion, and failure of ion-exchange channels, setting the stage for reperfusion injury (15). Post-reperfusion, innate and adaptive immune responses are activated by reactive oxygen species and damage associated molecular patterns, resulting in a sterile inflammation (16–19). Ischemia reperfusion injury causes structural and functional damage to renal tubules by inducing tubular cell death which manifests as a clinical spectrum of acute kidney injury ranging from transient acute kidney injury to primary non-function (PNF). When the transient acute kidney injury is severe enough, and the patient needs dialysis in the first week after transplantation, delayed graft function (DGF) occurs. An association between DGF and acute rejection has been reported (20) and this might affect long-term graft function as persistent inflammation in scarred areas after T-cell mediated rejection has been associated with chronic scarring and fibrosis due to maladaptive injury responses (21).

This injury process leaves marks, e.g., representing epithelial cell disruption and tubular injury that might be detected as biomarkers in the perfusate (22, 23).

Hypothermic Kidney Perfusion

In hypothermic conditions, options to assess kidney function are limited. Indeed, the metabolic rate at 4°C is limited to 10% of that at physiological temperature with a 40% lower rate of chemical reactions (24). Furthermore, in the majority of cases there is no active oxygenation during hypothermic kidney perfusion in which case aerobic metabolism is not supported (25). Focus has therefore been on identifying associations between markers of injury and post-transplant outcome.

Perfusate Injury Markers

Injured tubular cells release proteins into the perfusate during hypothermic perfusion where they can be detected. Today, there

is good quality evidence that perfusate injury markers should not be used to assess viability of kidneys during standard hypothermic organ perfusion. In a systematic review, Guzzi et al. summarized the findings of 29 clinical studies assessing the association between PNF, DGF, and long-term graft survival and perfusate injury markers measured during hypothermic perfusion of DCD and DBD kidneys (26). Only four studies were identified as good quality prospective studies (27–30).

Glutathione S-Transferase (GST) concentrations during hypothermic perfusion have been well-studied with an independent association with DGF, however, the predictive accuracy of GST for DGF is moderate at best and no correlation with long-term outcome has been found (27–29). Similar to GST, perfusate lactate dehydrogenase independently associates with DGF and PNF but predictive accuracy is low (27, 31). While heart-type fatty acid binding protein (H-FABP) showed to be an accurate biomarker of kidney injury after transplantation in preclinical studies (32), clinical studies showed only moderate predictive power of perfusate H-FABP for DGF (27, 31). Neutrophil gelatinase-associated lipocalin (NGAL), released by renal tubular cells in response to ischemic injury, is a recognized biomarker of acute kidney injury (26, 30, 31, 33), but no reliable association of NGAL release during hypothermic perfusion with post-transplant outcomes has been found (31). Some studies assessing perfusate lipid peroxidation and perfusate interleukin-18 (a pro-inflammatory cytokine) show little promise as viability markers (28, 31). Associations between other biological parameters, like lactate, N-acetyl-D-glucosamine, Kidney injury molecule 1, and others were either not significant, not accurate, or described in single studies. Growing interest in microRNA's in multiple disease processes draws our attention for their use in viability assessment during hypothermic perfusion (34).

Whether predictive accuracy of perfusate injury markers is improved when the perfusate is actively oxygenated, is not known and subject of ongoing research (www.cope-eu.org). This is an important question as hypothermic oxygenated perfusion is already finding its way into clinical practice (e.g., the Netherlands) after it was recently shown that older DCD kidneys benefit from active oxygenation in the cold (25).

Perfusion Parameters

Since the early days of hypothermic kidney perfusion, it has been hypothesised that kidney viability is associated with perfusion parameters. Indeed, at a stable perfusion pressure, a lower renal flow indicates a higher intrarenal resistance and reflects increased vascular injury or interstitial oedema. A correlation between perfusion parameters and DGF and PNF has been shown in retrospective studies that suffered from selection bias as kidneys were discarded based upon perfusion parameters(35–38). A large randomized controlled prospective trial, without selection bias, has shown that renal resistance at the end of hypothermic perfusion is an independent risk factor for DGF and 1-year graft survival but the predictive accuracy is low (39). These findings have been confirmed by Parikh et al. in a large prospective cohort (30).

While perfusion parameters, such as renal resistance on the pump, provide additional information on quality of the graft, they should not be used as clinical decision making tools. When the perfusate is actively oxygenated, endothelial cell integrity might be improved. This might change perfusion parameters and their predictive power which is the subject of ongoing research (www.cope-eu.org). In addition, in relating Ohm's Law to fluid flow (**Eq. 1**), it is important to remember that exact flow or resistance values will depend not only on the kidney but also on the perfusion device (pressure or flow driven) and the settings (e.g., pump pressure chosen) that are used. Perfusion parameter read-outs, and therefore any defined thresholds, are not necessarily transferable from one perfusion device to the other.

$$\Delta P/F = R \quad (1)$$

where ΔP is the driving pressure of perfusion pressure as set by the pump (in mmHg) in case of a pressure-controlled system, F is renal artery flow (ml/min), and R is the renal resistance (mmHg/mL/min).

Normothermic Kidney Perfusion

The advantages of normothermic perfusion with regard to viability assessment relate to the use of a perfusate based on oxygenated red blood cells or oxygen carriers at physiological temperatures, meaning the graft can be fully metabolically active. In addition to assessing injury markers and perfusion parameters, normothermic perfusion would therefore allow to evaluate kidney function. Indeed, e.g., creatinine can be added to the perfusate and in this way a creatinine clearance from the perfusate over time can be calculated. In contrast to hypothermic perfusion, normothermic perfusion requires considerable technical expertise with the potential of dramatic consequences in case of technical failure as the graft would be exposed to warm ischemia.

Normothermic perfusion as mostly been developed to be used as a "resuscitation tool." This means a short period (1–2 h) of normothermic perfusion immediately before transplantation following static cold storage (40). Results of a first randomised controlled phase II trial assessing the effectiveness of normothermic perfusion as a resuscitation tool compared to static cold storage in controlled DCD kidneys are awaited (41). Meanwhile, experimental data show the feasibility, and possible benefit, of prolonged normothermic perfusion preservation starting immediately after kidney procurement (42, 43).

Initial evidence that normothermic perfusion could be used as a platform to assess viability pre-transplantation was provided by Hosgood et al. when a discarded kidney was transplanted after evaluation during a short period of normothermic perfusion (44). In a further series of kidneys, that were considered unsuitable for transplantation, a kidney quality score during normothermic perfusion was derived. This score is based on the macroscopic aspect of kidneys during perfusion, the arterial flow, and volume of urine production. Kidneys with a score ≥3 out of 5 were considered transplantable (**Table 1**) (44–46). The clinical studies leading up to development of the score suffered from selection bias because not all kidneys were transplanted. The score remains

TABLE 1 | Kidney quality assessment score as defined by Hosgood et al.

Kidney quality assessment score parameter	Point
Macroscopic assessment	
Grade I: Excellent perfusion (global pink appearance)	0
Grade II: Moderate perfusion (patchy appearance)	1
Grade III: Poor perfusion (global mottled and purple/black appearance)	2
Renal Blood flow	
Threshold ≥50 ml/min/100 g	0
Threshold <50 ml/min/100 g	1
Total urine output	
Threshold ≥50 ml/min/100 g	0
Threshold <50 ml/min/100 g	1

Scores range from 1 to 5, 1 indicating the least injury to 5 the most severe. Reproduced from (95) with permission under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0).

to be validated in large series. In that light, it is important to realise that the majority of the evidence on the use of normothermic perfusion as a viability assessment platform has been obtained from kidneys that were perfused on a custom made circuit. Therefore, the threshold flow values as proposed by Hosgood et al. depend on the perfusion pressure (**Eq. 1**) (47) and are not directly transferable to settings using different perfusion pressures. Also, although the physical properties of the filter remain the same when a healthy kidney is perfused *ex situ*, the perfusate composition and perfusion pressures (pump pressures) will change oncotic and hydrostatic pressures and therefore influence filtration and ultimately "urine" production during kidney perfusion (48). Adding tubular injury markers to the kidney quality assessment score might improve its accuracy and this has been explored (49).

Importantly, Schutter et al. recently showed that early functional assessment may not reflect actual physiology. In a pig model of normothermic perfusion kidneys were mainly centrally perfused in the first 2 h of perfusion, while it took time for the outer cortex to reach its physiological dominant perfusion state (50). Before that, the functionally important renal cortex appeared severely underperfused, meaning longer perfusion times might be needed for reliable viability assessment. This point was also raised by Hosgood et al. who recently published a report on a pair of kidneys that had passed the quality assessment test but still developed PNF (51).

LIVER

In contrast to kidney perfusion, liver perfusion has not yet reached the stage of wide-spread clinical implementation. Building on the pioneering work of Starzl and others (52–54), both hypothermic and normothermic liver perfusion are now the topic of several clinical studies investigating the value of perfusion as a preservation method but also as a platform for organ viability assessment. The need for optimized preservation and reliable viability assessment is high as an increasing number of DCD livers, at higher risk of PNF and post-transplant cholangiopathy, are offered for transplantation (7, 8). Like in the kidney, ischemia reperfusion injury in the liver causes cellular injury. Hepatocellular injury leads to a spectrum of clinical presentation, marked by increased transaminases. When severe, early allograft dysfunction occurs which is associated with increased mortality and graft loss (55–57). When irreversible, in the case of PNF, recipient mortality is high (58). While the liver regenerates, it remains difficult to assess what level of injury a liver can tolerate while still providing life sustaining function to the recipient. Furthermore, cholangiocyte injury and injury to the peribiliary plexus can lead to post-transplant cholangiopathy, a vexing complication leading to increased morbidity and reduced graft survival (59, 60). Liver perfusion offers a window of opportunity to gather additional information on both the level of injury sustained and the remaining liver function.

Hypothermic Liver Perfusion

Like in kidney, options to assess liver function during hypothermic perfusion are likely limited because metabolic rate is severely reduced. However, in contrast to kidney, hypothermic liver perfusion is nearly always actively oxygenated and mitochondrial respiration continues (61). A short period of hypothermic oxygenated perfusion of the liver has been described to have immunomodulatory effects, preserve the endothelial cell glycocalyx and the peri-biliary vascular plexus and glands, and improve post-transplant outcomes (61–65). Recent studies have shown less post-transplant hepatocyte injury and reduced cholangiopathy rates with hypothermic oxygenated perfusion (65, 66).

Perfusate Injury Markers

In the first clinical series of hypothermic liver perfusion, Guerrera et al. already described a correlation between perfusate and post-transplant serum transaminases (63, 67). These findings were confirmed by Patrono et al. but none of the injury markers were independently associated with outcomes (68). The detection of mitochondrial flavin mononucleotide (FMN), an integral part of mitochondrial complex I, in the perfusate might be a surrogate marker for impaired cellular energy production.

There is evidence that the release of FMN occurs independently of the other hepatocellular enzymes (69). A strong correlation of FMN with post-transplant peak transaminases and coagulation factors was found in addition to correlation of FMN with hospital stay, post-transplant complications, and graft failure within 3 months (69). FMN was also predictive of early allograft dysfunction (69). The correlation of FMN with early allograft dysfunction was also described by Patrono et al. though not found to be significant (62). Currently there is too little evidence to conclude whether injury markers measured during hypothermic oxygenated liver perfusion are helpful in predicting viability. With the completion of the first large trials, further evidence on the proper value of these markers is likely to become available in the near future (NCT01317342) (65).

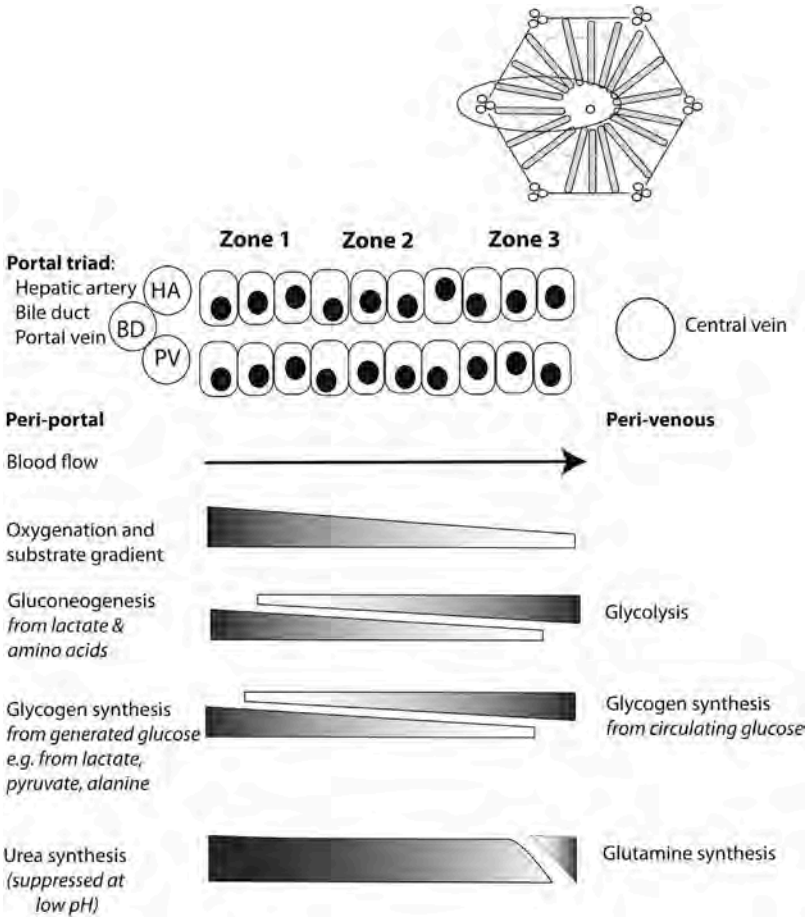

FIGURE 2 | Schematic overview illustrating metabolic liver zonation with reference to glucose and ammonia metabolism. Blood entering the liver lobule *in vivo* through hepatic artery (HA) and portal vein (PV) branches is rich in hormones, nutrients and oxygen. Periportal (zone 1) metabolic processes will include those requiring such conditions, while perivenous (zone 3) hepatocytes may preferentially include those metabolic processes that are less dependent on high levels of oxygen, for example, or those requiring products made in the periportal hepatocytes, such as urea. Reproduced from (84) with permission under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0).

Perfusion Parameters

Very little is known about the relationship between hepatic artery or portal vein flow and resistance during hypothermic oxygenated liver perfusion. Like in the kidney, an increase in flow over time and a decrease of hepatic artery resistance are observed (65, 70). Patrono et al. observed a slower decrease in hepatic artery resistance in livers that developed early allograft dysfunction but larger series need to be analysed to understand the value of perfusion parameters as predictor of post-transplant viability (70).

Normothermic Liver Perfusion

In contrast to normothermic kidney perfusion, normothermic liver perfusion is more widely studied. In a randomised study, normothermic liver perfusion has been shown to reduce post-transplant graft injury, measured by hepatocellular enzyme release, compared to cold storage (71). Despite these findings, no differences were seen in graft or patient survival, hospital stay and bile duct complications. Remarkably, a 50% lower rate of organ discard was noticed in the perfusion arm, confirming the need for pre-transplant viability assessment to increase the number of liver transplants. It must be noted that this trial was not designed to address organ utilization and selection bias because of the non-blinded nature might have been present. Trials with organ utilization as primary outcome should randomise as early in the process as possible, ideally at the time of the organ offer or even at the time of listing the patient for transplant (72). A short period of normothermic liver perfusion to test viability has been explored by a number of groups (73–82). Encouraging results have led to the implementation of normothermic liver perfusion as a viability assessment tool in expert centres although there is considerable variability in both indications and assessment criteria (83). Because the liver is metabolically active, liver function might be assessed during normothermic perfusion. In this light it is important to remember that both hepatocytes and

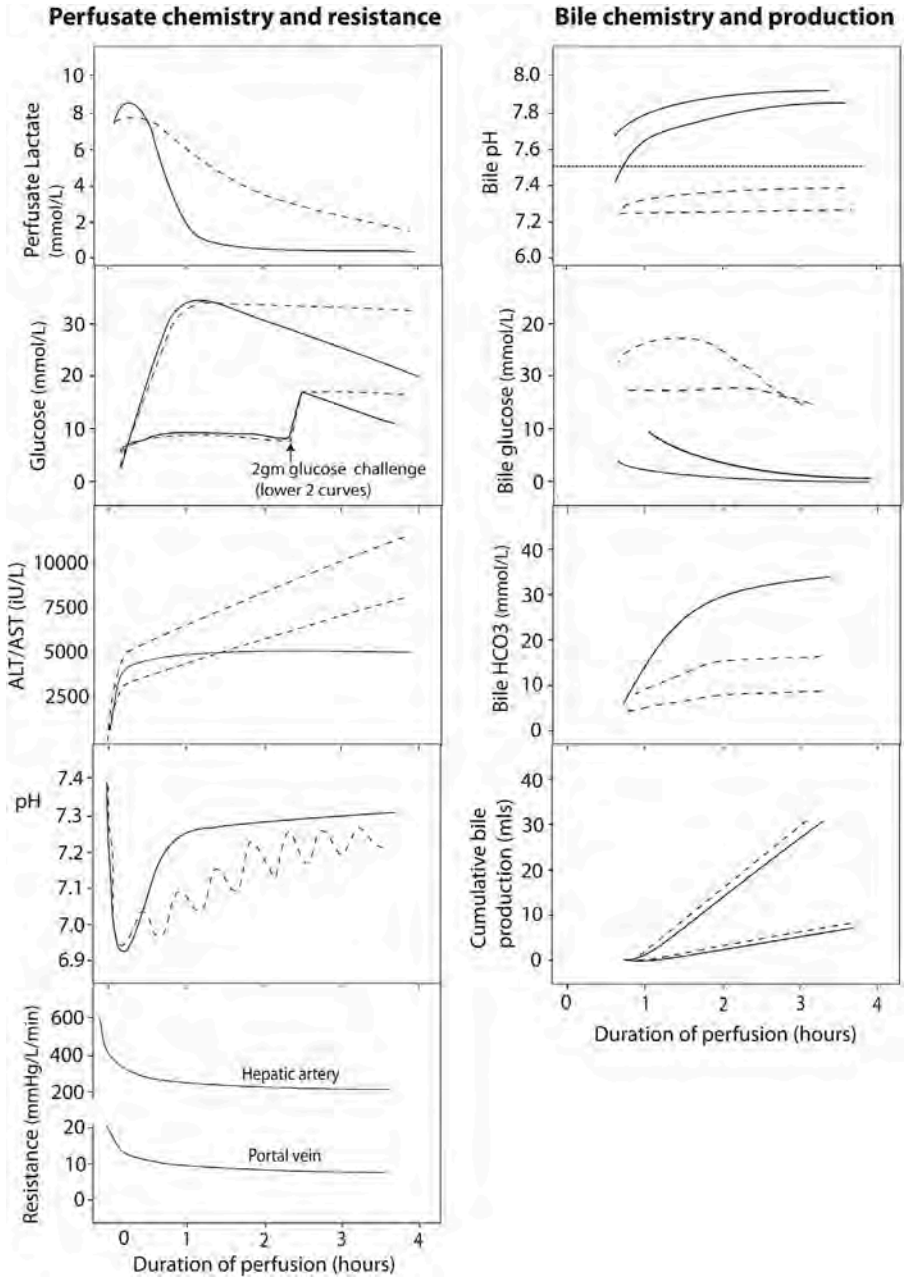

FIGURE 3 | Typical normothermic perfusate profiles of liver perfusion. The figure shows schematic graphs with typical biochemical and resistance profiles during normothermic liver perfusion with an interpretation regarding viability given according to current state of knowledge. Profiles of viable hepatocellular compartment livers are denoted by solid black lines, while dashed lines denote grafts where viability might be in doubt, due to a slow lactate clearance, persistently raised perfusate glucose, rising perfusate transaminase concentration or requirement for continued bicarbonate support to maintain pH. The graphs also show the different biochemical profiles of bile depending on the viability of the ducts, where viable cholangiocytes producing bile with an alkaline pH, low glucose (especially relative to the high perfusate glucose) and increasing bicarbonate levels. To date, there is no clinical evidence in support of bile production or hepatic resistance thresholds for viability. Reproduced from (84) with permission under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0).

cholangiocytes need to be functioning for sustained graft function and survival.

Markers of Hepatocyte Injury and Function

In assessing the hepatocyte compartment, the zonation of the hepatocytes helps when interpreting the meaning of several perfusate markers (84). As oxygen concentrations are the highest in the periportal zone, zone 1 hepatocytes are differentiated to carry out processes that require high oxygen concentrations (**Figure 2**). Near the central vein, zone 3 hepatocytes are adapted to the low oxygen concentrations that are present.

Perfusate Transaminases

Perfusate transaminases (as opposed to postoperative systemic levels of transaminase) have been used to determine the viability of a particular graft for implantation. In viable livers, perfusate transaminases seem to plateau over time. Most livers will reach this plateau by 2 h (76, 77, 82) therefore continued transaminase increase is suggestive of ongoing injury during perfusion (**Figure 3**). It must be noted that transaminase levels may be influenced by the age of the donor, steatosis, ischemia time, among other factors (72). Perfusate transaminases should be normalized for liver weight and perfusate volume to allow comparability with other perfusion systems and different livers (72). Because aspartate aminotransferase may also rise from haemolysis on the circuit, alanine aminotransferase might be more representative of the degree of hepatocellular damage (76, 77, 85).

Perfusate transaminases seem to be correlated with post-transplant systemic levels of transaminases (77) though the usefulness of this correlation in helping predict outcome is unclear. Indeed, postoperative levels of transaminases are influenced by the perfusion itself and the large volume of perfusate (wash-out) (72). Additionally, bilirubin and INR seem to have a stronger predictive capacity for patient and graft survival compared to AST, indicating that hepatocyte injury with little involvement of the biliary tree has a more benign course (86). The usefulness of the current definition of early allograft dysfunction (using peak transaminases in the first week, total bilirubin and INR levels) (55) in case of livers transplanted after perfusion is unclear and the definition might need revisiting (86, 87).

Perfusate Lactate

A slow clearance of lactate is associated with severe parenchymal injury where viability may be in doubt (71, 73, 77, 84, 85). Indeed, lactate metabolism occurs mainly in the periportal hepatocytes (zone 1), so a viable rim of zone 1 hepatocytes can metabolise the lactate in the relatively small volume of perfusate, even in the presence of severe parenchymal damage in zone 2 and 3 (**Figure 3**). Therefore, lactate is not recommended as a single viability marker.

Perfusate Glucose

Glycogenolysis is an ATP-independent process that continues during cold storage, evidenced by increasing perfusate glucose levels early during normothermic perfusion (**Figure 3**). A normal level of glucose during normothermic perfusion may reflect minimal ischemia, but may point out glycogen exhaustion or extensive liver injury (77). Over time, a viable liver will re-incorporate this glucose into glycogen during perfusion (**Figure 3**) (77).

Acid-Base Homeostasis During Perfusion

Regulation of the hepatic acid-base balance depends, among others, upon the differential metabolism of glutamine along the lobule (88). Healthy livers tend to have a better pH regulation and stabilisation (**Figure 3**). Analysing pH and the need for external regulation by bicarbonate replacement could help assessment viability of the hepatocyte compartment (76, 77).

Coagulation Factors During Perfusion

In a preclinical study, severely injured livers have low perfusate levels of anticoagulant and coagulation factors compared to those that are minimally injured livers (89). Little information on the value of perfusate (anti)coagulation factors in human settings is available. Such proteins are detectable but no correlation between (anti)coagulation factors and severity of post-transplant injury has been shown (89, 90). Whether low factor concentrations are predictive of outcome remains to be investigated (89).

Bile Production During Perfusion

Bile production is an important function of the hepatocyte and the volume of bile produced during normothermic perfusion has been associated with hepatocyte injury (91). However, the absence of bile production during perfusion is not necessarily a feature of a non-viable graft (71, 92).

Markers of Cholangiocyte Injury and Function

The importance of assessing cholangiocyte viability was recently demonstrated by Mergental et al. who selected livers, thought unsuitable for transplantation on static cold storage, based on hepatocyte viability criteria. Of 31 initially discarded livers, 22 (71%) met hepatocyte viability criteria were successfully transplanted with no PNF cases. However, three out of ten (30%) DCD livers developed biliary complications requiring retransplantation (80). Indeed, while the hepatocyte is responsible for producing bile, the healthy cholangiocyte ensures an alkaline composition of bile with low glucose levels (**Figure 3**) (92, 93). Watson et al. and Matton et al. provide suggested cut off values for bile pH, glucose, and bicarbonate concentrations that need validation in large series (77, 78, 85, 94). As for kidney, clinical studies identifying these cut-off values suffer from selection bias as not all livers were transplanted, though pathological assessment of the intra-hepatic bile ducts of some of the non-transplanted livers were correlated with bile biochemistry (77).

Perfusion Parameters

Hepatic artery and portal vein resistance decrease quickly during perfusion to reach a steady state (**Figure 3**). Little is known about the meaning of these findings though Watson et al. observed no correlation of these parameters with outcome or biochemical markers of hepatocellular injury (77).

CONCLUSION

Organ perfusion has demonstrated it can serve as a viability assessment tool with current evidence suggesting normothermic perfusion is better suited. Indeed, although good quality evidence shows that injury markers and perfusion parameters during hypothermic kidney perfusion predict graft outcome, these markers lack the predictive accuracy needed in clinical practice. Little is known about the association of liver perfusate injury markers and perfusion parameters during hypothermic perfusion and this deserves further investigation. The recent large clinical trials, where livers were transplanted regardless of perfusate markers, provide valuable cohorts.

Normothermic perfusion, with a metabolically fully active organ, has been shown to be able to select viable grafts from

those that were thought unsuitable for transplantation. Nevertheless, to date, there are no clear, validated and accurate markers to allow routine implementation of the technique in clinical settings. Data from larger studies are needed. Ideally, selection bias should be avoided by transplanting all organs that are perfused and blinding clinical teams to the viability assessment findings. However, as these studies would involve organs of doubtful viability, and therefore a reasonable chance of post-transplant failure, this obviously poses ethical concerns exposing patients to an increased risk of complications. One way would be to accumulate cases in large international registries so that a high enough number of cases with an undesirable outcome can be analysed together.

AUTHOR CONTRIBUTIONS

IJ conceptualized the paper. LV and IJ reviewed the literature. LV drafted the manuscript. IJ performed the critical revision.

CONFLICT OF INTEREST

IJ has received speaker's fees from XVIVO perfusion paid to her institution.

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

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REVIEW
published: 17 March 2022
doi: 10.3389/ti.2022.10108

Liver Transplantation as a Cornerstone Treatment for Acute-On-Chronic Liver Failure

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Acute-on-chronic liver failure (ACLF) is a distinct clinical syndrome, characterized by acute decompensation (AD) of liver cirrhosis, severe systemic inflammation, intra- and extrahepatic organ failures, and a high short-term mortality. Liver transplantation (LT) is a potentially life-saving treatment for patients with decompensated liver cirrhosis and, due to the high mortality rates, particularly for ACLF patients. In the last decade, a plethora of studies has produced compelling evidence in favor of LT in ACLF, demonstrating high post-LT survival rates and excessive waitlist mortality. The importance of LT in these patients is underscored by the fact that no specific therapy for ACLF is available yet, rendering expeditious life-saving LT to be the only feasible treatment option for some ACLF patients. This review aims to provide an overview on pathophysiology, clinical trajectory, and clinical management of ACLF and to delineate the current literature regarding perspectives and limitations of LT as a life-saving treatment option for ACLF patients.

Keywords: liver transplantation, decompensated cirrhosis, liver cirrhosis, ACLF, acute-on-chronic liver failure

INTRODUCTION

Liver cirrhosis constitutes a significant public health burden worldwide. It is associated with a high morbidity and a significant loss of disability-adjusted life-years (1–3). Acute decompensation (AD), defined by the onset of cirrhosis-related complications and hospitalization, is a watershed moment in a patient's clinical course and is associated with a marked decline in survival (4). Recent studies have suggested that AD defines a heterogeneous syndrome with distinct clinical phenotypes and not a unidimensional continuum, ending in ACLF (5–7). While clinical trajectories significantly differ between these phenotypes, a considerable fraction of patients with AD progress to pre-ACLF or present manifest acute-on-chronic liver failure (ACLF). Severe systemic inflammation (SI) is the hallmark of ACLF, a crucial driver in disease progression (8, 9). ACLF is defined by acutely decompensated cirrhosis with development of extra- and/or intrahepatic organ failures and it is associated with a median transplantation-free 28-day mortality of 32.8% (10). Moreover, a recent study demonstrated ACLF to be highly prevalent worldwide in patients admitted to the hospital with AD (see **Figure 1**) (11, 12). Although patients with defined ACLF undergo liver transplantation (LT), to date, presence of ACLF and ACLF severity are not specifically prioritized in organ allocation.

Due to scarcity of donor organs, strong competition exists for patients on the waiting list for liver grafts, and patients with decompensated cirrhosis must also contest with patients listed for other indications with time-sensitive match MELD score, especially with hepatocellular carcinoma (HCC). MELD score-based allocation systems were designed to stratify waiting list patients with decompensated cirrhosis and to allocate liver grafts following the 'sickest first' principle.

OPEN ACCESS

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Received: *12 October 2021*
Accepted: *27 January 2022*
Published: *17 March 2022*

Citation:
Schulz MS, Gu W, Schnitzbauer AA and Trebicka J (2022) Liver Transplantation as a Cornerstone Treatment for Acute-On-Chronic Liver Failure. Transpl Int 35:10108. doi: 10.3389/ti.2022.10108

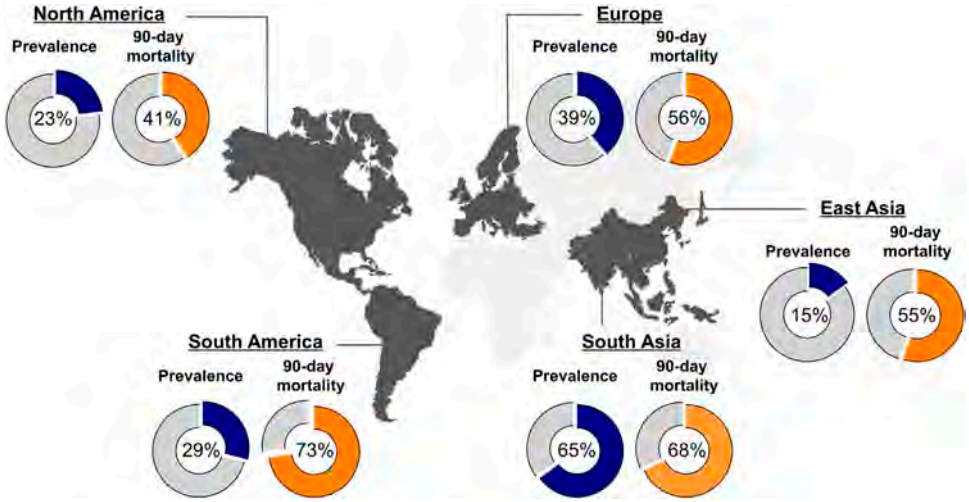

FIGURE 1 | Global prevalence and 90-day mortality of ACLF. Figure displays the global prevalence of ACLF (blue piechart), according to EASL-CLIF criteria, and 90-day mortality rates (orange piechart) depending on the geographical region as reported by Mezzano et al. (11).

However, in recent years, limitations of these allocation systems have become apparent, particularly because current prognostic models do not adequately take into account that prognosis and dynamics differ distinctly between AD phenotypes and ACLF.

In the last decade, several studies have evaluated post-transplant outcomes in patients receiving LT with ACLF. The aim of this review is to provide an overview of our current understanding on pathophysiology, clinical trajectory, treatment options and prognosis of ACLF and to review recent literature on LT as a life-saving treatment option in patients with ACLF.

DEFINITIONS OF AD AND ACLF

AD is defined by the onset of cirrhosis-related complications, such as development of ascites, gastrointestinal hemorrhage, hepatic encephalopathy or bacterial infection leading to hospitalization (6). The development of AD constitutes a decisive time point and a "prognostic watershed" in the clinical course of cirrhosis (13). The trajectory of end-stage cirrhosis is commonly shaped by these decompensating events, whereby the first episode of AD leads to a significant reduction of the median survival time from 12 to less than 2 years (4, 14). In 30% of patients, AD progresses to development of hepatic and/or extrahepatic organ failures, which, together with a severe systemic inflammatory response, are the hallmarks of ACLF (10, 15, 16). ACLF is considered a distinct clinical syndrome, highly prevalent worldwide, and it is associated with a high short-term mortality, rendering it a global public health problem (17). The Chronic Liver Failure Acute-on-Chronic Liver Failure in Cirrhosis (CANONIC) study determined major risk factors in patients with AD, which were associated with a high short-term mortality. Derived from the findings of the pioneering CANONIC study, the Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA) score and the Chronic Liver Failure Consortium Organ Failure (CLIF-C OF) score were developed (10, 18) (see **Table 1**). According to the EASL-CLIF definition, patients present manifest ACLF in case of:

1. Single kidney failure (serum creatinine ≥2 mg/dl)
2. Single organ failure combined with kidney dysfunction (serum creatinine ranging from 1.5 to 1.9 mg/dl) and/or mild-to-moderate HE
3. Presence of two or more organ failures

This definition provides a higher mortality than sepsis in cirrhosis, which clearly defines a severe clinical situation. After ACLF development, the clinical course of patients varies. While some show rapid clinical deterioration, others improve towards resolution of ACLF. Recently, a large meta-analysis of global epidemiological data found that ~35% of patients admitted to hospital due to acutely decompensated liver cirrhosis, in fact presented defined ACLF at admission, according to EASL-CLIF criteria (11). These findings underscore the global impact of ACLF and the challenge that its clinical management poses to hepatologists and ICU physicians. Outcome is largely determined by ACLF severity, which is defined by the presence and the number of organ failures. Patients with ACLF grade 1 show a 90-day mortality of 41%, while patients with two organ failures (ACLF grade 2) or three and more (ACLF grade 3) show an even higher mortality rate of 55% and 78%, respectively (19). In contrast, 90 day-mortality in patients with AD is reported to be 14% (7). **Table 1** displays thresholds for defined organ failures according to the CLIF-SOFA score, while **Figure 2** shows clinical constellations of organ failures in ACLF and their respective ACLF grading (20).

Although no globally accepted homogenous definition of ACLF has been established to date, the principles of the different operating definitions according to the geographic region, the European EASL-CLIF, the North American

TABLE 1 | – CLIF- Sequential Organ Failure Assessment (SOFA) score defining thresholds for organ failures (bold) to assess ACLF severity (20).

CLIF-sequential organ failure assessment (SOFA) score					
Organ failure	**0**	**1**	**2**	**3**	**4**
Liver (bilirubin, mg/dl)	<1.2	≥1.2-<2.0	≥2-<6.0	≥6.0-<12.0	**≥12.0**
Kidney (creatinine, mg/dl)	<1.2	≥1.2-<2.0	**≥2-<3.5**	**≥3.5-<5.0**	**>5.0 or RRT**
Cerebral (HE grade)	No HE	HE grade I	HE grade II	**HE grade III**	**HE grade IV**
Coagulation (INR or PLT count)	<1.1	≥1.21 < 1.25	≥1.25-<1.5	≥1.5-<2.5	**>2.5 or PLT count ≤20.000**
Circulatory (MAP, mmHg and vasopressors)	≥70	<70	**Dopamine ≤5* or dobutamine or terlipressin**	**Dopamine >5* or E ≤0.1* or NE ≤0.1***	**Dopamine >15* or E >0.1* or NE >0.1***
Lung					
PaO2/FiO2	>400	>300–≤400	>200–≤300	**>100–≤200**	**≤100**
SpO2/FiO2	>512	>357–≤512	>214–≤357	>89- ≤214	**≤89**

TABLE 2 | Clinical characteristics, prognosis and therapy options for patients with SDC, UDC, pre-ACLF and ACLF, according to findings of the PREDICT study (6).

	Stable decompensated cirrhosis (SDC)	**Unstable decompensated cirrhosis (UDC)**	**Pre-ACLF**	**ACLF**
Systemic inflammation	Minor	Moderate	Severe	Highly severe
Complications	Benign clinical course	Primarily portal hypertension-driven complications	Incipient organ dysfunctions	Manifest (multi-)organ failure(s), sepsis, IMC/ICU
Prognosis	Recompensation, discharge	Readmission due to AD	Development of ACLF after approx. 14 days	Organ failures, intensive care
Therapy	Out-patient clinic	Management of complications, consider LT evaluation	Evaluation for LT	Rapid LT, possibly ELS as briding-to-transplant
LT within 12 months	11.8%	16.7%	15.1%	—
1-year mortality without LT	9.5%	35.6%	67.4%	—

NACSELD or the Asian Pacific APASL-AARC definition, mirror the differences in clinical practice while highlighting similar principles of organ failures, SI and high short-term mortality. In future years, the community needs to develop and homogenize a uniform definition, which will be acceptable worldwide.

Precipitating Events of Acute Decompensation

The development of AD and ACLF is frequently caused by precipitating events, most commonly bacterial infection, alcohol-induced hepatitis, gastrointestinal bleeding or toxic encephalopathy (21). In most cases, patients show either bacterial infection and/or severe alcoholic hepatitis, alone or in combination, upon onset of decompensation. Importantly, recent data shows that the type of precipitating event does not determine patient outcome but, instead, the number of precipitants does. The PREDICT study found that ACLF patients with two or more precipitants showed a significantly higher 90-day mortality (63.4%) than patients with one (49.7%) or no determinate precipitant (42.2%) (21). In up to 60% of AD patients and 29–40% of ACLF patients, no precipitant could be identified (5, 21).

A large meta-analysis published shortly before the multicentric PREDICT study found bacterial infections to be the most prevalent precipitating event world-wide. This meta-analysis included 30 studies, analyzing data of 140,835 patients with AD and 43,206 with ACLF, defined by EASL-CLIF criteria (11). Interestingly, the authors were able to map geographical heterogeneity of ACLF prevalence and mortality rates as well as preceding trigger events. In line with the findings of PREDICT, this study also reported alcohol to be the second most frequent ACLF trigger in European study cohorts after bacterial infection, while in other geographical regions, namely East Asia and North America, alcohol consumption was the most common trigger. Viral infections played a minor role as ACLF triggers in Asia (10–12%) but were almost non-existent in other regions of the world (0–1%).

Clinical Courses of AD and ACLF

In recent years, large prospective studies, such as CANONIC and PREDICT, have provided corroborating data on the proposed systemic inflammation hypothesis, suggesting SI to be a major driver in the progression of AD to ACLF and a crucial determinant of a patient's clinical course (6, 10, 15, 22). The development of cirrhosis-related complications and organ failures depend on a shared pathophysiological background, which is largely determined by progression of SI (7, 9). Importantly, the grade of SI is not only associated with disease severity but also with overall patient survival (23, 24).

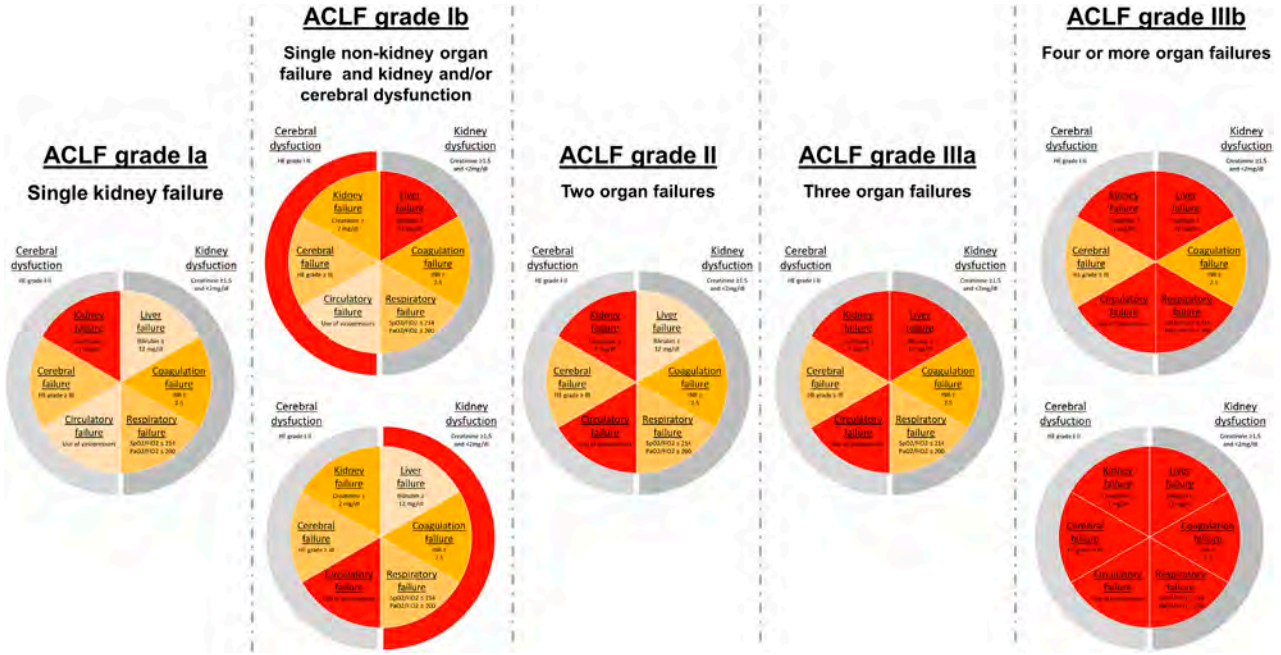

FIGURE 2 | ACLF grades according to EASL-CLIF criteria. Classification of ACLF grades based on organ failure assessment by the adapted Chronic Liver Failure—Organ Failure (CLIF-OF) score (18). Pie charts show organ failure constellations in ACLF patients and the corresponding ACLF grade. Central slices display organ failures (OF), while outer slices represent relevant organ dysfunctions. Cut-off values for defined OFs, according to the CLIF-OF score, are displayed in each slice.

Recently, the PREDICT study revealed that AD constitutes a heterogeneous clinical condition with distinct clinical phenotypes (6). These clinical phenotypes are characterized by a distinct pathophysiology and are associated with a markedly different prognosis. Therefore, a novel classification has been proposed by the authors of the PREDICT study, dissecting these distinct clinical courses of AD. Patients with stable decompensated cirrhosis (SDC) represent most patients admitted with AD. These patients show detectable but low SI, present cirrhosis-associated complications less frequently, are more likely to be recompensated quickly and have a lower 1-year mortality risk (6, 25).

In contrast, patients with unstable decompensated cirrhosis (UDC) suffer primarily from portal hypertension-driven complications, show a higher risk of recurrence of AD and a significantly increased risk of death (6). Although, compared to SDC, UDC is associated with higher SI, data suggests that severe PHT is the main pathophysiological driver and the hallmark of UDC. Interestingly, UDC patients present a higher prevalence of bacterial infections, such as spontaneous bacterial peritonitis, which can in turn perpetuate decompensating events and negatively affect the further clinical course. The third clinical course of AD determined by the PREDICT study is pre-ACLF, which constitutes a distinct clinical phenotype and is characterized by development of ACLF within 90 days. These patients show rapid progression of SI compared to UDC and significantly higher short-term mortality (6). It is now well recognized that SI is a crucial driver of disease progression, possibly acting in synergy with other organ-specific pathomechanisms to mediate organ dysfunctions, ultimately facilitating the development of ACLF, which, indeed, is demonstrated by the newly described clinical entity of pre-ACLF (6, 7, 10).

Pathomechanisms in AD and ACLF

In recent years, an emerging body of evidence has established SI as a key driver in AD and ACLF disease progression (7, 9, 26–28). While clinically significant portal hypertension (PHT) is the main driver in compensated advanced chronic liver disease (cACLD), recent studies suggested extensive activation of SI as determining further disease progression, aggravating and accelerating development of organ failures and ACLF (29). PHT-associated congestion as well as splanchnic endothelial dysfunction further aggravate gut epithelial barrier permeability (26, 30–32). Translocation of bacterial components and their metabolites is considered to cause bursts of SI by systemic exposure to gut microbiome-derived pathogen-associated molecular patterns, so-called PAMPs, presumably triggering acute decompensation events and ACLF (33–35). Indeed, emerging evidence has identified PHT-driven gut epithelial permeability as the critical driver of SI (9, 34).

A recent study demonstrated progressively increasing pro-inflammatory cytokine concentrations among different AD phenotypes, being most severe in pre-ACLF (see **Figure 3**) (27). Patients with manifest ACLF showed high concentrations of pro-inflammatory biomarkers, such as interleukin (IL)-1ra, IL-6, IL-8, tumor necrosis factor (TNF)-α or irreversibly oxidized albumin (HNA2), which positively correlate with poor short-term survival

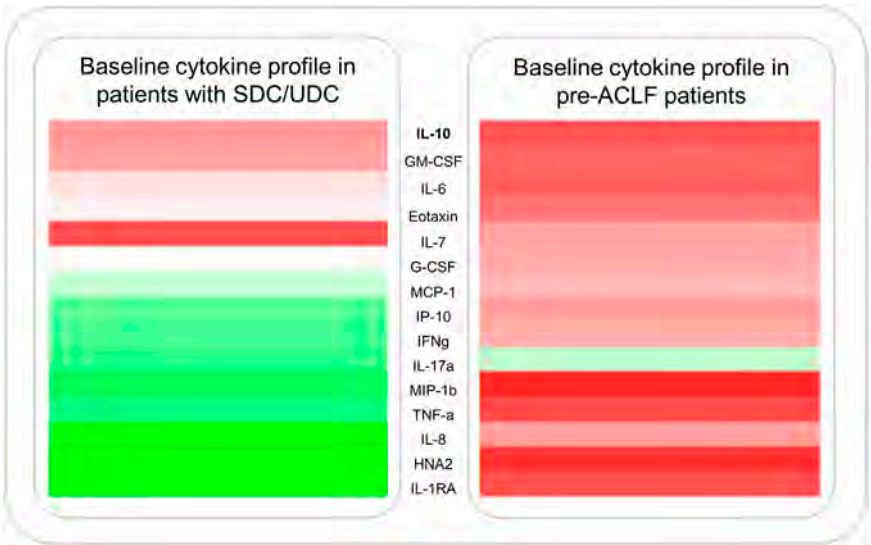

FIGURE 3 | Cytokine expression profiles displayed as a heatmap in patients with AD and pre-ACLF. Figure shows median plasma levels of various pro-inflammatory cytokines at enrollment of 503 patients admitted with SDC/UDC or pre-ACLF. Data published by Trebicka et al. (27).

(27). A sustained systemic inflammatory state is an energetically highly expensive process. Metabolome analysis in patients with decompensated cirrhosis has demonstrated inflammation-driven systemic catabolism, whiles manifest ACLF is characterized by severe disruptions of cell and energy metabolism and a severe catabolic state (9, 28). ACLF patients show disrupted lipid metabolism and impaired β-oxidation as well as disrupted oxidative phosphorylation and ATP synthesis (28). Thus, accumulating free fatty acids (FFAs), reactive oxygen species (ROS) and other metabotoxins presumably promote mitochondrial dysfunction, thereby accelerating metabolic disruption and cellular dysfunction. Inflammation-induced metabolic disruption and mitochondrial dysfunction, resulting in hypometabolism of peripheral organs, is presumed to complement traditional organ-specific mechanisms to perturb organ function, thereby promoting the development of organ failures and ACLF progression through these immunopathologic effects (9, 36, 37). The systemic inflammation hypothesis has severely broadened our pathophysiological understanding by complementing traditional paradigms of acute decompensation. This is paralleled by the developments in the last decade, not only regarding the changing etiologies of cirrhosis but also by a decrease in PHT-driven complications and an increase in SI-mediated decompensation (3).

Management of ACLF Before LT

Despite the high short-term mortality of ACLF, no specific treatments are available to improve patients' clinical course. The main principle in management of ACLF is to identify and treat the precipitating event, diagnose and treat associated complications, provide supportive therapy and, in some cases, facilitate organ support (20, 38). In patients with a determinable precipitant, early identification and adequate therapy is paramount (5). Ideally, patients with ACLF should be monitored. While monitoring is more feasible in IMC or ICU units, remote monitoring, e.g., of heart rate variability, may also be a solution in these patients as shown recently in a collaborative study (39). As the PREDICT study has demonstrated, bacterial infections and severe alcoholic hepatitis are the most frequent precipitating events in European patient populations (21). Thus, 50% of ACLF patients show infection, either as a precipitating event or as a complication, and in patients with ACLF grade, prevalence of bacterial or fungal infection increases up to 70% (5). Once bacterial infection is confirmed, early initiation of a broad empirical treatment, with consideration of local resistance spectrums, is most critical (40, 41). A globally increasing prevalence of multidrug-resistant organisms (MDROs), particularly multidrug-resistant Gram negative bacteria, poses additional challenges for clinical management (13). Notably, patients with ACLF more frequently present infections with extensive drug-resistant organisms and also show a lower infection resolution rate (42). Ideally, empirical antibiotic therapy should be adjusted to microbiological results as soon as possible.

The second most frequent precipitant in ACLF, according to the PREDICT study, is severe alcoholic hepatitis. These patients show a similar clinical course and comparable outcomes to patients with precipitant bacterial infections (21). For patients with severe alcoholic hepatitis, initiation of prednisolone therapy is often indicated. However, steroid response rates are negatively correlated to the number of organ failures at baseline (43). The Lille score can be used to identify patients who lack response to steroids early on in treatment (44). Nevertheless, in specific programs, transplantation may present an option in therapy of refractory severe ASH (45).

In cases of acute variceal hemorrhage, a new treatment option in transjugular intrahepatic portosystemic shunt (TIPS) placement has emerged, complementing the standard medical treatment of early administration of a vasoconstrictor (e.g.,

terlipressin or octreotide) and endoscopic therapy (20). A recent multicenter observational study identified ACLF at admission to be an independent predictor of mortality and risk of rebleeding in patients with acute variceal bleeding (46). In these patients, pre-emptive (early) TIPS placement showed a significant benefit in 42-day and 1-year survival (46). This is a clear demonstration that PHT plays a crucial role as a driver of AD.

Depending on the geographical region, viral hepatitis can constitute a rather frequent cause of ACLF, particularly in Asian countries (11). In cases of hepatitis B virus infection or reactivation, an immediate initiation with a nucleoside or nucleotide analogue is indicated.

Conventional dialysis devices are highly effective in restoring fluid homeostasis and removing toxic hydrophilic substances from the circulation. However, these devices are unable to eliminate non-hydrophilic compounds, which accumulate in the body in the context of liver failure and ACLF (47). Therefore, extracorporeal liver support systems (ECLS) were developed, which can eliminate albumin-bound compounds. ECLS can be considered as a bridging strategy, especially in patients eligible for liver transplantation, but also in selected patients as a definite treatment to improve organ function. However, more evidence in needed. Two systems, albumin dialysis (MARS®) and fractionated plasma separation and adsorption system (Prometheus®) have been evaluated in large randomized controlled trials (RCTs) among ACLF patients. In both controlled trials, data did not show a significant benefit in overall patient survival (48, 49). Notably, at the time when these initial studies were conducted, the current EASL-CLIF definition of ACLF had not been established yet. Furthermore, it has to be mentioned that in a subgroup analysis of the Prometheus study, patients with a MELD score >30 showed improved survival (48).

In one recent meta-analysis, which assessed available evidence on ECLS in ACLF of 25 RCTs by the GRADE approach, the authors reported a reduction in mortality (RR 0.84, 95%CI 0.74–0.96) with moderate certainty (50). More recently, a Bayesian network meta-analysis, which included 16 RCTs on artificial and bioartificial support systems in ACLF, concluded that available evidence indicates plasma exchange (PE) in ACLF to be the best treatment option currently available among all support systems (51). In cumulative ranking, PE ranked first and was associated with a significantly increased 3-month overall survival and 3-month transplant-free survival in ACLF patients compared to standard medical treatment. In contrast, other artificial support systems did not reach statistical significance in this meta-analysis (51). Overall, several studies have indicated that PE might be a feasible treatment strategy in ACLF (52, 53). However, due to the low quality of evidence, larger RCTs are required, which are currently undertaken, for example by the APACHE trial.

Taken together, currently available evidence does not support a general recommendation for the use of extracorporeal liver support systems in ACLF patients outside of clinical trials, although under specific circumstances, it could be considered as an option to bridge-to-transplantation (20, 54).

Transplant Allocation in ACLF

LT is a potentially life-saving treatment for patients with ACLF. This is underscored by the fact that current principles in management of this severe syndrome rely on identification and treatment of the precipitant and supportive care for specific organ failures. Given the high short-term mortality among ACLF patients and in light of the unavailability of specific disease-modifying drugs as well as negative studies regarding albumin dialysis, rescue transplantation emerges as a critical and life-saving option for severe ACLF patients. Data of recent years have accelerated the formation of consensus among societies that patients with ACLF grade 1 and 2 should be listed for LT. In fact, ACLF patients benefit from rapid evaluation and listing, which is highlighted by the observation that even patients who recover from the index ACLF event are still at risk of a recurrent decompensation and more severe ACLF in the future (55). Even after recovery from ACLF, inherent 6-month mortality ranges from 40% to 50% (15, 56).

Patients with AD listed for LT show a waitlist mortality of 15% (57). However, the PREDICT study demonstrated that UDC and pre-ACLF are associated with a significantly higher short-term mortality compared to stable AD, which is not necessarily reflected in current prognostic models. These patients are at risk of developing organ failures and progression to ACLF, resulting in rapid deterioration of their clinical condition (6). Upon progression to ACLF, patients show a 28-day transplant-free mortality ranging from 30 to 40% (10, 15). In patients with ACLF grade 3, 28-day mortality increases to 68%, whereas patients with 4-6 organ failures show an even higher mortality rate of up to 88.9% in 28 days, according to data from the CANONIC study (5). In line with these findings, data analyzed by the United Network for Organ Sharing (UNOS) database showed that patients with ACLF grade 3 had a significantly higher waitlist mortality within 14 days after listing than listed patients with 1a-status (58).

Importantly, this high short-term mortality in severe ACLF patients is not fully reflected by current scoring tools used for transplant allocation. Conventional prognostic models for assessment of mortality risk in patients with cirrhosis are the Model for End-Stage Liver Disease (MELD), MELD-sodium (MELD-Na) and Child-Pugh scores (59, 60). In fact, these scores predict both, progression to ACLF and survival among ACLF patients (61). Most countries have adopted MELD or MELD-Na score-based allocation policies to prioritize most severe patients with decompensated liver cirrhosis for LT. However, these scores lack important clinical determinants of short-term mortality among ACLF patients. For one, no surrogates for SI, such as white blood cell count, CRP or ferritin, are taken into account, although SI is considered the main driver in ACLF progression and strongly correlated with mortality rates (8, 24, 27, 62). Furthermore, neither score incorporates surrogates for portal hypertension or presence of respiratory or circulatory failure to estimate patients' mortality risk, although recent data suggests that pulmonary failure in particular is an important determinate of mortality in ACLF patients [own unpublished observation]. Also, neither MELD nor MELD-Na score are incorporating cerebral dysfunction/HE.

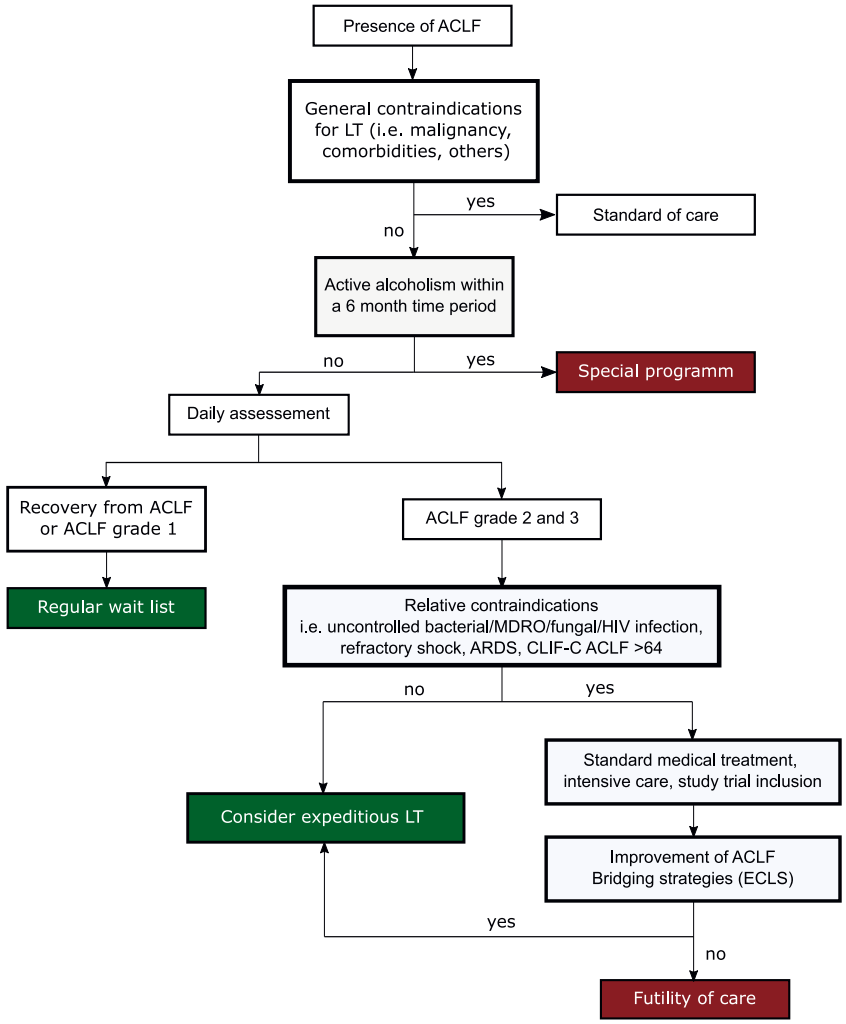

FIGURE 4 | Decision algorithm to assess eligibility of ACLF patients for LT.

However, the assessment of this clinical parameter could be considered compromised by the subjective nature of its assessment.

In view of these limitations, the CANONIC study specifically designed the CLIF-C ACLF score to assess mortality risk in patients with ACLF (18). The CLIF-C ACLF score incorporates the number of organ failures, reflected by the CLIF-OF score, age and white blood cell (WBC) count as a surrogate for severity of SI (18). These parameters have been determined as crucial predictors of short- and long-term survival in ACLF patients but are not included in other models. In recent years, several studies have corroborated that the CLIF-C ACLF score shows a significantly higher predictive accuracy than other prognostic models for short-term mortality in ACLF patients (18, 63, 64). A CLIF-C ACLF score from 64 to 70 points is regarded as the threshold to futility of care and may thereby be helpful to identify patients in whom supportive care must be critically discussed if rescue LT is not a valid option.

In fact, limitations regarding current MELD score-based risk stratification allocation policies are underlined by a recent study analyzing data from the UNOS database, which showed that patients with ACLF grade 3 and MELD-Na score <25 have a higher waitlist mortality than patients without ACLF and a MELD-Na score >35 (65).

Importantly, a recent study assessed mortality rates in 18,979 patients with ACLF and demonstrated that the MELD-Na score markedly underestimates the 90-day mortality of ACLF patients (66). Moreover, several studies reported a declining predictive accuracy of the MELD score over the last decades of MELD score-based LT allocation. Initially, this became apparent when comparing predictive performance of the MELD score in former studies with reports from current patient populations (16, 18, 60, 67). This observation was corroborated in a recent analysis of 120,156 patients listed for LT between 2002 and 2016 with data provided by UNOS network, displaying a declining MELD score c-statistic of 0.8 in 2002 and only 0.71 in 2016 (68). Multiple reasons for this observation have been proposed, whereby epidemiological shifts in the landscape of cirrhosis with changing prevalence of etiologies and accelerated listing

of more highly advanced patients with liver cirrhosis are considered to be major contributors (57). Data suggests that high mortality rates of an increasing number of listed patients with rapid decompensation and ACLF might not be adequately reflected with current prognostic tools. These considerations emphasize the increasing need to improve MELD score-based models to better reflect waitlist mortality and possibly modify and improve LT allocation policies for ACLF patients in the future.

Patient Selection and Contraindications for LT

In light of these challenges and due to the limited supply of organ donors, optimal patient selection, identification of relative contraindications and timing of LT appears to be critical. A decision algorithm for LT evaluation in ACLF patients is shown in **Figure 4**.

Recently published data from a large multicentric study identified four independent pretransplant risk factors among patients who received LT in ACLF grade 3 (69). The authors were able to use these risk factors, namely age ≥53 years, lactate level ≥4 mmol/L, mechanical ventilation with pulmonary failure (PaO2/FiO2 ≤ 200 mmHg) and leukocytes ≤10G/L, to develop and validate a prognostic model to predict posttransplant survival in ACLF grade 3 patients. The transplantation for ACLF-3 model (TAM) score assigns 1 scoring point for each criterion met and allows for stratification into two groups: recipients with a TAM score of >2 showed a poor post-LT outcome with a 1-year mortality of almost 84%, while a TAM score of ≤2 was associated with a mortality rate <10% (69).

Although this study provided a novel clinical tool to assess a window of transplantability, one of its major limitations was the fact that the derivation cohort for the TAM score consisted of only 22 patients, who met the inclusion criteria of death within 1 year. A recent single-center study has since retrospectively assessed the TAM score in 100 patients (70). The authors found that the TAM score was efficiently discriminating between ACLF grade 3 post-LT survivors and non-survivors if assessing patients at the time of LT or directly before LT. In contrast, the score did not show any reliable prediction for patient outcome at ICU admission or 2 days after admission (70). Interestingly, a recent study observed that ACLF grade 3 patients, who showed improvement of ACLF severity prior to LT also showed higher post-LT survival rates (71).

The recently published ECLIS study assessed 234 patients receiving LT for ACLF, also reporting pre-LT lactate levels to be predictive of post-LT outcome (72). Furthermore, this study found renal replacement therapy at LT and recent MDRO infection to be independent predictors of poor post-LT outcomes (72). Independent of MDRO status, uncontrolled bacterial infections, fungal infections and severe sepsis are generally considered a contraindication to LT, since post-LT immunosuppression may exacerbate the infection. For a similar reason, uncontrolled human immunodeficiency virus (HIV) infections should also be regarded as relative contraindications to LT (56). However, bacterial infections are the most common ACLF precipitant and, furthermore, are frequent complications upon ACLF progression.

Active alcoholism is considered a contraindication to LT and abstinence for 6 months is a requirement for LT listing in many countries. The 6 month rule is implemented to allow liver recovery, decrease post-LT relapse rates and reduce allograft loss. However, severe alcoholic hepatitis constitutes a major precipitant to ACLF. Due to the high short-term mortality of ACLF, a considerable fraction of patients die within this 6 month period, disregarding whether these patients would otherwise be feasible candidates for LT. This is a controversial topic, since several studies have found that outcomes in LT recipients with alcoholic hepatitis, who received LT in under 6 months showed a high 1-year post-LT survival of 74–94% and relapse rates of 10–17% (73). Evidence is indicating that selective use of LT in patients with alcoholic hepatitis, who meet specific psychosocial requirements, might be a feasible strategy (74, 75). A recent study has introduced the prognostic Sustained Alcohol Use Post-LT (SALT) score, which can be used to identify patients with a low risk of alcohol relapse. The score is comprised of four pre-transplant variables: patients drinking pattern at presentation (>10 drinks/day, +4 points), prior failed rehabilitation attempts (≥2, +4 points), history of alcohol-related legal issues (+2 points) and history of prior non-THC substance abuse (+1 points) (76). In the study cohort, a SALT score of <5 had a 95% negative predictive value and high sensitivity for sustained alcohol use after LT, showcasing that individual patient assessment and selective LT in suitable candidates could be a new approach in LT allocation in the future (76).

Outcomes After LT

In recent years, several studies have demonstrated high post-LT survival rates among ACLF patients, although data show some geographical variability due to heterogeneity of study populations. Initially, analysis from the CANONIC population showed a 1-year post-LT survival of 75.3% in a small number of 25 ACLF patients receiving LT, which was lower than in the study's overall population (15).

In the following years, single center retrospective studies have reported 1-year post-LT survival rates ranging from 70% to 87%, depending on patient population and ACLF severity (77-80). A retrospective study conducted by Levesque et al. demonstrated that ACLF patients presenting ACLF grade 1 and 2, according to EASL-CLIF criteria, showed a high 90-day post-LT survival of 85.3% and 83.3%, respectively, while patients transplanted with ACLF grade 3 only had a 90-day survival of 60% in the study population (79). In contrast, a larger multicenter European study including 250 ACLF patients found 1-year post-LT survival of 83.9% in patients with ACLF grade 3 (80), presumably because ACLF grade 3 patients were carefully selected for LT in this study. Acute respiratory distress syndrome (ARDS), uncontrolled sepsis, active gastrointestinal bleeding and hemodynamic instability were considered contraindications to LT in these patients (80). This underlines the importance of patient selection, but urges us not to regard ACLF grade 3 as an absolute contraindication for LT. Moreover, this study strikingly contrasted the 1-year post-LT survival of 83.9% in

patients with ACLF grade 3 compared to only 7.9% in the non-LT control group with ACLF grade 3, underlining that LT often is the only life-saving option for patients with severe ACLF.

A recent extensive retrospective analysis has since clearly provided robust data, showing that all ACLF patients, including ACLF grade 3, significantly benefit from LT. Sundaram et al. analyzed data from over 50,000 patients included in the UNOS database and found even higher 1-year survival rates post-LT in ACLF grade 1 (89.1%), ACLF grade 2 (88.1%) and ACLF grade 3 (81.9%) (65). Interestingly, this study found that mechanical ventilation at LT and a donor risk index >1.7 were independently associated with poorer post-LT survival. The donor risk index (DRI) was established to quantitatively assess donor-specific factors to predict the risk of graft failure and is comprised of seven donor characteristics, most importantly donor age, donation after cardiac death or split/partial graft (81).

A more recent study assessing a European cohort of 2,677 patients showed similar results regarding survival, with survival rates being >80% among all ACLF grades (72). Similar results were also found in other prospective and retrospective studies of European cohorts (15, 78). Depending on the study population, risk factors for post-LT mortality in ACLF were mechanical ventilation, circulatory failure and four or more organ failures (65), need for renal replacement therapy, as well as infection with MDROs as precipitating events or as complications (72).

In summary, these studies demonstrate that ACLF patients strongly benefit from LT, and that post-LT survival does not significantly differ from that in patients without ACLF. Furthermore, data urge us to not generally regard ACLF grade 3 as an absolute contraindication for LT, instead patients must be carefully selected.

CONCLUSION

In conclusion, various recent studies have demonstrated that LT is a feasible and life-saving option for ACLF patients with excellent post-LT outcomes. In many cases, patients with severe ACLF have no other treatment option than expeditious LT and a clear survival benefit can be shown if patients are carefully selected. Importantly, increased mortality rates among ACLF patients are not fully reflected in current prognostic tools used for transplant allocation. Further studies will be necessary, but data demand a critical reflection of current transplant allocation systems to improve risk stratification in patients with this severe syndrome. In clinical management of decompensated cirrhosis, patient progression to ACLF should trigger early decision-making and rapid transplant evaluation, as suggested for patients with acute liver failure, to stay within the narrow window for transplantation.

AUTHOR CONTRIBUTIONS

MS and JT: concept and design, literature research, drafting of the manuscript. WG and AS: critical revision of the manuscript for important intellectual content.

FUNDING

JT is supported by grants from Deutsche Forschungsgemeinschaft (SFB TRR57 to P18), European Union's Horizon 2020 Research and Innovation Programme (Galaxy, No. 668031 and MICROB-PREDICT, No. 825694), Societal Challenges–Health, Demographic Change and Wellbeing (No. 731875), and Cellex Foundation (PREDICT). The funders had no role in study design, data collection and analysis, decision to publish, or preparation 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.

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GLOSSARY

ACLF acute-on-chronic liver failure

AD acute decompensation

ARDS acute respiratory distress syndrome

ASH alcoholic steatohepatitis

ATP adenosine triphosphate

cACLD compensated advanced chronic liver disease

CANONIC study EASL-CLIF Acute oN chrONIC liver failure study

CI confidence interval

CLIF consortium chronic liver failure consortium

CLIF-C ACLF score CLIF-Consortium ACLF score

CLIF-C OFs CLIF-Consortium Organ Failure score

CLIF-SOFA score CLIF-sequential organ failure assessment score

CRP C-reactive protein

DRI donor risk index

EASL European Association for the Study of the Liver

ECLIS study ELITA/EF-CLIF collaborative study

ECLS extracorporeal liver support systems

FFAs free fatty acids

GRADE approach Grading of Recommendations Assessment, Development and Evaluation approach

HCC hepatocellular carcinoma

HE hepatic encephalopathy

HIV human immunodeficiency virus

HNA2 irreversibly oxidized albumin

ICU intensive care unit

IL-1ra interleukin-1 receptor antagonist

IL-6 interleukin-6

IL-8 interleukin -8

IMC intermediate care unit

LT liver transplantation

MELD score Model for End-Stage Liver Disease score

MELD-Na score Model for End-Stage Liver Disease–sodium score

MRDOs multidrug-resistant organisms

PAMPs pathogen-associated molecular patterns

PE plasma exchange

PHT portal hypertension

Pre-ACLF pre-acute-on-chronic liver failure

RCT randomized controlled trial

ROS reactive oxygen species

RR relative risk

SALT score Sustained Alcohol Use Post-LT

SDC stable decompensated cirrhosis

SI systemic inflammation

TAM score transplantation for ACLF-3 model

THC tetrahydrocannabinol

TIPS transjugular intrahepatic portosystemic shunt

TNF-α tumor necrosis factor

UDC unstable decompensated cirrhosis

UNOS United Network for Organ Sharing

WBC white blood cell

ESOT | Transplant International

MINI REVIEW
published: 18 March 2022
doi: 10.3389/ti.2022.10084

Inequitable Access to Transplants: Adults With Impaired Decision-Making Capacity

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Inequitable access to deceased donor organs for transplantation has received considerable scrutiny in recent years. Emerging evidence suggests patients with impaired decision-making capacity (IDC) face inequitable access to transplantation. The "Ethical and Legal Issues" working group of the European Society of Transplantation undertook an expert consensus process. Literature relating to transplantation in patients with IDC was examined and collated to investigate whether IDC is associated with inferior transplant outcomes and the legitimacy of this healthcare inequality was examined. Even though the available evidence of inferior transplant outcomes in these patients is limited, the working group concluded that access to transplantation in patients with IDC may be inequitable. Consequently, we argue that IDC should not in and of itself be considered as a barrier to either registration on the transplant waiting list or allocation of an organ. Strategies for non-discrimination should focus on ensuring eligibility is based upon sound evidence and outcomes without reference to non-medical criteria. Recommendations to support policy makers and healthcare providers to reduce unintended inequity and inadvertent discrimination are set out. We call upon transplant centres and national bodies to include data on decision-making capacity in routine reporting schedules in order to improve the evidence base upon which organ policy decisions are made going forward.

Keywords: transplantation, ethics, capacity, law and policy, equitable access

OPEN ACCESS

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Received: *30 September 2021*
Accepted: *11 February 2022*
Published: *18 March 2022*

Citation:
Thom RL, Dalle-Ave A, Bunnik EM, Krones T, Van Assche K, Ruck Keene A and Cronin AJ (2022) Inequitable Access to Transplants: Adults With Impaired Decision-Making Capacity. Transpl Int 35:10084. doi: 10.3389/ti.2022.10084

Abbreviations: IDC, Impaired decision-making capacity; EBPG, European Best Practice Group; QoL, Quality of Life; QALY, Quality Adjusted Life Year; aHR, adjusted hazard Ratio; CI, Confidence Interval; OCEBM, Oxford Centre for Evidence based medicine.

INTRODUCTION

Issues of scarce resource allocation and inequitable access to medical treatment have long-since been the doctor's dilemma. Deceased donor organs for transplants are a scarce resource, and it is widely agreed that equitable access to transplantation must be prioritised. In recent years transplant professionals and advocacy groups have highlighted how those who may have impaired legal decision making capacity (IDC) have historically faced inequitable access to transplant waiting lists and organ allocation (1–3). This has led to multiple United States jurisdictions instituting specific legislation, however such changes are yet to be seen in Europe (1).

Those who may have IDC include patients with 1) intellectual disability, 2) a mental health condition, including for example disorders affecting reasoning such as psychosis, 3) cognitive impairment that may be due to neurological disease or a single acquired deficit (e.g., stroke or head injury) and finally 4) disorders or consciousness such as persistent vegetative or minimally conscious states. Cognitive impairment is of particular importance as up to 70% of patients aged over 55 receiving dialysis have moderate to severe cognitive impairment (4) and there is emerging evidence which suggests such patients have a lower likelihood of being listed for transplantation (5).

In this paper we interrogate the relationship between 1) apparent lack of mental capacity to make relevant decisions and 2) equitable access to deceased donor organ transplantation. We seek to explain why lacking the mental capacity to consent to transplant should not itself per se be a barrier to access to and allocation of an organ for transplant. We do this with reference to four key transplant outcome measures and specifically interrogate whether, and if so to what extent, the concerns raised by these four key transplant outcome measures are supported by published empirical evidence. We highlight ethical considerations and legal issues, and, finally, set out recommendations and guidelines for clinicians and policy makers to help overcome perceived barriers and avoid unintentional discrimination.

MATERIALS AND METHODS

The "Ethical and Legal Issues" working group of the European Society of Organ Transplantation undertook an expert consensus process between October 2020 and March 2021. This took the form of extensive online discussions between clinical transplant, ethics, and legal experts. Discussions were informed by a review of the published literature relating to transplantation in persons with IDC.

For the purpose of this paper relevant literature was identified by a search of MEDLINE accessed through PubMed. Search terms used were (organ transplantation) AND (mental incapacity OR intellectual disability) between September 2010 and September 2020. We included peer reviewed publications from scholarly journals. Our key purpose was to identify whether strong evidence existed to support the view that transplant outcomes are inferior in persons with IDC.

Our search generated 66 papers. The titles and abstracts of all English language papers were screened. 16 papers relevant papers were identified. One paper was excluded as it was a case study. Seven papers were primary research- six retrospective cohort studies and one online survey. The remainder were literature reviews, ethical analyses or editorials. Further sources were identified through cited materials. In addition, primary and secondary legal sources from LexisNexis and Westlaw databases and public policy documents were analysed.

TRANSPLANT OUTCOME MEASURES AND INEQUITABLE ACCESS TO TRANSPLANTATION

Four key transplant outcome measures emerge in the literature as relevant clinical concerns and to varying degrees cut across all the groups we have identified as at risk of lacking the mental capacity to make relevant decisions as regards to medical treatment and transplantation. These are 1) medication adherence, 2) graft outcome, 3) patient outcome and 4) quality-of-life (QoL). While medication adherence is not itself a transplant outcome measure, we observe that medication non-adherence is assumed to have a causal effect on transplant outcomes. As post-transplant medication non-adherence is taken to negatively impact organ and patient survival and quality of life, the *prognosis* of non-adherence is mentioned in the literature as a reason not to list a patient or not to allocate an organ.

We assessed whether, and, if so, to what extent, the concerns raised by these four inter-related key transplant outcome measures are supported or actively refuted by the published empirical evidence. We included outcome data relating to living donor transplantation because limited evidence was available on deceased donor transplant outcomes in persons with IDC. A summary of this empirical assessment is set out in table one (**Table 1**) and is followed by an ethical and legal analysis of the concerns raised by each transplant outcome measure and by their assumed causal dependency.

In the empirical and theoretical literature found to date disorders of consciousness and their implications for potential transplant recipients have not received attention. This lack of empirical evidence has led us to exclude them from our further discussion, although their position would benefit from further theoretical analysis as they seem to be a group who are subject to distinct concerns.

Medication Adherence

Non-adherence to prescribed medication is common, transplantation is no exception. The estimated prevalence of non-adherence in transplant recipients is between 36 and 55% (6). There are multiple factors which have been shown to be associated with non-adherence, including "youth (<50 years old), male, low social support, unemployment, low education, >3 months post graft, living donor, >6 comorbidities, >5 drugs/day, >2 intakes/day, negative beliefs, negative behaviour, depression and anxiety (7)"- however, many of these factors may

TABLE 1 | Summary of empirical evidence relating key transplant outcome measures to each group with potentially impaired decision making capacity.

Group with potentially impaired DECISION-MAKING capacity	Key transplant outcome measures			
	Adherence with medical therapy	**Graft outcome**	**Patient outcome**	**Quality of life**
Intellectual disability	Cohort studies suggesting adherence is comparable. OCEBM[a] level 3 (1, 17)	Multiple cohort studies suggesting graft outcomes are comparable. OCEBM level 3 (1, 13–19, 33)	Multiple cohort studies suggesting non-graft outcomes are comparable. OCEBM level 3 (1, 13–19, 33)	Evidence is that in general quality of life is improved by transplantation (25) OCEBM level 1 Small number of cohort studies showing QOL benefit in this group. OCEBM level 3 (26, 33)
Severe mental health conditions	Evidence of increased non-adherence in those with depression (7) OCEBM level 3 but not in other conditions in particularly in those with psychosis/mania (8, 9) OCEBM Level 3	Evidence of poorer outcomes in those with depression (24) OCEBM level 1. Otherwise conflicting evidence from cohort studies of other psychological conditions OCEBM Level 3 (2, 8, 9, 22)	Evidence of poorer outcomes in those with depression (24) OCEBM level 1 Otherwise conflicting evidence from cohort studies of other psychological conditions OCEBM Level 3 (2, 8, 9, 22)	Evidence is that in general quality of life is improved by transplantation (25) OCEBM level 1
Cognitive impairment	Evidence from cohort studies of reduced adherence in older age groups of transplant recipients OCEBM level 3 (11, 12)	Cohort studies indicate worse outcomes (23) OCEBM level 3	Cohort studies indicate worse outcomes (23) OCEBM level 3	Cohort study evidence that QoL benefit is consistent in over 65s (those most at risk of cognitive impairment on dialysis)(28) OCEBM level 3
Permanent disorders of consciousness	No concern as adherence would be assured by caregiver	No evidence available	No evidence available	Theoretical reason to believe QoL outcomes would be significantly different from the general population of transplant recipients

[a]*Oxford Centre for Evidence Based Medicine 2011 levels of evidence are included to indicate the degree of certainty with which the authors make these assertions.*
This table has drawn on evidence relating to intellectual disability from the paediatric literature. However in this paper we do not consider children as a discrete category, as they are treated differently where they are considered too young to have the legal capacity to make the relevant decisions, whether or not they have any intellectual disability or mental disorder.

be equally present in patients who have decisional capacity as in those who lack it.

Non-adherence is frequently linked to those with mental health disorders (2). However, in a study of 955 transplant recipients it was found that those with a pre-existing mental health diagnosis and those with pre-transplant non-adherence were not necessarily groups which overlapped (8). Studies looking specifically at adherence in severe mental health disorders which may result in IDC (e.g., psychosis) are scarce. However Molnar used percentage of days covered by immune suppression prescriptions for a cohort of 442 post-transplant patients with a history of psychosis and mania and found that these did not differ significantly between those with a psychiatric history and those without (9).

In contrast it could be argued that those with intellectual disability may already have strong social support networks and committed carers which act as protective factors against non-adherence (1, 10). Samelson-Jones in a case review of five adults with intellectual disability who received cardiac transplants found only one instance of significant non-adherence which was primarily due to a deterioration in the ability of the caregiver rather than the patient (10).

Finally, it is widely acknowledged that in the general population those with advanced age and co-morbidity face specific barriers to adherence. Polypharmacy, visual loss and cognitive impairment may all contribute to difficulty adhering with complex medication regimes. One study which attempted to assess if these general concerns were replicated in the transplant population showed non-adherence to be alarmingly high in older transplant recipients affecting 86% (11). With another showing that age >60 was found to be significantly associated with worse adherence (12).

The limited evidence available is inconclusive with regards to whether adherence in persons with IDC is reduced when compared to the general population. It is therefore not possible to assert that IDC can legitimately be used as a surrogate marker for post-transplant non-adherence. Concerns related to post-transplant medication non-adherence may be alleviated when committed caregivers and social support networks are available.

Graft and Patient Outcomes

Cohort studies have shown that patients with intellectual disability receiving a variety of solid organ transplants have equal survival to those without (1, 13–20). A literature review of transplant outcomes in those with intellectual disability found 18 published studies with a mixture of solid organ transplants included, mostly but not exclusively in paediatric recipients (1). The largest cohorts are found in kidney transplant recipients where 5-year graft survival ranged from 75 to 100% (1) and when compared to matched populations without intellectual disability there is no difference in acute rejection or graft survival (13).

Meta-analysis have shown depression to be associated with increased graft loss and all-cause mortality RR1.65 (CI:1.21–2.26) (21) although a causative factor is not considered and a large retrospective cohort study of 4582 patients in Ontario has shown a hazard ratio (HR) = 1.494 [95% confidence interval (CI) = 1.168–1.913] of post-transplant death in patients with a diagnosis of "psychological conditions" which was independent of age (22). However, this represents a very heterogenous group. In contrast cohort studies of patients with psychosis or mania do not reveal an association with increased rejection or graft loss (8, 9) although there is likely to be selection bias as those transplanted were likely stable prior to transplantation.

Cognitively impaired recipients in a retrospective study of 864 patients at two centres in North America showed that there was a substantially higher all cause graft loss than in those without impairment in living donor recipients- aHR 5.40 (CI 1.78–16.34, $p < 0.01$) and in deceased donor recipients with severe cognitive impairment aHR 2.92 (CI1.13–7.50, $p = 0.03$) but no statistically significant difference in those with any stage of cognitive impairment (23).

Quality of Life (QoL)

There is a wealth of evidence supporting the assertion than kidney patients' QoL is greatly improved by transplantation, particularly when compared to remaining on dialysis (25). This is the principal reason transplantation is considered to be the gold standard treatment of kidney failure. However, there remains considerable debate over the best measures to judge QoL. For example, a major criticism of the objective Quality Adjusted Life Year (QALY) measure, which gives weight to quantity and utility of life as well as quality, is that it is inherently biased against those with limited life expectancy and that the "Quality" factor is often not measured by self-assessment but by third-party assessment although it is widely recognized that QoL is a subjective rather than an objective dimension.

Chen et al. directly address this with regard to patients with intellectual disabilities and argue that there is "bias, subjectivity and stigma frequently associated with clinicians QoL assessments of patients with intellectual disability [which must] not be used to categorically exclude patients from lifesaving and life-enhancing surgery" (1). They go on to cite evidence that perceived QoL of recipients with intellectual disability and QoL of the principle carer improved post transplantation (26), showing that those with intellectual disability also benefit from transplantation. When considering psychological disorders while psychiatric comorbidity and particularly depression remain common in patients post transplant (27) it does not follow that patients with these diagnoses would be excluded from the benefit to QoL offered by transplantation. Similar criticisms of ableism may be levelled at clinician attitudes towards those with advanced age and cognitive impairment even though again limited evidence would show that QoL improvements from transplantation are consistent even in older age groups (28).

From available evidence on these four interrelated outcomes, one can conclude that there is very limited evidence on non-adherence of persons with IDC, only very weak evidence of worse outcomes of renal transplants with regards to graft and patient survival and QoL in persons with cognitive impairments and/or persons suffering from depression, but not in patients with intellectual disabilities and other psychological conditions.

ETHICAL ISSUES

Clinical decision-making regarding access to or allocation of deceased donor organs for transplant is constrained by scarcity, and so prompts considerations of justice. Justice implies that equals should be treated equally: when patients are similar in medically relevant respects, they ought to be treated equally, as all persons are considered as having the same right to life and health. However, reasonable persons may commit to different ethical theories on what equal treatment entails. Consequently, there is no consensus on the principles of fair allocation of scarce healthcare resources (29).

In living donation, by contrast, the issue of fair allocation does not usually arise, as the recipient brings his or her own donor and does not lay claim to a public pool of scarce organs. That is not to say that there are no ethical concerns regarding equal access in living donation. For instance, access to living donors may not be equally distributed among patients with impaired decision-making capacity. Also, our literature reveals data suggesting significantly inferior outcomes in living donor kidney transplantation in cognitively impaired patients. These concerns merit further investigation, but are beyond the scope of this manuscript.

The most prominent ethical theories of justice are utilitarian and egalitarian. Utilitarian principles aim to maximise the aggregated benefits produced by scarce resources, while egalitarian principles strive for equity or equal opportunity, regardless of aggregated outcomes, and/or for giving priority to the worst-off. These principles for allocation almost always stand in tension with each other, as giving priority to the worst-off often reduces overall utility, and vice versa.

Applying either theory, patients with IDC should be assessed and might even be prioritized, to ensure equal opportunity to a life-saving treatment. It seems reasonable to assume that for all potential recipients, regardless of decisional capacity, transplantation would offer significant QoL benefits, and that assumptions to the contrary may be subject to negative bias. Even from a utilitarian perspective, differentiated treatment of patients with and without relevant decision-making capacities is warranted only when there are (measurable) differences in transplant outcomes between the two groups. The evidence base would have to be as solid and the estimated risk of shorter survival or QoL would have to be as low as in other patients who are currently not being assessed for organ transplant, for example patients with significant cardiovascular or neoplastic disease. Given the current state of knowledge, we conclude that there is no sound ethical justification not to list patients with IDC who (presumably) want to be listed.

Further research is recommended to confirm whether graft or patient outcomes are inferior in patients with impaired decision-making capacity. Evidence on transplant outcomes is needed to guide decision-making about listing for transplantation.

However, as long as there is no evidence to conclude that transplant outcomes measures are (much) lower in persons with impaired decision-making capacity, there is no medical or ethical reason to exclude these patients from organ transplantation.

LEGAL ISSUES

The critical legal issue is how to secure individuals with IDC effective legal protection against discrimination on the basis of disability, as this is contrary to the United Nations Convention on the Rights of Persons with Disabilities (CRPD), the European Convention on Human Rights, and many national Constitutions. The CRPD explicitly imposes an obligation upon States party to it to prevent discriminatory denial of health care or health services on the basis of disability (Article 25(f)), as part of those States' recognition that persons with disabilities have the right to the enjoyment of the highest attainable standard of health without discrimination on the basis of disability. Whilst the European Convention on Human Rights does not include an express right to health, it enshrines in Article 14 the right not to be discriminated against (including on the basis of disability) in the enjoyment of rights under the Convention, including the right to life (Article 2) and the right to physical integrity (Article 8). These obligations are mirrored in non-discrimination provisions enshrined in many national Constitutions. In some of these Constitutions, such as the German Constitution (Article 3 (3)), discrimination on the ground of disability is explicitly prohibited. In short, making eligibility for organ transplantation contingent upon the person's decision-making capacity would amount to unjustified differential treatment on the basis of intellectual disability, which would be in violation of non-discrimination obligations under human rights and constitutional law. However, existing international guidelines on transplantation do not expressly address the potential for discrimination upon the basis of disability (30–32).

Our concern is that when making decisions about listing or allocation, clinicians might look to the absence of decision-making capacity rather than to the possible relevant medical implications of that incapacity, and, no doubt inadvertently, risk discrimination. That a person may have an intellectual disability means that they may not ask to be put forward for transplantation, but it says nothing about whether they should medically qualify for it.

Therefore, we suggest that transplant wait listing and allocation decisions should take into account decisional incapacity only to the extent that it influences relevant medical criteria, such as the state of that person's health or the outcome of the transplantation. Also, clinicians should proceed on the basis that a patient without the relevant decisional capacity would wish to be considered for a transplant unless there is good reason to believe to the contrary. This means that focus is then placed upon whether there is a *medical* reason for not putting the person forward.

Further, securing the rights of those with disabilities requires tailoring of care plans, and identifying strategies to support their adherence. Ironically, many of those who lack decisional capacity are in fact in situations where adherence can be maximised, if not guaranteed: for instance those with profound impairments needing continued and intensive care. The most creative of these strategies may be required where a person has fluctuating capacity, for instance as a result of a mental health condition. In some jurisdictions, these strategies could include the approval by court of a care plan aimed at optimising outcome.

Crucially, adopting such strategies (and our recommendations below) will not mean that individuals with impaired decision-making capacity will automatically jump the allocation queue; rather, it means that they are given their proper place in the queue.

KEY RECOMMENDATIONS

The purpose of these recommendations is to promote equitable access to transplantation and ensure that patients without the relevant decisional capacity will be considered for transplantation.

1. That the person does not have the mental capacity to make relevant decisions ("the relevant decisional capacity") should not in and of itself be an absolute or relative contraindication to transplantation
2. There should be a general assumption that patients without the relevant decisional capacity should have equitable access to organs for transplant and would want to be considered for a transplant unless there is proper reason to believe to the contrary.
3. Decision-making regarding access to transplantation for patients with impaired decisional capacity should as far as possible include the potential recipient, their families and carers. Such decision-making should specifically include 1) identification of the wishes and feelings of the patient towards transplantation; and 2) where it is understood that the patient would wish access to transplantation, drawing up a care plan which would maximise the chances of a successful transplant outcome.
4. When it is being determined that a person without the relevant decisional capacity is not eligible for transplant this must be based on sound medical reasons and evidence. It should not be on the assumption that the lack of capacity in and of itself would affect transplant outcome measures.
5. When a patient without the relevant decisional capacity has been judged not to be suitable for a transplant it is the clinician's responsibility to inform them and their family/carers honestly and transparently about the basis upon which the decision was made.
6. In order to overcome perceived barriers and avoid unintentional discrimination, transplanting centres and national bodies should include data on decision-making capacity in their routine transplant reporting schedule in order to improve the evidence base upon which organ policy decisions are made going forward, and develop a suitable operational framework that facilitates

transplantation in persons with impaired decision-making capacity.
7. International guidelines on transplantation should include, in their provisions on prohibiting discrimination in organ allocation, an explicit reference to discrimination based on disability.

Conclusion

This paper arose out of a concern on the part of the expert group as to the place of decisional capacity in considerations of access to and allocation of organs for transplants, and, in particular, a concern that such capacity–a cornerstone of autonomy–could inadvertently give rise to unintended discrimination upon the basis of disability. In the paper, we have outlined the ways in which the evidence does not support some of the assumptions which on occasion appear to have underpinned thinking in this area, examined the ethical arguments, and framed matters by reference to international and regional human rights instruments.

We recognise that this paper is just a first start in identifying the problem. We tentatively suggest that our recommendations may assist both in delineating it fully and resolving it. A systematic review to interrogate the issues we have raised further alongside a programme of research investigating transplant outcomes would be useful. Finally, while our focus in this paper has been access to deceased donor organs for transplantation we would like to acknowledge that issues related to living donor transplantation also require attention. In particular, determining whether, and if so to what extent, patients with cognitive impairment have inferior transplant outcomes should be a priority and could help guide clinicians in identifying individuals who may not be suitable for transplantation.

AUTHOR CONTRIBUTIONS

All contributing authors have participated in the consensus process, design and writing of the manuscript. AC/RT led the manuscript and led on the transplant outcomes. AK and KA led on the legal section. AD-A, TK and EB led on the ethics section. All authors agreed on the categories of patients with impaired decision making. AC was senior author with oversight of the entire manuscript and is chair of the "ethical and legal issues" working group of ESOT.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We thank the European Society for Organ Transplantation for facilitating the consensus process.

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SYSTEMATIC REVIEW AND META-ANALYSIS
published: 17 March 2022
doi: 10.3389/ti.2022.10315

Is Preemptive Kidney Transplantation Associated With Improved Outcomes when Compared to Non-preemptive Kidney Transplantation in Children? A Systematic Review and Meta-Analysis

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Main Problem: Preemptive kidney transplantation (PKT) is performed prior to dialysis initiation to avoid dialysis-related morbidity and mortality in children and adolescents. We undertook a systematic review to compare clinical outcomes in PKT versus kidney transplantation after dialysis initiation in paediatric patients.

Methods: The bibliographic search identified studies that compared paediatric recipients of a first or subsequent, living or deceased donor PKT versus non-preemptive kidney transplant. Methodological quality was assessed for all studies. Data were pooled using the random-effects model.

Results: Twenty-two studies (n = 22,622) were included. PKT reduced the risk of overall graft loss (relative risk (RR) .57, 95% CI: .49–.66) and acute rejection (RR: .81, 95% CI: .75–.88) compared to transplantation after dialysis. Although no significant difference was observed in overall patient mortality, the risk of patient death was found to be significantly lower in PKT patients with living donor transplants (RR: .53, 95% CI: .34–.83). No significant difference was observed in the incidence of delayed graft function.

Conclusion: Evidence from observational studies suggests that PKT is associated with a reduction in the risk of acute rejection and graft loss. Efforts should be made to promote and improve rates of PKT in this group of patients (PROSPERO).

Systematic Review Registration: https://clinicaltrials.gov/, CRD42014010565

Keywords: outcomes, meta-analysis, systematic review, paediatric, preemptive kidney transplantation

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Received: *20 December 2021*
Accepted: *12 January 2022*
Published: *17 March 2022*

Citation:
Rana Magar R, Knight S, Stojanovic J, Marks SD, Lafranca JA, Turner S, Dor FJMF and Pengel LHM (2022) Is Preemptive Kidney Transplantation Associated With Improved Outcomes when Compared to Non-preemptive Kidney Transplantation in Children? A Systematic Review and Meta-Analysis. Transpl Int 35:10315. doi: 10.3389/ti.2022.10315

Abbreviations: CI, confidence interval; DD, deceased donor; DGF, delayed graft function; eGFR, estimated glomerular filtration rate; ESKD, end-stage kidney disease; HD, haemodialysis; HRQoL, health-related quality of life; KT, kidney transplantation; LD, living donor; nPKT, non-preemptive kidney transplantation; PD, peritoneal dialysis; PKT, preemptive kidney transplantation; RR, relative risk; SD, standard deviation; SDS, standard deviation score.

INTRODUCTION

Kidney transplantation (KT) is the treatment of choice for children with end-stage kidney disease (ESKD) as it offers better survival and quality of life compared to treatment with dialysis (1, 2). Preemptive kidney transplantation (PKT) is performed before the initiation of dialysis to avoid the morbidity and mortality associated with dialysis (3, 4). Whether or not PKT also leads to improved clinical outcomes has been addressed by several studies but these report mixed findings. A USA registry analysis showed significantly better 5-year patient and graft survival rates in children transplanted preemptively vs. non-preemptively (nPKT) (5), whilst a multicentre retrospective cohort study from Japan found no difference in either patient survival or 5-year graft survival between these groups (6). Likewise, a number of single centre studies also show inconsistent results (7–10).

Historically, some centres believed that children with chronic kidney disease had to progress to ESKD requiring dialysis before being offered KT. The experience of dialysis would give children a sense of what life was like on dialysis leading to improved adherence post-transplant (11). This practice is no longer supported in most paediatric nephrology centres.

Paediatric ESKD patients differ from adult patients in terms of causes of ESKD, donor-recipient size mismatch, post-transplant complications, medication non-adherence, growth and development complications, and co-morbidities associated with the lower urinary tract (12). Therefore, it is important to evaluate the benefits of PKT specifically for the paediatric population. We undertook a systematic review to determine whether it is beneficial for paediatric patients to undergo KT before dialysis is initiated.

MATERIALS AND METHODS

Registration of Protocol

This study was designed and reported according to the PRISMA guidelines (13). The protocol was prospectively registered with PROSPERO (CRD42014010565) (14).

Inclusion Criteria

Type of studies: Any study design, including registry analyses, cohort studies, case-control studies and case series comparing PKT with nPKT, were eligible for inclusion. Case reports, and narrative reviews, editorials without primary data and non-English studies were excluded. We included both full articles and congress abstracts, and also checked for overlap in case abstracts were later published as full texts.

Type of participants and intervention: Eligible studies included those that compared paediatric recipients of a first or subsequent, living donor (LD) or deceased donor (DD) PKT versus nPKT. We included studies that described their population as paediatric or reported an age range of up to 18 years. PKT was defined as transplantation prior to any initiation of peritoneal dialysis (PD) or haemodialysis (HD). nPKT refers to transplantation after any given period of PD or HD. No restrictions were imposed on pre-transplant dialysis duration (dialysis vintage). Studies reporting on recipients with either a history of a previous organ transplant other than kidney or recipients of multi-organ transplants were excluded.

Type of outcomes: The outcomes of interest were overall graft loss (non-censored for death), death-censored graft loss, patient death (from all causes), delayed graft function (DGF), incidence of acute rejection (any definition, including clinically suspected and biopsy-proven acute rejection), renal function [serum creatinine or estimated glomerular filtration rate (eGFR)], primary non-function, quality of life, return to school after transplantation, height/growth measures, and incidence of cardiovascular morbidity, infections and malignancy.

Search Strategy

As this review was part of a larger study that reviewed the available evidence for both paediatric and adult KT patients, a broad bibliographic search was carried out up to 31 July 2020 using a mixture of free text and controlled vocabulary terms (**Supplementary Table S1**), which retrieved references for both paediatric and adult studies. Five electronic databases including EMBASE, MEDLINE (OvidSP), Cochrane Central Register of Controlled Trials (CENTRAL), Web-of-science and Google Scholar were searched. No limits for date of publication or language were applied. The references of identified studies or review articles were scanned to find potentially eligible studies that may have been missed during the literature search. Attempts were made to contact the study authors in case of missing data or unclear study information.

Selection of Studies

The study selection was carried out in two stages by independent reviewers (RRM, LP, ST, and JL). Initially, titles and abstracts of the retrieved studies were screened against the inclusion/exclusion criteria, followed by full-text review of potentially eligible papers and final selection of the studies to be included in the review. Discrepancies between reviewers were resolved by consensus.

Data Extraction

Two reviewers (RRM and LP) independently extracted the data using a standardized data extraction sheet. Discrepancies between reviewers were solved by discussion. Where there was more than one publication of the same study, data were only extracted from the publication that had the most complete data or the largest sample size. We extracted data on general study information and demographics, and primary and secondary outcomes. Where possible, data for LD and DD were extracted separately.

Assessment of Methodological Quality

The methodological quality of the included studies, published as full text papers, was assessed by two independent authors (RRM and LP) using the Downs and Black checklist (15). Two out of the

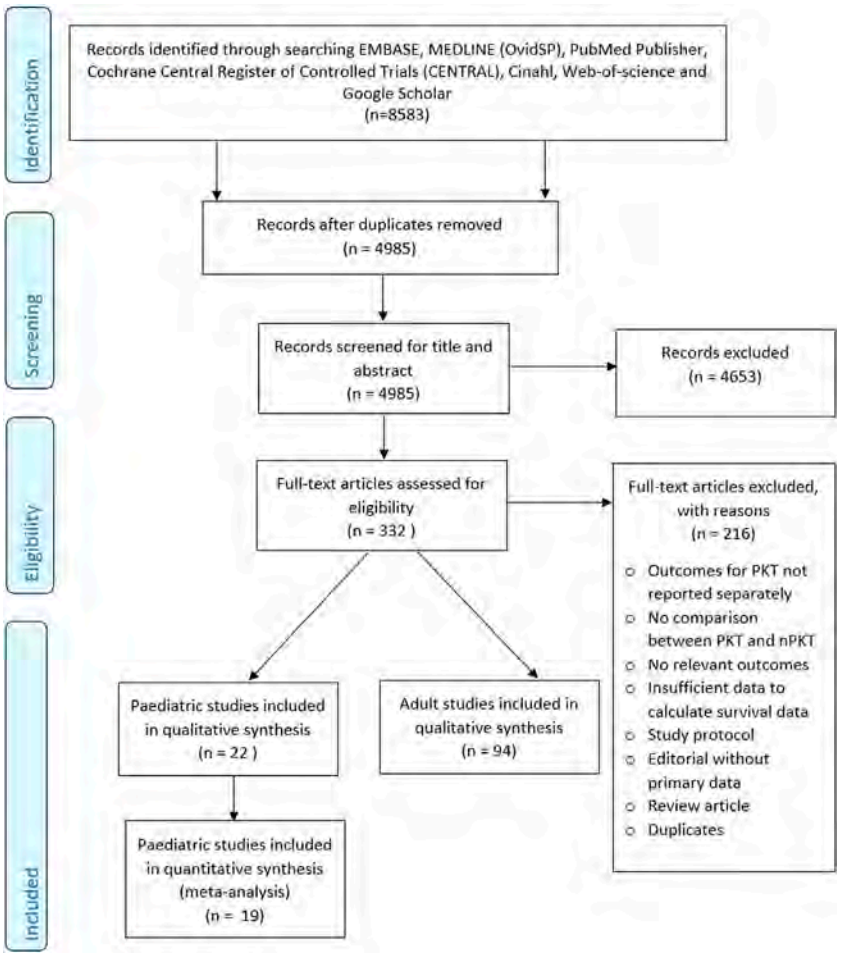

FIGURE 1 | PRISMA flow diagram.

27 items from the checklist were removed, i.e., the items relating to intervention compliance and the power of the study, as these were considered irrelevant or could not be calculated.

Statistical Analysis

Where at least three studies reported on an outcome, meta-analysis was performed using the statistical software R version 3.6.3. Data were pooled using the random-effects model to calculate the relative risk (RR) with a 95% confidence interval (CI). We planned to analyze data according to LD vs. DD, however, this was not always feasible as most studies combined LD and DD in their analyses. Hence, data were pooled regardless of whether they were LD and/or DD. Patient or graft survival rates were converted to the number of deaths and graft losses. Data on graft loss were categorized as either overall graft loss or death-censored graft loss. If a study neither defined graft loss nor specified whether the graft loss data was death-censored or non-censored for death, we categorized graft loss as being non-censored for death. We calculated a pooled estimate for the nPKT group if the study reported the results for nPKT according to different dialysis durations or separately for PD or HD. If a single study reported an outcome at more than one time point, the most recent follow-up data was used. Data were pooled for any duration of follow up. In order to account for the role of confounders in the analysis of the overall graft loss, we also calculated a pooled ratio consisting of adjusted ratios either calculated or directly extracted from the studies. Secondary analyses were conducted excluding smaller studies with overlapping countries and study periods to avoid duplicate use of data. If less than three studies reported on an outcome we summarized the results in a narrative review.

Heterogeneity was analyzed using the I^2 statistic (16). Where heterogeneity was significant ($I^2 \geq 50\%$), a mixed effect analysis was performed to explore its potential causes.

RESULTS

Included Studies

The literature search retrieved 8,583 references. Following full-text analysis of 332 studies, 216 studies were excluded (**Figure 1**). Of the remaining 116 studies that met the inclusion criteria, 22

TABLE 1 | Characteristics of the included studies.

Author (year); country	Study design and setting	Paediatric definition	1st Tx only	Number of patients					% Of HD in nPKT	HLA mismatch (Mean ± SD)		Duration of follow up	
	Period when Tx was received			LD		DD		Total					
				PKT	nPKT	PKT	nPKT			PKT	nPKT	PKT	nPKT
Amaral (5) (2016); United States	Retrospective registry analysis; multicentre January 2000–September 2012	<18 y	Yes	1,104	2,266	564	3,593	7,527	NR	3.26	3.79	NR	NR
Atkinson (24) (2020); United States	Prospective cohort study; multicentre March 2006–January 2017	<17 y	Yes	50	41	29	50	170	41.7	—	—	Median: 3.8 y IQR: 1.8–5.8 y	NR
Butani (25) (2011); United States	Retrospective registry analysis; multicentre January 1995–December 2000	<17 y	Yes	730	1,354	273	1,249	3,606	47.6	2.8 ± 0*	—	5 y	5 y
Cransberg (17) (2006); Europe Cransberg (18) (2000); Netherlands	Retrospective registry analysis; multicentre January 1990–January 2000	<16 y	Yes	86	132	70	825	1,113	NR	2.3 (LD); 2.6 (DD)	2.1 (LD); 2.5 (DD)	Mean = Median = 5.3 y Range: 0–14.1 y	Mean = Median = 5.3 y Range: 0–14.1 y
Cuervo (19) (2007); Mexico	Cohort study; single centre January 1995–December 2003	NR	NR	17	13	2	6	38	NR	—	—	NR	NR
Duzova (32) (2009); Turkey	Retrospective cohort studies; single centre 2000–2008	NR	NR	13	17	4	12	46	NR	—	—	5 y	5 y
Fitzwater (30) (1991); United States	Retrospective cohort studies; single centre Until 1987	<18 y	Yes	13	17	0	16	46	75.8	—	—	Mean: 24 m	Mean ± SD: 19.5 ± 7 m
Flom (26) (1992); United States	Retrospective cohort studies; single centre January 1984–December 1990	NR	No	26	40	0	0	66	32.5	—	—	Median: 3.5 y Range: 0.5–7.1 y	Median: 4.35 y Range: 0.6–7.3 y
Garcia (9) (2015); Brazil	Retrospective cohort study; single centre January 2000–December 2010	NR	NR	49	109	32	133	323	26.4	—	—	Median: 36 m IQR: 13–68 m	Median: 42 m IQR: 17–69 m
Harada (6) (2001); Japan	Retrospective cohort studies; single centre August 1987–December 1998	≤18 y	NR	9	20	—	—	29	45.0	2.2 ± 0.70	2.3 ± 0.87	Mean ± SD: 42.4 ± 19.4 m	Mean ± SD: 68.3 ± 39.8 m

(Continued on following page)

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

Author (year); country	Study design and setting	Paediatric definition	1st Tx only	Number of patients					% Of HD in nPKT	HLA mismatch (Mean ± SD)		Duration of follow up	
	Period when Tx was received			LD		DD		Total					
				PKT	nPKT	PKT	nPKT			PKT	nPKT	PKT	nPKT
Kaya (20) (2018); Turkey	Retrospective cohort study; single centre 2005–2017	NR	NR	—	—	—	—	230	NR	—	—	Median: 7.23 y Mean ± SD: 4.71 ± 2.61 y	Median: 7.23 y Mean ± SD: 5.88 ± 9.38 y
Kim (27) (2019); Canada	Retrospective cohort study; single centre January 2000–December 2015	<18 y	No	54	98	21	151	324	51.0	—	—	1 y	1 y
Kramer (21) (2012); Europe	Retrospective registry analysis; multicentre January 1988–December 2007	>3 and <18 y	Yes	321	435	123	950	1829	NR	—	—	8 y	8 y
Mahmoud (22) (1997); France	Retrospective cohort study; single centre April 1987–December 1994	NR	NR	8	8	32	55	103	82.5	3.3	3.3	Mean: 3.3 y Range: 0.8–7.0 y	Mean: 3.2 y Range: 0.4–7.8 y
Marlais (28) (2018); United Kingdom	Retrospective registry analysis; multicentre January 2000–December 2015	<18 y	NR	607	—	—	—	2038	44.9	—	—	NR	NR
Naderi (10), (2017); Iran	Retrospective cohort study; single centre 1989 to 2013	≤18 y	No	—	—	—	—	314	89.2	—	—	Mean ± SD: 15.9 ± 4.0 y Range: 0.5–20 y	Mean ± SD: 15.9 ± 4.0 y Range: 0.5–20 y
Nevins (7) (1991); United States	Retrospective cohort study; single centre July 1979–October 1987	<6 y	Yes	31	24	2	13	70	56.8	—	—	5 y	5 y
Offner (8) (1993); Germany	Retrospective cohort study; single centre January 1970–September 1991	NR	Yes	14	14	14	14	56	NR	—	—	5 y	5 y
Reydit (29) (2017); France	Retrospective cohort study; multicentre 1995–2013	≤18 y	Yes	-	-	-	-	1920	NR	—	—	Median: 7 y	Median: 7 y
Sinha (31) (2010); United Kingdom	Cross-sectional study; single centre May 1993–November 2006	NR	NR	16	46	23	44	129	42.2	1.83	2.14	Median: 4 y Range: 1–12 y	Median: 4 y Range:1–15 y

(Continued on following page)

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

Author (year); country	Study design and setting	Paediatric definition	1st Tx only	Number of patients					% Of HD in nPKT	HLA mismatch (Mean ± SD)		Duration of follow up	
	Period when Tx was received			LD		DD		Total					
				PKT	nPKT	PKT	nPKT			PKT	nPKT	PKT	nPKT
Splinter (33) (2018); Netherlands, Belgium and Germany	Cross-sectional study; multicentre October 2007–December 2014	8–18 y	NR	-	-	-	-	150	NR	—	—	N/A	N/A
Vats (23) (2000); United States	Retrospective registry analysis; multicentre 1992–1996	NR	Yes	466	890	159	980	2,495	60.4	—	—	Mean ± SD: 28.6 ± 19.5 m	Mean ± SD: 27.3 ± 19.0 m

*PKT, preemptive kidney transplantation; nPKT, non-preemptive kidney transplantation; HD, haemodialysis; Tx, transplant; DD, deceased donor; LD, living donor; *, standard error; SD, standard deviation; IQR, interquartile range; y, years; m, months; NR, not reported; N/A, not applicable.*

were identified as paediatric studies reporting on a total of 22,622 patients (**Table 1**). Cransberg (17) and Cransberg (18) were considered as one study due to insufficient data on the extent of overlap between the studies. Only the estimate for adjusted graft survival was extracted from Cransberg (18).

Methodological Quality

The methodological quality of the included studies varied with quality scores ranging from 10 to 19 out of a maximum possible score of 26 (**Supplementary Table S2**). Eleven studies adjusted for confounders in their analysis.

Patient Death

Ten studies (5–8, 17, 19–23) reported data on patient deaths. The pooled analysis showed no significant difference in the risk of patient death for PKT vs. nPKT (n = 13,490; RR: .77; CI: .53–1.11; p = .16; **Figure 2**). Heterogeneity was not significant (I^2 = 35.13%). The difference in the risk remained nonsignificant after excluding four studies (8, 17, 20, 22) with overlapping countries and study periods (n = 11,988; RR: .86; CI: .53–1.39; p = .53; I^2 = 57.94%; **Supplementary Figure S1**).

Patient death for LD transplants was reported in three studies (5, 6, 17). The pooled analysis revealed a significantly lower risk of patient death in PKT patients (n = 3,617; RR: .53; CI: .34–.83; p = .0054; I^2 = 0%; **Supplementary Figure S2**).

Two studies (5, 17) reported data on patient survival for DD. Amaral et al (5) reported a significantly higher 5-year patient survival in the PKT versus nPKT group (97.5% vs. 95.0%; p = .004). However, in the Cransberg et al (17) study, patient survival at 6 years following transplantation was similar between these groups.

Graft Loss

Sixteen studies (5–10, 17, 20, 22–29) reported on overall graft loss. The meta-analysis revealed that the risk of graft loss following PKT was significantly lower than that of nPKT (n = 20,212; RR: .57; CI: .49–.66; p < .0001; I^2 = 51.24%; **Figure 3**). Results were similar after excluding four (8, 24–26) studies with overlapping countries and study periods (n = 16,314; RR: .54; CI: .47–.62; p < .0001; I^2 = 32.22%; **Supplementary Figure S3**). Eight of the 16 studies reported ratios adjusted for various confounders, using multivariate analyses or by matching the PKT and nPKT group (5, 6, 8, 9, 18, 22, 25, 29). Pooling of these adjusted ratios showed a similar result (n = 16,715; RR: .61; CI: .40–.92; p = .018; I^2 = 60.7%; **Supplementary Figure S4**). The adjusted ratios and confounders are presented in **Supplementary Table S3**.

In an attempt to explain the heterogeneity between studies for overall graft loss, a mixed-effect analysis was performed which looked at the role of four moderator variables: the percentage of HD patients in the nPKT group, length of follow-up, percentage of LD, and the year of publication (**Supplementary Figures S5–S8**). None of these variables were found to significantly influence the relative risk of graft loss. It may be worth noting that on visual inspection of the forest plot, the heterogeneity is in the size of effect rather than the direction of effect.

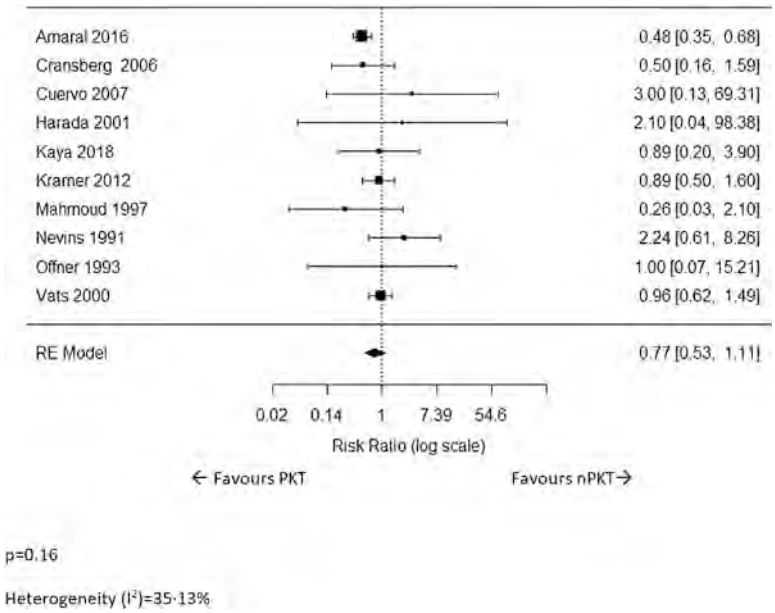

FIGURE 2 | The relative risk of patient death for PKT vs. nPKT.

Five studies (5, 6, 23, 26, 27) reported on overall graft loss for LD, and the pooled analysis showed that PKT significantly reduced the risk of graft loss (n = 4,973; RR: .57; CI: .46–.69; p < .0001; I^2 = 0%; **Supplementary Figure S9**).

Two studies (5, 23) reported on overall graft survival in DD recipients. Amaral et al (5) reported a significantly higher 5-year graft survival rate in PKT patients compared to nPKT patients (85.4% vs. 76.4%; p < .001). However, Vats et al (23) reported similar 3-year graft survival in PKT versus nPKT (PD and HD) patients.

Death-censored graft loss was reported in two studies (9, 30) for LD and DD data combined. Garcia et al (9) reported a higher 12-, 36-, 60- and 90-month death-censored graft survival rate, adjusted by donor type, in PKT patients compared with nPKT patients (97% vs. 87%; 92% vs. 79%; 86% vs. 72%; 76% vs. 65%, respectively). The difference was significant at 90 months (p < .05); however, the study did not clearly report if the differences were significant at the other time points. The study by Fitzwater et al (30), found no significant difference in the 2-year death-censored graft loss between PKT and nPKT.

Delayed Graft Function

DGF was reported in three studies (17, 25, 27). The RR for the incidence of DGF was .57 (n = 4,871; CI: .22–1.50; p = .26; **Supplementary Figure S10**). Heterogeneity was high (I^2 = 81.51%). We could not explore heterogeneity as the number of studies was too small.

DGF for LD was reported in two studies (17,27). Cransberg et al (17) showed a slightly higher incidence of DGF in PKT patients (3.5% vs. 2.4%), but did not report if this difference was significant. No significant difference was observed between PKT vs. nPKT in terms of DGF in the study by Kim et al (27).

The only study that reported on DGF in DD patients was Cransberg et al (17), which observed no difference in the DGF rate between PKT and nPKT.

Acute Rejection

Incidence of acute rejection was reported in seven studies (6, 17, 22, 25–27, 30). The pooled analysis revealed that the risk of acute rejection in PKT patients was significantly lower than that of nPKT patients (n = 4,897; RR: .81; CI: .75–.88; p < .0001; **Figure 4**). Heterogeneity was low (I^2 = 0%). Similar results were observed after excluding Fitzwater et al (30) from the analysis due to overlapping country and study period (n = 4,851; RR: .81; CI: .74–.87; p < .0001; I^2 = 0%; **Supplementary Figure S11**). Of the seven studies, only two (6, 22) adjusted for confounders; hence, a pooled estimate of the adjusted acute rejection rate could not be calculated.

Three studies (6, 26, 27) reported on the rate of acute rejection for LD. Although the effect size was similar to the overall analysis, it did not reach statistical significance (n = 247; RR: .79; CI: .55–1.15; p = .22; I^2 = 0%; **Supplementary Figure S12**).

Cransberg et al (17) was the only study that included data on acute rejection for DD. In the study, a significantly higher percentage of patients remained acute rejection-free following PKT than after nPKT (52% vs. 37%; p = .039) at 3 years.

Cardiovascular Morbidity, Infections and Malignancy

Two studies reported cardiovascular morbidity outcomes (17, 31). Cransberg et al (17) measured the incidence of severe hypertension between PKT vs. nPKT at one, three and 5 years post-transplant, and found significantly lower incidence of severe hypertension in the PKT group in the third year (40% vs. 64%; p =

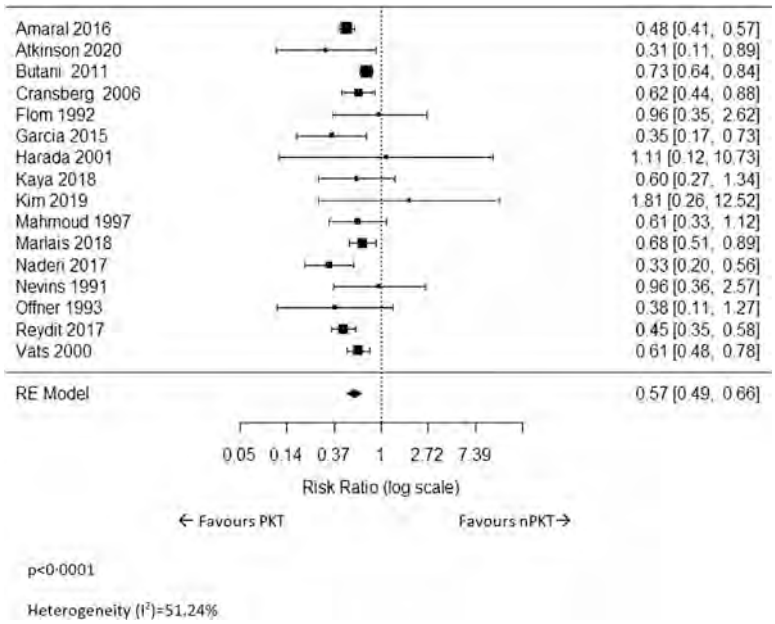

FIGURE 3 | The relative risk of overall graft loss for PKT vs. nPKT.

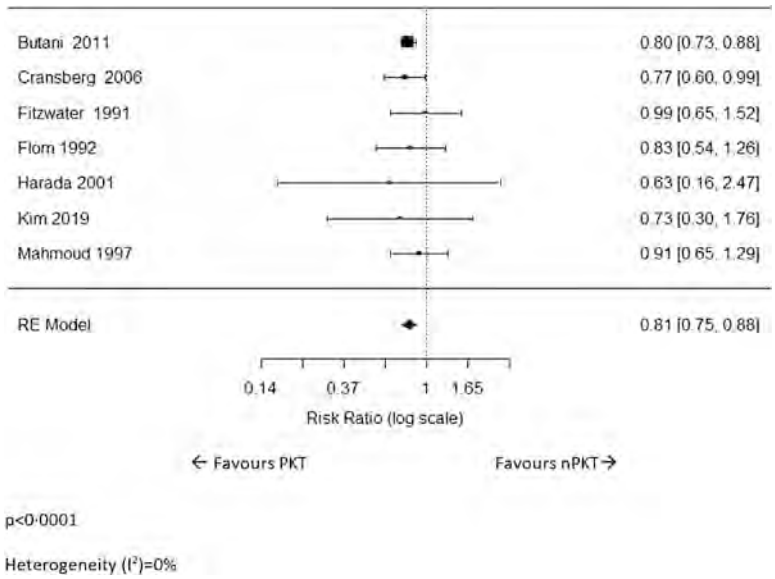

FIGURE 4 | The relative risk of acute rejection for PKT vs. nPKT.

.016), among patients with DD transplants. The study by Sinha and Marks (31) also showed a significantly lower incidence of hypertension in the PKT versus nPKT group (31% vs. 53%; p = .02) for combined LD and DD data. No studies reported on infections and malignancy.

Renal Function

Renal function was reported in six studies as either eGFR or serum creatinine, with four studies (20, 22, 30, 32) reporting on LD and DD data combined. Mahmoud et al (22) evaluated the mean GFR at one and 4 years post-transplant, and found no statistical differences in the GFR values between the PKT and nPKT group at both follow-ups. The study by Kaya et al (20) also showed no significant difference in the mean GFR between these groups within a median follow-up of 7.23 years. Duzova et al (32) measured the mean GFR values at one, two, three and 5 years after transplantation, and reported a significantly lower mean GFR in the PKT group only in the third year (mean ± standard

deviation (SD): 86 ± 31 ml/min/m^2 vs. 101 ± 31 ml/min/m^2; $p <$ .05). Likewise, Fitzwater et al (30) reported no statistical differences in the serum creatinine levels between PKT and nPKT at 1 month, 3 months, 6 months, 1 year and 2 years post-transplant.

Two studies (26, 27) reported renal function for LD only. Kim et al (27) reported no differences between PKT and nPKT in the median GFR at 1 month and 1 year. Flom et al (26) reported a higher mean (±SD) GFR for PKT (68 ± 28 ml/min/1.73 m^2) versus nPKT (HD and PD) (both 60 ± 26 ml/min/1.73 m^2), calculated over a median follow-up of 3.5, 3.6 and 5.1 years for PKT, PD and HD respectively. However, the study did not report whether this difference was significant.

Primary Non-Function

No studies reported on primary-non function.

Quality of Life

Quality of life was reported in only two studies (6, 33). Splinter et al (33) assessed the health-related quality of life (HRQoL) of patients who spent at least 6 months on their treatment modality, using the PedsQL™ questionnaire. The PedsQL™ consisted of five major domains, including physical health, emotional functioning, social functioning, school functioning, and psychosocial health. The mean ± SD HRQoL scores for physical health was significantly higher in the PKT vs. nPKT group (78.6 ± 18.0 vs. 70.4 ± 20.5; $p <$ .05), but showed no differences between the groups for the other domains. Harada et al (6) asked patients about the benefits and disadvantages of renal transplantation. The percentage of patients that reported feeling satisfied with the improvement in their physical condition was significantly higher in the PKT vs. the nPKT group ($p <$ .01). On the other hand, a significantly higher percentage of patients in the nPKT group reported satisfaction related to the freedom from restrictions of liquid intake, daily diet and time spent on dialysis, following renal transplantation ($p <$ .01). No significant differences were observed between the two groups regarding disadvantages felt due to renal transplantation, which included anxiety about the fate of the renal graft and annoyance resulting from frequent hospital visits and daily medications.

Return to School

No studies reported data on return to school.

Height/Growth

Three studies (6, 8, 31) reported findings on the height/growth of patients. Harada et al (6) assessed the mean ± SD heights of the patients at transplantation and at one and 3 years post-transplant, using the national cross-sectional standard growth chart for boys and girls. The study showed significantly better mean ± SD height in the PKT vs. nPKT group at transplantation (−.84 ± 0.73 vs. −2.86 ± 1.93; $p <$ .05) and at 3 years post-transplant (−.53 ± 1.65 vs. −3.22 ± 1.94; $p <$ 0.05), only for patients less than 15 years old. Sinha and Marks (31), who measured the height of the patients at the last clinical visit (range 1–15 years) using the median standard deviation score (SDS), found no significant differences in the scores between the two groups. Similar results were reported by Offner et al (8), who also used the median SDS to measure the height of the patients at 1 year post-transplantation.

Primary Kidney Transplant

Secondary analyses comparing PKT versus nPKT patients with primary KT are presented in **Supplementary Figures S13–S15**.

DISCUSSION

The available evidence from observational studies suggests that PKT significantly lowers the risk of graft loss and acute rejection compared to nPKT. PKT patients with LD transplants are seen to benefit from a reduced risk of patient death as well as overall graft loss. Most studies in our review showed nonsignificant differences in post-transplant renal function between PKT and nPKT patients. Regarding other outcomes, such as cardiovascular morbidity, quality of life and height/growth, it was not possible to draw firm conclusions due to the limited evidence available. However, with regard to quality of life, patients reported improvement in physical condition better in the PKT than the nPKT group. There were not enough data to draw firm conclusions regarding different outcomes for DD and LD kidney transplantation.

Our results agree with the findings of the systematic review by Abramowicz et al (34), which looked at a combination of paediatric and adult KT recipients and suggested PKT offers better allograft survival. The same benefit has been observed in studies performed on adult PKT patients (35, 36). Research explaining the reasons for this benefit, especially specific to paediatric patients, is scarce. It is possible that several confounding factors have accounted for some or all of this observed survival advantage. Studies have shown that rates of PKT are significantly higher in children who are white versus other races, and males versus females (37–39). This may result in selection bias, which in turn may affect graft survival.

We attempt to explain the association between PKT and higher graft survival by analysing data in adult studies because of the lack of data on paediatric patients. It should, however, be noted that it remains unclear to what extent these adult data can be applied to the paediatric patients. Firstly, some authors have speculated that the association of between PKT and a reduced risk of graft loss may have been influenced by higher residual renal function of native kidney observed in PKT patients at transplantation, compared to nPKT patients. However, three studies have found that PKT with higher pre-transplant eGFR is not linked to better graft survival (40–42), suggesting that pre-transplant residual renal function may not be one of the major factors affecting graft survival. Secondly, the survival benefit of PKT may be due to the avoidance of comorbidities, such as cardiovascular disease, that are associated with dialysis (43). A study by Prezelin-Reydit et al (44), however, found that the adjusting for cardiovascular comorbidities and diabetes mellitus did not alter the link between PKT and the reduction in the hazard

of graft failure. This agrees with our subgroup analysis of adjusted risks, which still showed a graft survival advantage for PKT. Lastly, as PKT take place earlier in a patient's natural history of disease compared to nPKT, there are concerns that this "lead time" may bias observational studies to favour PKT as the optimal treatment modality (11, 45). However, Gill et al (36) demonstrated that PKT and nPKT patients with at least 2 years of allograft survival established similar baseline GFR levels at 6 months post-transplant, disapproving the hypothesis that the graft survival benefit linked to PKT may be a consequence of lead time bias due to earlier transplantation of PKT patients with preserved native kidney function.

Another significant finding in our meta-analysis is a lower incidence of acute rejection in PKT patients which may be explained by the biological differences observed in the immune reactivity of PKT versus nPKT patients (11). These differences are not yet well understood and are somewhat counterintuitive; therefore, further in-depth immunological studies into T cell senescence and allo-immunity in both groups are warranted.

This study had several weaknesses. It only included observational studies, which by nature are frequently subject to confounding and bias, which may lead to false-positive findings (46). Additionally, although current paediatric kidney transplantation guidance advises PKT whenever possible, in reality, some non-adherent children may be initiated on dialysis before receiving a transplant. This practice introduces a bias and it may be an additional unaccounted confounder in our results. The small number of studies in some of the pooled analyses preclude finding convincing evidence for the outcomes, for example for delayed graft function. Heterogeneity was high for some of the outcomes, and could not always be explored due to the small number of studies. Definitions of reported outcomes were not clearly stated for some studies, e.g., overall graft survival or death-censored graft survival. We were unable to perform separate analyses for LD versus DD patients for most outcomes due to limited number of studies that presented these data separately. It was also unclear from some of the included studies whether there were any pre-emptive second transplants included in the study populations. Although we attempted to address the possible role of confounding variables, such as socio-economic status, health literacy, psychosocial support, lead time bias and recurrence of primary ESKD, on overall graft survival by pooling adjusted ratios, this is limited to the adjustments used in the original analyses and additional confounders may still be present. Another limitation is the inconsistent reporting of dialysis vintage, making it difficult to assess the impact of different durations of dialysis on transplant outcomes.

Our systematic review also highlights the inconsistent and poor reporting of certain outcomes that are relevant to paediatric ESKD patients, such as cardiovascular disease and quality of life. Studies have shown that absence from school, social engagement, symptoms (feeling ill or pain), hospitalisation, poor sleep and fatigue are important to children with ESKD (47–49), however, these outcomes were poorly reported or not reported at all by the studies included in the review. Future studies should report the core outcomes established by the SONG-Kids initiative (50) to ensure that outcomes relevant to children are included in research proposals.

In conclusion, systematic review of observational studies showed that paediatric PKT patients have a lower risk of overall graft loss and acute rejection than nPKT patients. While no difference was seen in overall patient mortality, PKT appeared to significantly lower the risk of patient death in LD patients. Therefore, it is important to develop pathways that ensure PKT options for as many paediatric ESKD patients as possible, especially emphasising on living donation. With education of paediatric patients and carers early in the disease process about LD PKT, a timely transplant or timely waitlisting for DD KT (in absence of LD options) can be achieved for many patients. This also calls for a redesign of the default renal replacement therapy pathway, which unfortunately is still set to dialysis before transplantation.

AUTHOR'S NOTE

The abstract of the paper received the 2021 Marius Renard Award for Best Abstract on Paediatric Transplantation (a joint ESOT and IPTA (International Pediatric Transplant Association) award) during the 2021 ESOT congress.

AUTHOR CONTRIBUTIONS

RRM: Literature search, data collection, methodological quality assessment, data analysis, data interpretation, writing, and project administration. SK: data analysis, data interpretation, revising, and writing. JS: data interpretation, revising, and writing. SM: data interpretation, revising, and writing. JL: Conceptualization, literature search, data collection, data interpretation, revising, and writing. ST: Conceptualization, literature search, data collection, data interpretation, revising, and writing. FD: Conceptualization, data interpretation, revising, writing, and supervision. LP: Conceptualization, literature search, data collection, methodological quality assessment, data analysis, data interpretation, revising, writing, project administration, funding acquisition, and supervision.

FUNDING

The study was supported by a grant from the Philip Allison Foundation. The funder had no role in the study design, data collection, analysis, interpretation of the data, decision to publish and/or the preparation 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.

ACKNOWLEDGMENTS

We would like to acknowledge Wichor Bramer, Biomedical Information Specialist, Erasmus Medical Centre, Rotterdam, The Netherlands, for designing and running the searches.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10315/full#supplementary-material

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ORIGINAL RESEARCH
published: 10 March 2022
doi: 10.3389/ti.2022.10107

The Suitability of Potential Organ Donors Using Real Case-Scenarios; Do we Need to Create a "Donor Board" Process for Donors Perceived as Unlikely Suitable?

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Introduction: Despite availability of selection criteria, different interpretations can lead to variability in the appreciation of donor eligibility with possible viable organs missed. Our primary objective was to test the perception of feasibility of potential organ donors through the survey of a small sample of external evaluators.

Methods: Clinical scenarios summarizing 66 potential donors managed in the first year of our Organ Recovery Center were sent to four critical care physicians to evaluate the feasibility of the potential donors and the probability of organ procurement.

Results: Potential donors procuring at least one organ were identified in 55 of the 66 cases (83%). Unanimity was reached in 38 cases, encompassing 35 out of the 55 converted and 3 of the non-converted donors. The overall agreement was moderate (kappa = 0.60, 95% CI: 0.37–0.82). For the organs finally procured for transplantation, organ donation was predicted for the majority of the cases, but high discrepancy was present with the final outcome of organs not procured (particularly liver and kidney).

Conclusion: The assessment of a potential donor is a complex dynamic process. In order to increase organ availability, standardized electronically clinical data, as well a "donor board" structure of decision might inform future systems.

Keywords: organ procurement, potential donors, transplant, critical care, system

OPEN ACCESS

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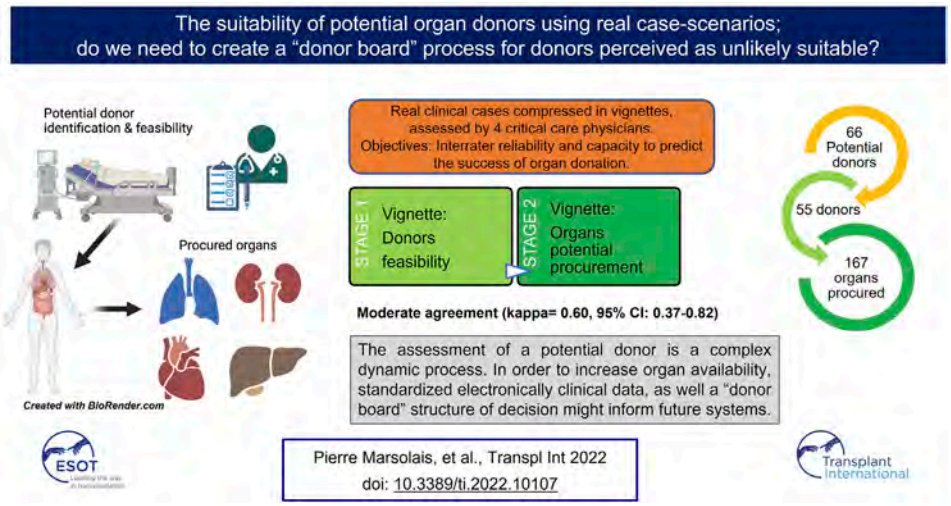
***Received:** 12 October 2021*
***Accepted:** 11 January 2022*
***Published:** 10 March 2022*

Citation:
Marsolais P, Larouche G, Lagacé A-M Williams V, Serri K, Bernard F, Rico P, Frenette AJ, Williamson D, Albert M and Charbonney E (2022) The Suitability of Potential Organ Donors Using Real Case-Scenarios; Do we Need to Create a "Donor Board" Process for Donors Perceived as Unlikely Suitable? Transpl Int 35:10107. doi: 10.3389/ti.2022.10107

Abbreviations: DBD, donation after brain death; DCD, Donation after Cardiac Death; OPO, Organ Procurement Organization's; ORC, Organ Recovery Center.



GRAPHICAL ABSTRACT |

INTRODUCTION

Worldwide organ shortage is a major issue in the field of organ transplantation (1, 2). In 2018, with 20.6 donors by million population, a total of 2,782 transplants were performed while 223 people died waiting for an organ in Canada (3). In recent years, numerous initiatives have improved the yield of organ donation, including campaigns targeting the adherence of the general population to organ donation, promotion of presumed consent and living donation, guidelines for donor management (4, 5), organisational structure (6, 7), introduction of liver splits, reintroduction of Donation after Cardiac Death (DCD) (8, 9) and amongst others, extended criteria (10). Despite these significant efforts, the gap between the number of organs offered and the demand remains.

The critical care physician is a central stakeholder in the optimization of organs, while the entire process leading to organ donation from an identified potential donor relies often on numerous caregivers, including the Organ Procurement Organization's (OPO) personal and the transplantation programs (11). The identification of donors, medical staff attitudes and institutional culture have been identified as sources of missed opportunities (12, 13). Unfortunately, despite the presence of selection criteria, different interpretations can lead to important variability in the appreciation of the eligibility of a donor, with many viable organs missed (14, 15). Several studies have reported cases of organs being first refused by an institution and then successfully transplanted after acceptation by another (16–18), reflecting the variability in acceptance on the transplant team side. Even if the relation between success of donation, communication of donor information and processes of decision making seems important, it may be difficult to isolate specific culprits in a complex and fragmented system.

Preliminary reports suggest the impact of a dedicated team on organ donation, applying organ management to increase the conversion of patients into donors (19, 20). However, little is known regarding the supporting donor team, particularly regarding how critical care physicians assess potential donors and feasibility of organ donation. As summarized by Tong et al, qualitative studies are required in order to understand the process of decisions, central to the improvement of transplant care (21). The overall objective of the present study was to evaluate the perception of feasibility of a group of potential organ donors, through the survey of a small sample of external evaluators. Our hypothesis was that the blind comparison of the evaluator's perceptions of feasibility with the outcome of organ donation would allow us to identify barriers or potential directions to improve donor's realization.

MATERIALS AND METHODS

Sources of Data

The study was conducted in 2016–2017 and retrospectively selected a group of potential donors referred to our Organ Recovery Center (ORC) in its first year of activity (June 2013–June 2014) (19). The study data sources were the medical chart, scanned electronic documents, resource nurse's donor files, and the provincial OPO coordinator dataset. Laboratory, radiology and investigations (i.e., bronchoscopy, echocardiography, pathology) results were collected. The study was approved by the institutional research ethic board (CER 2014–1049).

Population and Pre-Defined Level of Donors' Potentiality

All potential donation after brain death (DBD) and DCD admitted during the first year of our ORC activity, either directly from our ICU or transferred from other centers, were

TABLE 1 | Potential donors' characteristics.

	N = 66
Age (years), median (range)	57 (17–84)
Female/Male, N	29/37
Deceased neurologically, N (%)	59 (89)
Causes of brain injury, N (%)	
Brain Anoxia	19 (29)
Cerebral Hemorrhage	29 (44)
Ischemic Stroke	3 (4.5)
Brain Trauma	14 (21)
Cerebral tumor	1 (1,5)
PoDo References, N (%)	
From our center	29 (44)
From other centers	37 (56)
Converted Donors, N (%)	55 (83)
Female/Male, N	24/31
Age (years), median (range)	53 (17–84)
DBD, N (%)	49 (89)
DCD, N (%)	6 (11)
Converted Donors from other centers, N (%)	30 (55)

Results are displayed as N (%) or Median (Range).
PoDo, Potential donors; ORC, Organ Recovery Center; DBD, Donation after Brain Death; DCD, Donation after Cardiac Death.

TABLE 2 | *A priory* feasibility according to Organ Recovery Center (ODC).

ORC categories	Converted, N = 55	Not converted, N = 11
A. Feasible, N = 39	38	1
B. *Unfeasible, N = 27	17	10

**Identified in regard to either OPO guidelines, suspicion of neoplasia or identified legal/ administrative barriers.*

included. As for any case entered into the database, they were categorized into 2 categories: 1) those who had no obvious problem to be converted (feasible donors) because they completely matched the local OPO criteria and requirements; 2) those who had been identified by the ORC as unlikely to be converted (a priori unfeasible) after the initial assessment but still supported. The latter were identified in regard to either OPO guidelines, suspicion of neoplasia or identified legal/ administrative barriers. This assessment was done *a priori* according to the perception of the ORC team and collected systematically at admission. As for the definite outcome of donation for transplant, two sub-categories were defined, namely converted (at least one organ transplanted) vs none converted donors.

Clinical Case Scenario Vignettes Development

Using the collected information from the 66 potential donors, we developed clinical vignettes in the form of clinical case-scenarios (22). The presented information was anonymized and standardized to protect the privacy of patients. The vignettes were built in two parts (example in **Supplementary Material**): the first page contained a short description of the potential donor at the time of admission in the ORC (if transferred) or at the time of consent for organ donation in our center. The second page described the clinical information, radiological assessments and the physiological evolution of the following organs: heart, lungs, kidneys and liver, excluding the pancreas. We did not consider the pancreas because of the very restrictive criteria for this organ based on the age below 50 years, Body mass index under 30 and the absence of diabetes.

The content of each vignette was examined by the investigators, who reviewed the clarity and comprehensiveness of items, individually or in group, until an agreement was reached regarding the format and content. Two internal evaluators (intensivists working in our center), who were not part of the study, were sent a random sample of 10 vignettes, to assess the format and the content, comprehensiveness, clarity or the inaccuracy of information. Based on their comments, controls of information extracted from the patient's file for all the vignettes were made, as well as complements or modifications suggested after internal review. Modification of the format and items display were made according to their feedback.

Statistics

The sample size calculation was based on a kappa null value set at 0.4 and an expected significant difference to be 0.2, with a kappa of 0.6 for reached agreement. Considering an expected proportion of mean positive rating at 0.7 (to the question of feasible candidate or not) and power of 80%, the number of comparisons needed were 191. Of the 200 comparisons (4 × 50 vignettes), agreement testing was analyzed using Gwet kappa coefficient and the level of agreement scaled (23). Results were reported using descriptive statistics as proportions of categorical or ordinal variables and kappa were reported with 95% confidence intervals. A Fisher's exact test was used to compare proportions. p Value was deemed significant if < 0.05. Statistics were processed using IBM SPSS 20.

Design of Rating and Assessment Processing

The 66 vignettes were evaluated, with four blocks of a random sample of 50 vignettes sent to 4 critical care physicians from centers outside of the ORC service corridor. We aimed to establish the interrater reliability as primary objective and their capacity to predict donation outcome as the secondary objective. Each vignette was then evaluated by three physicians, except for two vignettes evaluated by the four physicians (Total sample of 50 × 4 = 200; 66 vignettes × 3 = 198). These physicians were involved in organ donor management on a regular basis and affiliated to the 4 hospitals with the highest volume of Quebec OPO referral. We capped the evaluation at 50 vignettes, to maximize their adherence to the process (4 × 50 = 200 assessments) and according to the sample size calculation. Initially, we sent the first page (**Supplementary Material**) detailing the general description of the patient after consent for organ donation. Clinicians were asked to state if they thought the potential donor presented on the vignette was a feasible organ donor, within the framework of OPO guidelines.

TABLE 3 | Proportion of potential donors rated as feasible by external clinicians.

Organ donation outcome	Feasibility rating	A (N = 50)	B (N = 50)	C (N = 50)	D (N = 50)
Converted donors (N = 55)	N/total (%)	33/40 (82)	41/41 (100)	27/43 (63)	34/42 (81)
Not Converted donors (N = 11)	N/total (%)	3/10 (30)	9/9 (100)	3/7 (43)	6/8 (75)
	Deemed feasible, proportion (%)	36/50 (72)	50/50 (100)	30/50 (60)	40/50 (80)
*Kappa** (95% interval)		0.69 (0.51–0.86)	0.78 (0.66–0.91)	0.37 (0.15–0.60)	0.60 (0.42–0.78)
PoDo converted assessed, N/total (%)		40/55 (73)	41/55 (75)	43/55 (78)	42/55 (76)
PoDo not converted assessed, N/total (%)		10/11 (91)	9/11 (82)	7/11 (64)	8/11 (73)

**Agreement* Kappa *between the converted donors and each clinician rating (p < 0.0001). The proportion of PoDo converted or not, received for assessment by each clinician are reported in the lower part of the table.*

The first column (upper part of table) give the absolute numbers of potential donors (PoDo) converted or not. The proportion of PoDo deemed feasible by each clinician for these two categories are the displayed in the four last columns.

ORC, Organ Recovery Center.

They received the instruction to give an answer based only on the information available, their knowledge, their judgement and their usual work environment. If they answered *yes*, they had to rate the probability of the organ donation outcome, as low, medium or high. After returning their answer, they were sent the second page with organs data, only for the cases they had deemed feasible. Based on the description, the clinicians rated their perception of suitability for transplantation (**Supplementary Material**); if they thought that it was the case, they had to rate the likelihood on an ordinal scale of categorical percentages (<20%, 20–40%, 40–60%, 60–80%, 80–100) for every organ separately. They were not aware, at any time, of the final organ donation outcome.

RESULTS

Potential Donors' Characteristics

During the first year of activity of the ORC, we managed 66 potential donors with a median age of 57 years. The majority of them were referred from other centers (56%) and cerebral hemorrhage was the most frequent brain injury (44%), as shown in **Table 1**. The number of potential donors converted in organ donors was 55/66 (83%), of which 6/55 (11%) were DCD.

All of the cases deemed feasible after the initial evaluation by the ORC were converted except one, whereas 63% of the cases deemed unfeasible were converted (**Table 2**). The causes for non-conversion of the potential donors were cancer (N = 3), infection (N = 1), circulatory collapse (N = 1), family withdrawal of consent (N = 1), no suitable organ (N = 3) and age related (N = 1).

Rating and Assessment of Potential Donors

Clinicians deemed the potential donors as feasible, for various proportions of the vignettes received (A: 72%, B: 100%, C: 60%, D: 80%). The feasibility rating of potential donors by clinicians is presented in **Table 3**. Of the 66 vignettes (first part), one case was rated feasible by none, 14 were rated feasible by one, 13 by two, 38 by three or more clinicians. Therefore, unanimity was reached in 38 cases, encompassing 35 out of the 55 converted and 3 of the non-converted donors. The overall agreement, for the same cases assessed, between clinicians was moderate (kappa = 0.60, 95% CI: 0.37–0.82). Three clinicians reported weak feasibility for 2.5–4% of their realistic cases, and one for 27% of them. The aggregation of the weak probability of feasibility category with "not feasible" did not increase their agreement level. The agreement between the converted donors and rating of the clinicians were good for two, moderate for one and fair for one (*Kappa*, **Table 3**).

Taking the final outcome as reference, the sensitivity of clinician to predict a converted potential donor was 87%, and specificity 31%. The positive predicted value was 86.5% and negative predictive value was 41%. Regarding the subgroup of predefined unfeasible potential donors, the clinicians (at least one) rated them feasible in more than 50% for those finally converted (median 66.5%; range 36–100%), but less than 50% for those not converted (median 43.5%; range 13–100%). Of the 17 cases deemed initially unfeasible by the ORC team but finally converted, 10 potential donors were deemed feasible by more than one clinician. For the feasible subgroup, their feasibility rate assessment was the highest (median 91%; range 76–100%).

Perceived Barriers of Converted Potential Donors

The number of converted potential donors assessed by each clinician was very similar (**Table 3**). Various proportions were deemed not feasible by clinicians (A: 18%, B: 0%, C: 37%, D: 19%; **Table 3**, first line). Of the 55 converted donors, clinicians deemed 20 cases (36%) not feasible (10 were by one clinician, 9 were by two clinicians and 1 by three clinicians).

The presence of non-admissible criteria according to the OPO and pathology that could be perceived as a barrier were present in the 10 cases, where at least two clinicians had declined feasibility. Despite the opportunity and request to describe a reason for non-feasibility, only four cases had comments written by the clinicians. All of the 10 potential donors had multiple organ failure at the time of support initiation (circulatory shock, acute renal failure, shock liver, coagulopathy or high lactate). Two cases were in a situation where the coroner was involved. One had a 9 mm suspicious lung nodule and another had multiple suspicious mediastinal adenopathies on CT-scan. Four of the potential donors had an aspiration pneumonia with significant lung infiltrates, with one of them also having an urosepsis; another had an endocarditis with

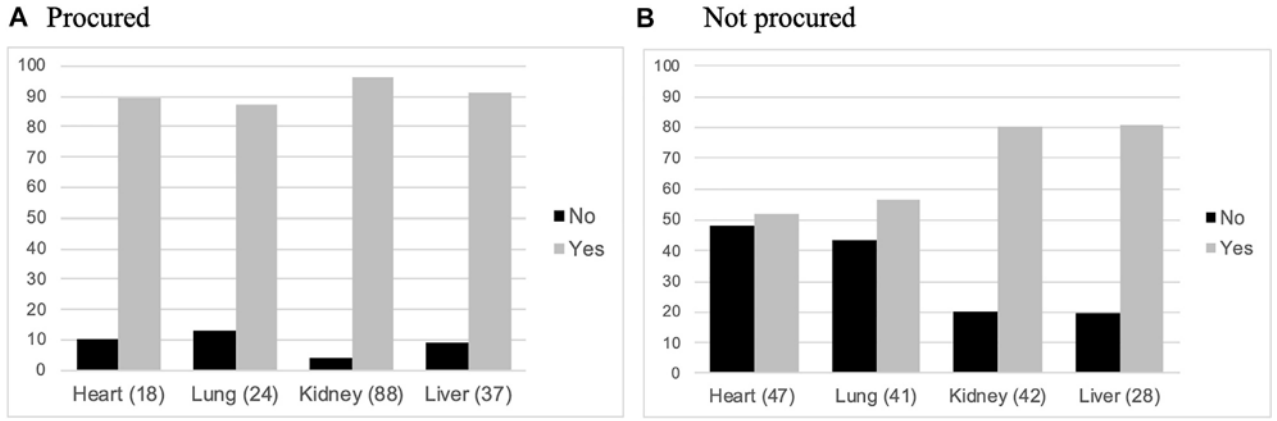


FIGURE 1 | Perceived feasibility of organs for transplantation. Histograms representing relative proportions (%) of organs perceived as potentially feasible or not for transplant by the clinicians. Procured organs **(A)** and not procured organs **(B)**.

cerebral embolization. Finally, one patient had trisomy and one had an HIV positive screening test (false positivity revealed later).

Potential Donors Deemed Feasible by Clinicians but not Converted

We found 6 potential donors deemed feasible by at least two clinicians, but not converted. One had positive Hepatitis C, with no suitable receiver once offered through the OPO; one had a highly suspected renal carcinoma and two others had non-resolving multiple organ failure with refractory shock. One transferred patient failed the criteria for DBD after assessment by the ORC team and was not suitable for DCD. Finally, consent was withdrawn by the family of a last one.

Organ Feasibility Rating

In the evaluation of organ suitability for transplant, 65 vignettes (second page) were transmitted to the clinicians and considered to be the denominator assessed (given on case rejected by all, after the first page assessment); they received a various proportion of their initial 50 cases that they had deemed feasible (A: 72%, B: 100%, C: 60%, D: 80%). It means that 13 vignettes were evaluated by two clinicians, 14 by one only and 38 of the 65 (58%) were evaluated by three clinicians, at this stage. Lung was considered as a whole, given the fact that on the 24 transplantation, a single lung was taken only in two cases. For the kidney, the total of the 88 (45 right + 43 left) were accounted for; both were procured in 39 and only one in 10 donors. For the organs, which were finally procured for transplantation, they predicted organ donation for the majority of the cases (**Figure 1A**), with less than 10% considered not feasible; they were more confident for kidneys, than for other organs (**Supplementary Figure S1A**). For organs, which were not procured, their predictions were more discrepant with the final outcome (**Figure 1B**), but in a lesser magnitude for hearts and lungs; they still thought that a majority of kidneys and livers could be procured for transplantation (**Supplementary Figure S1B**).

The reasons for final refusal of kidneys and livers by the OPO or the transplant team were multiples: mostly medical (e.g., past-medical history, suspect findings, compatibility with receiver, age, vascular anomalies, pathology findings per-op), consent changes or limitation by families, and no interest for the organ proposed.

DISCUSSION

The organ donation process is complex, resources-demanding and highly emotional, while the assessment of organ procurement feasibility is a challenging and dynamic process (13). In our study, we showed a high variability (moderate agreement) in a small sample of clinician's assessment, despite the fact that they deemed the majority of their assessed cases feasible. At the stage of the initial short description of the potential donor, up to 36% of the potential donors could have been discarded, depending on who would have managed the case.

To initiate donation support process, critical care physicians must perceive a fair likelihood of reaching donation and at least provide one acceptable organ for transplantation. Critical care physicians conduct organ support and then expect potential recovery of organ failure (2, 11). In contrast, the OPO local coordinator collects specific clinical parameters (i.e., left ventricular ejection fraction, hepatic enzymes, creatinine level, oxygenation), at different time points and communicate them to the transplant teams. The latter have also their own perspectives: priorities, age of potential donors, matching, perceived quality of the organs, access to operating rooms, transplant team availability.

As illustrated by our study, despite a high sensitivity to predict a converted potential donor, opinions were far from unanimous. Potential barriers could not be collected in detail, but many causes could be mentioned: the clinician's level of experience, the degree of confidence in the potential for maintenance or recovery of the organ function, the comfort in approaching family representatives, the perception of time needed for support and the access to eventual expertise in donor management (24, 25). Above all, we can also hypothesize that in an area as sensitive as organ donation, the perception of acceptability by colleagues and the institution is highly influenced by regional and institutional policies (26).

The perception of specific transplantable organs by the critical care physicians was high for the organ finally procured, but a more variable opinion was present for the organs not procured. An intriguing finding from our study is the discrepancy between clinician's perception of kidney or liver transplant feasibility and reality. A large proportion of these two organs were finally not offered, considered or accepted for transplantation. Except for the presence of multiple organ failure in a few cases, we could not identify consistent items after the review of the medical chart, clarifying the barriers to donor conversion. As pointed out in an audit of the Spanish national registry donation process by external experts, a proportion of organs are sometimes excluded on the basis of medical contraindications deemed inappropriate (7). The central question is why?

Clinically, there are probably unclear boundaries on absolute and relative rules for eligibility, despite OPO efforts to generate criteria (27). In addition, this is a moving target with more and more borderline donors being considered. The latter generates the heterogeneity of potential organ donors, which despite critical care predetermined endpoints usually leading to more organ procured (20), complexifies the clinical assessment of eligibility. In the case of our vignette study, with the short clinical scenarios provided representing an initial snapshot, then a follow-up on organ investigations or support, the four clinicians could have missed the changes happening over time during the active organ support.

The acceptance of an organ by the transplant surgeon or team is usually conditional and the paradigm is skewed; a primarily accepted organ could subsequently be refused on the basis of new information, whereas a primary rejected organ is generally without appeal. It depends on the timing and the set of clinical information conveyed at that time; a critical care clinician with experience might be able to tailor the timing to allow organ recovery. Often, if the organs have already been refused and the offer is not renewed, enabling organ donation will require extra communication efforts. Historically, the principle of urgency for organ procurement was broadly applied. In France for example, organs are allocated with the condition that the transplant team proceeds within a 24-hour period. Although partly efficient, this approach excludes any possibility of giving a temporarily failing organ time to recover enough to be reconsidered.

In our study, granular arguments from the perspective of the OPO and transplant team were not available to enlighten their assessment of feasibility, other than generic decisions. For example, transplant nephrologist or hepatologist may decide, due to organ dimension, characteristics and various past-medical history of the donor, that the proposed organ is not suitable for a receiver (28, 29). A large proportion of organs deemed feasible by our external clinicians were finally not procured. Unlike overall critical care outcomes scores (30), organ function outcomes for donors are underdeveloped, despite the recent availability of decision's algorithms for liver or kidney acceptance based on risks (31, 32).

In light of these observations, we believe that part of the reasons making the perceptions and outcomes so variable is the complexity of organ attribution system and the related processes. The literature showing the variability of acceptance in different centers supports this observation (17, 18). Moreover, transmission of clinical data as well as communication between the support and the transplant teams, are fragmented. The organ dispatching depends on what and how information is transmitted, often over the phone, and may lead to timely decisions that are not reassessed. Besides the biological/blood group matching of the proposed organs, the actual system depends on the variables related to the elements of allocations: 1) the timing; 2) local vs regional or national offer; 3) matching with borderline receiver (concept present on the donor's side); 4) non-objective/non-systematic availability of donor medical information (verbally transmitted by OPO coordinators); 5) fragmentation of decisions, with stakeholders detached from the donor bedside. The current model of decision is based on urgency, with the sickest patient on the waiting list being considered first (33, 34). However, could the system consider offering refused organ to borderline receivers (or with less chance to go up the list)? The exact processes regarding decision-making are not always clearly defined or collected, thereby making difficult to precisely identify the present constrains.

To help us move forward, we would like to bring up in the discussion the example of decision' process in oncology, typically involving multiple stakeholders. In this case, the best option for patients' treatment and prognostication requires a multidisciplinary evaluation by an oncology board, including every decision-makers; the information is shared in a timely manner between a treating physician, a surgeon, an oncologist and radiotherapist, in order to decide for the best treatment applicable. It was demonstrated that these complex medical decisions, requiring the weight of medical information with the best option for a cancer treatment, can improve care (35, 36). In the case of potential donors, particularly those perceived as unlikely feasible, the medical information framework and the process of sharing could be better systematized, in order to avoid mislead decisions. The creation of a structured online canvas (similar to a registry of clinical data), where the patient's characteristics, parameters and evolution overtime can be systematically documented (and automatically uploaded), could help to avoid subjectivity in the transmission of medical information. One can imagine that the critical care physician in charge of the patient, collaborating with the OPO coordinator, could feed real time information, specifications and also provide answers to questions from the transplantation team in a standardized manner. The development of algorithms testing the interaction between donors and recipients risk factors could help the teams and support a more objective system (37). We also propose the idea that the ultimate step would involve a session for more challenging cases in the format of a "donor board," similar to an oncologic board meeting, in order to make consensual decisions and optimize the use of available organs. In addition, we believe that a dedicated donor supportive structure gives the possibility to allow time for evaluation, organ recovery and to enter a better window of opportunity where potential organs are optimized (19).

Our study has limitations, essentially regarding the small number of evaluators and the retrospective aspect of the design. It is nevertheless the only real-life data we could collect so far. In addition, we were not aware of the previous selection ratio of potential donors entering our system, adding potential bias in the number of borderline donors assessed. Furthermore, no emotional or cultural aspects were collected, regarding the approach to donor support. The four evaluators had however the possibility of assessing a high number of cases represented by real scenarios sufficient to test

their agreement. A central aspect, that we did not consider for the analysis of our findings, is the difference in experience or expertise among the evaluators.

We acknowledge that the decision to cap the evaluation at 50 vignettes was based on our assumption that it would maximize the chance of response from the evaluators. Indeed, the variability of perception could have been lessened by the evaluation of the 66 potential donors by all evaluators. Another point is the possibility that the patient's medical information extracted from the charts/database and transcribed to the vignettes lacked of informative precisions. First, the OPO was running its own inquiries (mostly through discussion with families) on the medical background of the potential donors, as well as the characteristics of potential receivers; secondly, the vignettes were built with summarized descriptions collected at the time of consent for organ donation; third, the new evolution of the potential donor medical condition, as well as the surgical assessment at the time of organ extraction was not reflected in the vignettes. Finally, the evaluations of the cases were done by physicians working in university hospitals, illustrating a limited representation of appreciation, since our province holds a large majority (>65%) of ICU beds in community hospitals. The opinion emanating from physicians outside of these centers could have provided a different variability of perceptions.

In conclusion, our study reveals that the support and assessment of a potential donor is a complex dynamic situation, involving different sources of medical information, with variability of perception in organ donation feasibility. To improve the overall system, we raise the possibility to standardize electronically the donor's clinical/laboratory characteristics available to the transplant team, as well as the idea to test a "donor board" structure of decision. Further research, looking at the impact of such an approach in different healthcare system, is warranted.

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 Hôpital du Sacré-Coeur research ethic board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GL, PM, A-ML, VW, KS, FB, DW, MA, and EC designed the study, collected and analyzed the data, interpreted the results, wrote the manuscript and reviewed the final version of the manuscript. PR participated to the vignettes development, interpretation of the results, and reviewed the final version of the manuscript. AF contributed to the statistical analyses, the interpretation of the results and reviewed the final 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.

ACKNOWLEDGMENTS

GL was the recipient of a summer student bursary from the University of Montreal.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10107/full#supplementary-material

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ORIGINAL RESEARCH
published: 17 March 2022
doi: 10.3389/ti.2022.10295

Information Needs of People With Limited Health Literacy Regarding a New "Opt-Out" Organ Donation System: A Qualitative Study in the Netherlands

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Background: In the Netherlands, new legislation on organ donation was implemented, based on a "opt-out" consent system, which means that all adults are presumed to consent for organ donation, unless they actively register their decision not to donate. A public information campaign preceded the law change. In the Netherlands, 29% of the population has limited health literacy (LHL). The aim of the study was to gain insight in the information needs of Dutch citizens with LHL regarding organ donation and the new legislation, as well as in their preferred information channels.

Methods: A qualitative study was performed; 30 people participated in four focus groups and six individual interviews. Transcripts were coded, interviews were thematically analysed.

Results: People with LHL need specific information to make an informed decision on organ donation. Relevant topics: 1) choice options, 2) eligibility, 3) role of partner and/or family, 4) impact on quality of care, and 5) process of organ donation. Information should be easy to understand.

Conclusion: Current standard materials are too difficult and abstract. People with LHL require personal support to tailor general information to their personal situation, and practical help to actually register their choice. Suggestions on how to improve information is provided.

Keywords: organ donation, health literacy, information, communication, opt out

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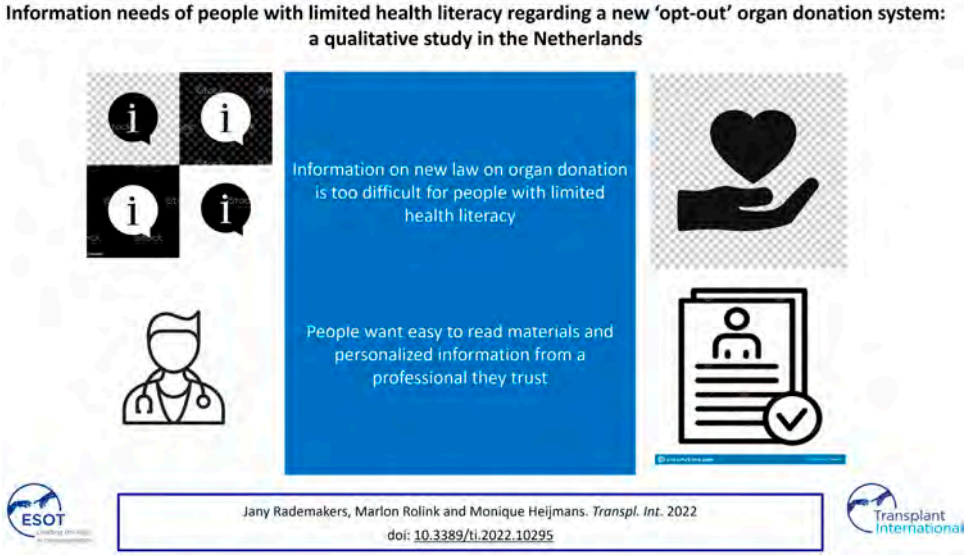
Received: *10 December 2021*
Accepted: *18 January 2022*
Published: *17 March 2022*

Citation:
Rademakers J, Rolink M and Heijmans M (2022) Information Needs of People With Limited Health Literacy Regarding a New "Opt-Out" Organ Donation System: A Qualitative Study in the Netherlands. Transpl Int 35:10295. doi: 10.3389/ti.2022.10295

INTRODUCTION

Globally, there is a shortage in organ donors to meet the demand for organ transplantation [1–3]. As a result, waiting lists of patients in need for organ or tissue transplantation are growing, and a substantial part of these patients die while on the list [1]. There are several pathways considered effective in increasing transplantation rates, one of which is the implementation of a legal system based on "presumed consent" or "opt-out" [1, 2]. The essential difference between an "opt-out" and an explicit consent or "opt in" system is that in the latter, citizens are not automatically considered



GRAPHICAL ABSTRACT |

organ and tissue donors unless they have actively registered as such. In an "opt-out" system, all citizens of 18 and older are presumed to be a donor, unless they choose to state otherwise. Several European countries have successfully implemented a legal 'opt-out' system: Austria, Belgium, Czech Republic, Croatia, Finland, France, Hungary, Italy, Latvia, Norway, Portugal, Slovakia, Slovenia, Spain, Sweden and the United Kingdom [3, 4].

In the Netherlands, a new Organ Donation Act based on "opt-out" came into force in 2020. Between September 2020 and March 2021, all adult Dutch citizens received a letter with information about the new Organ Donation Act and the request to enter their choice (online or through a paper form) in the Donor registry. In general, there are four choice options: 1) yes, I consent to being an organ donor, 2) no, I do not consent to being an organ door, 3) I want my partner or relatives to decide this after my death, 4) I want another (specified) person to decide this after my death. One can also exclude specified organs for donation. If -even after a reminder- people do not register their choice, they are automatically registered as an organ donor (option 1). Since the register was already in place, Dutch citizens could also have entered their choice earlier. In this case people were advised to check if their choice was still valid. In order to inform and prepare the general public, the Dutch government launched a national information campaign in 2019 through different channels (leaflets/brochures, advertorials, posters, television/radio commercials, short films, a website). On top of this national campaign, the Dutch Ministry of Health decided to develop and implement additional communication strategies for specific subgroups who might have different information needs and more difficulties in understanding and applying this information, f.e., people with cognitive impairments and citizens with limited health literacy (LHL).

Health literacy is the ability to access, understand, appraise and apply health information in the domains of healthcare, disease prevention, and health promotion [5]. It entails the capacity to read and write (functional health literacy), but also more advanced cognitive and social skills. In a recent review, three key elements of health literacy were discerned: 1) cognitive attributes (knowledge, functional skills, comprehension and understanding, appraisal and evaluation, critical thinking); 2) behavioural and operational attributes (seeking and accessing information, communication and interaction, application of information, citizenship); and 3) affective and conative attributes (self-awareness/self-reflection, self-control/self-regulation, self-efficacy, interest and motivation) [6]. Thus, health literacy is more than a cognitive skill or the "capacity to think." Especially if an active role of people is required, the "capacity to act" is even more important [7]. Regarding the Organ Donation Act, people not only need the skills to access and understand the given information, but also to develop a personal opinion about the different choice options and to actually enter their choice in the register. Given the fact that 29% of the Dutch population has inadequate or limited health literacy [8], providing clear information on the Organ Donation Act that fits the needs of this group and motivates them to actively register their personal choice was considered as a challenge by the Dutch government [8]. At the time the national communication campaign in the Netherlands was launched, there still was lack of evidence on how to best inform citizens with LHL about organ donation in general and about an 'opt-out' system in particular, neither in the Netherlands nor in most other European countries that have implemented such legislation [3, 4]. Wales was the only country that had acknowledged the information needs of specific subpopulations (elderly, people with limited reading skills, lower educated citizens, black/ethnic minority groups) and tailored their information and channels accordingly [4, 9]. Earlier studies in different domains (e.g., cancer screening, screening for Down in pregnant women, health decisions in general) had already demonstrated that people with LHL have more difficulties with understanding and applying health information and making

informed choices, and that they need specific and tailored information [10–16].

To get better insight in the information needs of Dutch citizens with LHL regarding the new Organ Donation Act as well as in their preferred information channels, the Dutch Ministry of Health requested the study presented in this article. The study was done in 2019, at which time the national information campaign was already running. The research questions were:

- What are the information needs of Dutch citizens with LHL regarding organ and tissue donation in general and the new Organ Donation Act in the Netherlands in particular?
- How do Dutch citizens with LHL want to receive information on these topics? Through which types of materials and channels do they want to receive the information?
- What do the results of this study imply for the information strategy towards Dutch citizens with LHL?

The article will end with giving specific suggestions for improving information on organ and tissue donation and the new Organ Donation Act.

MATERIALS AND METHODS

Given the explorative character of the subject and the possible difficulties of the target group with reading and writing, a qualitative study design was chosen.

Participants

Respondents were recruited through organisations that target specific subpopulations with LHL (limited reading and writing skills, minor intellectual disabilities, migrants). These organisations were selected because the research institute had previous worked with them and/or because they were partner organisations of the Dutch Alliance for Health literacy. Inclusion criterion was the subjective acknowledgement of the potential participant that (s)he regularly experienced difficulties with accessing and understanding health information (basic levels of health literacy). The organisations gave potential participants easy and understandable information about the study (on the topic of the study, the aim, methods of datacollection and practical aspects), and then asked people whether they would be willing to participate. The aim was to perform three focus groups or—if people would prefer that—individual interviews, with at least 24 participants (≥18 years) in total and/or till data saturation occurred. Data saturation means that no new topics or themes emerged during the interviews, and new interviews added no more insgihts. All respondents received a gift cheque of 15 euro for their participation.

Data Collection

A topic questionnaire was developed for the interviews and focus groups. Topics were: current level of knowledge and attitude about organ and tissue donation in general and the new Organ Donation Act in specific. Information needs were discussed, as well as preferred types of materials and channels for receiving that information. Also some existing materials on the new Organ Donation Act (an animated movie on the website www.donorregister.nl, television commercials and a leaflet that was distributed to every household in the Netherlands) were evaluated. The interviews and focus groups started with an explanation of the study purpose. All participants understood the specific research information and consented to participate in the study. Permission was asked and given to record the session and to use anonymized quotes from the interviews and focus groups. Data collection took place in July and August 2019.

Data Analysis

The (group and individual) interviews were audio-recorded, transcribed verbatim, and anonymized. Each transcript was coded by the researchers, using the research questions and topics as a general framework for thematic analysis. All transcripts and codes were discussed in the research team. Since there were no systematic differences in the topics that emerged from the focus groups and the individual interviews, we analysed them together and combined the information in the results.

Ethical Approval

This study does not fall within the scope of the Dutch Medical Research Involving Human Subjects Act and therefore does not require ethical approval. All respondents participated on a voluntary basis. They gave informed consent to use their answers for scientific research.

RESULTS

Participants

In total, 30 respondents (15 men and 15 women; age 18–65) volunteered to participate in the study. Four focus groups were held in different locations (with 3, 4, 6, and 11 participants respectively). Six respondents were unable or did not want to travel and were individually interviewed at their home. Two focus groups (N = 7) and all individually interviewed persons (N = 6) had difficulties with reading and writing and/or understanding health information. One group were migrants who—despite living in the Netherlands for several years—experienced additional problems with reading and understanding Dutch language (N = 11). One focus group were participants with minor intellectual disabilities (N = 6). At the end of the data collection, data saturation occurred and no new themes were identified. In the analysis phase, no specific differences between the different groups of respondents emerged, therefore we decided to present the data for all respondents with LHL together.

Knowledge Level and Attitude Regarding the Organ Donation Act

Many respondents—about a third–had not heard at all of the new Organ Donation Act. Those who were aware, lacked basic knowledge. Specifically, they did not really know what it entailed nor what was required of them. Most often they had seen or heard a television or radio commercial, but did not actively seek additional information. The different options were unclear for most respondents and they were not actively

thinking about which option would match their opinions and sentiments best. If they reflected on the choice, they were inclined to choose option 1 (to be a donor), as they felt more or less obliged to do so. If anything, thinking about death and organ donation made people anxious and uncertain.

> "*And it is also a little bit scary. I used to think if I register, I will be dead by next week. . .. Or suppose I am not complete dead and they are cutting in me?" (Female).*

Some respondents reported low levels of support for opt-out legislation; they felt angry, because they felt forced by the government to make a decision. Some respondents mentioned that this kind of law was unfair to people with limited (health) literacy skills, since if you do not understand what you have to do and don't register your personal choice, you will automatically become a donor.

> *"People [with LHL] are fooled by wrong links on websites. They are vulnerable and hardly dare to use the computer. Also because they don't understand everything, they can easily be pushed into making a choice which they actually don't support."(Male).*

In general, most respondents believed that they lacked specific and practical information to make an informed personal decision.

Information Needs

People with LHL expressed a desire for additional information on several key themes. The five main topics identified were:

(1) Choice options and the differences between them: whether it is an obligation to become an organ donor, what the choice options are and whether you can change your decision at a later point in time;
(2) Eligibility: if, on the basis of health or lifestyle, someone is suitable as a donor (e.g., when you smoke or when you have a chronic disease), and whether one's religion allows you to be a donor or not;

> *"I have diabetes, and, yes, I do not know about the parts in my body . . . how good they still are."(Male).*
>
> *"I am a religious woman. We talked about it in the community centre. Some people had difficulties with it. They wonder whether God would approve if I would donate my organs. They think that God would not want that." (Female).*

(3) Role of the partner and/or family: respondents want to know what happens if multiple relatives don't agree (in case of option 3), or when you are registered as a donor and your partner or relatives oppose to that;

> *"But your, father or mother, . . . they can say no, for example. Because you are automatically in [the register], if you do nothing. And then you are automatically a donor, when you die. Then your parents can say, she doesn't want it, even though she is in it. Right? Or not? How about that?" (female).*

(4) Impact on quality of care: some respondents expressed fears around medical mistrust. For example, they expressed concerns that the care they receive will be negatively affected;

> *"If I now say that I will be a donor, are they still going to help me well when I am ill, or do they think, she can better be dead, because then we have organs again.' (Female).*

(5) Process of organ donation: some respondents want to know what actually happens after your death, how much time there is before you are taken away, about the medical procedure itself and how the process impacts the funeral and its preparations.

> *"I want to know if, when I say yes, what will happen to my body? What happens then? Can you still say goodbye to someone in a decent way? (Female).*
>
> *"Somebody then scared me. They said that when you are dead, it takes very long before the family gets your body back. Because it is taken away. Because everything has to be taken out. I don't want that. Then I renounced it." (Female).*

Furthermore most respondents requested practical help in registering their choice through the website, since they have difficulties with or are unable to use computers.

> *"I prefer a little bit the old-fashioned, really filling out on paper. If I really have to, I could do it [digitally]. But I feel that I often have to be helped by someone, together, and watching with me."(Male).*

Also help in retrieving and filling out the paper form, which you can also use for registering, was desired.

Evaluation of Current Information Materials

The content of the information currently used was generally considered too difficult and too abstract to make an informed decision whether or not to be an organ donor. Three materials were specifically discussed with the respondents: an animated movie, television commercials and a folder that was distributed to every household in the Netherlands. In general, the respondents expressed a preference for information in a spoken form, in short movies or animations.

> *"A video is clear, because it clearly shows what is exactly happening. . . A paper, I would read but not understand at all. The letter is too much text." (Female).*

Nevertheless, only two of the participants had seen the animated movie before the focus group session, even though it

was broadly distributed by the government. Main advantages of the animated movie were that the information was spoken (not too quick), that much information was presented in a clear way and that the website address and phone number, where more information could be obtained, was visible long enough. Main disadvantages were that people were not aware of these movies, and that they were accessible through a website only. Suggestions for improvements were: 1) show examples of "real" people who tell why they decided to choose for one of the options (narratives) and 2) actually show the process of registering, step-by-step, how one should do that, and 3) make the movie available in different languages.

Most respondents mentioned seeing the television commercials. They remembered that famous Dutch persons played a role in it, and were positive of the diversity of characters in the commercials. They considered the commercials funny, and good for general awareness since television is an accessible medium. However, the main message of the commercials was not clear. They were too short, and didn't provide enough background information on the registration process. Information was not repeated and also the website address and telephone number were only shown briefly.

A leaflet in general was considered useful because it contains all relevant information and could be used as a reference. However, the content and language level of the leaflet that was distributed in all Dutch households was considered much too difficult: too much text, too long sentences. Words like organs, donation and donor register were difficult to comprehend.

> *"If you would ask me "what does 'donor' exactly mean?" Than I would not know that at all. I cannot explain what it is." (Female).*

In general, the leaflet was not readable for most respondents, especially those with limited reading skills. Respondents suggested to use more pictures and animations and less text in the leaflet. The fact that a logo of the Dutch government/Ministry of Health was clearly printed on the front of the leaflet raised ambivalent reactions. Though it was considered positive that you know who the sender of the leaflet is, most of the respondents back off if they get mail from the government, either because they know from personal experience that it will be difficult to comprehend, but also because mail from the government usually is bad news (e.g., taxes).

Preferred Information Materials and Channels

Most respondents suggested to make all materials on organ donation and the new law easier to comprehend in general, so that they could be used universally and no specific materials for people with LHL would have to be developed. However, since they do have additional information needs, some extra communication strategies seem warranted. In general, the respondents preferred simple movies and narratives of other people. An easy to read leaflet would be appreciated. Some respondents also use the Internet as a source of information, but for others this is too difficult.

The respondents stressed the importance of actual personal support, in order to understand and personalise the information, discuss the options and help with the actual registration. They suggested the involvement of organisations and professionals within their personal network, e.g., organisations that support people with intellectual disabilities, neighbourhood teams, schools, health care organisations and providers. Also the social network (family, friends) was considered very important to discuss this complicated topic with.

DISCUSSION

Main Findings

Many of the people with LHL in this study had not heard at all of the new Organ Donation Act that was to be implemented in the Netherlands. Those who were aware (usually through television or radio commercials) did not know what it entailed nor what was required of them. Participants lacked the information they needed to make an informed personal decision. They expressed a need for more specific information on organ donation and what the new law entailed. The five key themes that emerged were: 1) choice options and the differences between them, 2) eligibility, 3) role of the partner and/or family, 4) impact on quality of care and 5) the process of organ donation. Furthermore they expressed a need for practical help in registering their choice.

Current information on the new Dutch law on organ donation was generally considered too difficult and abstract. The importance of actual personal support was stressed, in order to understand and personalise the information, discuss the options and help with the actual registration. The respondents suggested the involvement of organisations and professionals they already have contact with, like their GP or a social worker. Also the social network (family, friends) was regarded an important source for help and support.

This study shows that people with LHL have more difficulties with understanding and applying health information and making informed choices, which has been demonstrated in many other studies and health contexts (e.g., cancer screening, screening for Down in pregnant women, health decisions in general) [10–16]. People with LHL need information that is easy to understand and relatable. Written information is often considered too difficult to comprehend, and generally too abstract. They are interested in experiences of others and narratives [17]. They also express a need for more practical information, e.g., a step by step explanation of what is to be done. Furthermore, they require more personal support in making health related choices and decisions. Our study confirms these results in the context of organ donation.

Strengths and Limitations of the Study

To our knowledge, this is the first study on the information needs and preferred information channels of people with LHL regarding organ donation. It is a strength of this qualitative study that representatives from this target group could express their own needs and preferences,

since they are often underrepresented in quantitative scientific studies. The participants in our study were recruited through different organisations, each with a focus on a specific subpopulation. This led to a representation of various subgroups of people with LHL in our study (people with reading and writing difficulties, migrants, people with minor intellectual disabilities). Though this is a qualitative study with only 30 participants, we think this diversity and the fact that saturation occurred makes the results generalizable to the larger group of people with LHL. The subjects selected acknowledged their difficulties with regard to understanding health information. This might generate some bias in the sample, as people with LHL who do not acknowledge these difficulties might have different needs. Another limitation of our study is that not in all focus groups, the current information materials were systematically discussed, due to time constraints and different discussion priorities of the participants. However, where it was done, reactions and answers all pointed in the same direction.

Implications for the Information Strategy for People With LHL

Some of the current materials were considered useful, but should be more accessible (e.g., the animated movie) or adapted to the reading level and information needs of people with LHL (e.g., the information leaflet). Including less text, long sentences and difficult, abstract words, and more specific information on the topics that were mentioned by the respondents. Co-creation and pre-testing such a leaflet with representatives from the target group is recommended. As information in spoken form was preferred, the respondents in our study also suggested to make special movies for specific target groups together with them, and distribute them through regular channels of the organisations they already have contact with and access to. All these materials would focus on knowledge, one of the cognitive attributes of health literacy [6]. For actual behaviour to take place, attention for the other (behavioural/operational and affective/conative) attributes is also important [6, 7]. The respondents also stressed that they require practical information and personal support. People with LHL often also have limited computer skills, so seeking information on the Internet, registering one's choice through a website or finding information on how to get a paper form is a special challenge with which they need support. People also need support in order to tailor the general information on organ donation to their personal situation. This can also help in reducing the anxiety and uncertainty that was expressed by many people in our study. This support can either be provided by professionals they already know (e.g., teachers, case workers or health care providers), by people from their own social network and/or by volunteers who are present in community centres where materials are distributed. It is important that the professionals actually coach the person with LHL in making the decision that best fits their situation and wishes, by providing understandable information on all options (and not only the one they would consider best) and through methods used in shared decision making, such as value elicitation. Since people with LHL heavily rely on persons they trust, it is important to remain neutral with respect to the options and refrain from "advising" in a certain direction.

CONCLUSION

People with LHL need specific information to make an informed decision on organ donation. This information should be accessible and easy to understand. Current standard materials are too difficult and abstract. Furthermore, they require personal support to tailor general information to their personal situation, and practical help to actually register their choice.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study does not fall within the scope of the Dutch Medical Research Involving Human Subjects Act and therefore does not require ethical approval. All respondents participated on a voluntary basis. They gave informed consent to use their answers for scientific research.

AUTHOR CONTRIBUTIONS

JR and MH designed the study. MR and MH performed the data collection. All authors were involved in the analysis of the data. JR drafted the manuscript, all authors contributed to the final manuscript.

FUNDING

This study was funded by the Dutch Ministry of Health, Welfare and Sport.

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
published: 23 March 2022
doi: 10.3389/ti.2022.10411

A Qualitative Study in Family Units on Organ Donation: Attitude, Influencing Factors and Communication Patterns

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This study aimed to analyze the attitude, influencing factors and communication patterns of organ donation in Chinses families. We conducted in-depth interviews with 97 participants from 26 families in China from August 2018 to October 2020. Interviews were audio-recorded, transcribed by the researchers. Thematic analysis was used to analyze the data and Nvivo 12 was used to catalog coded data. Thirty-eight participants indicated that they would like to be a donor while the majority were unlikely to donate. Among those who were willing to donate, some disagreed with family members to donate organs. Themes found included attitude, the timing of thinking, taboo and fear, traditional beliefs, ethics and family communication patterns. Lack of knowledge, fear, taboo, some traditional beliefs and mistrust may discourage donation. Altruism and policy which is good for the family seem to encourage donation. We also constructed three family communication patterns to provide a deeper understanding of the family in China. This is the first qualitative study that analyzed attitude, influencing factors and communication patterns based on family units in China mainland. Our findings showed that family comes first in Chinese. We suggest that family-based consent and incentives are more suitable for the Chinese social context.

Keywords: organ donation, family, attitude, communication patterns, ethics, qualitative research

OPEN ACCESS

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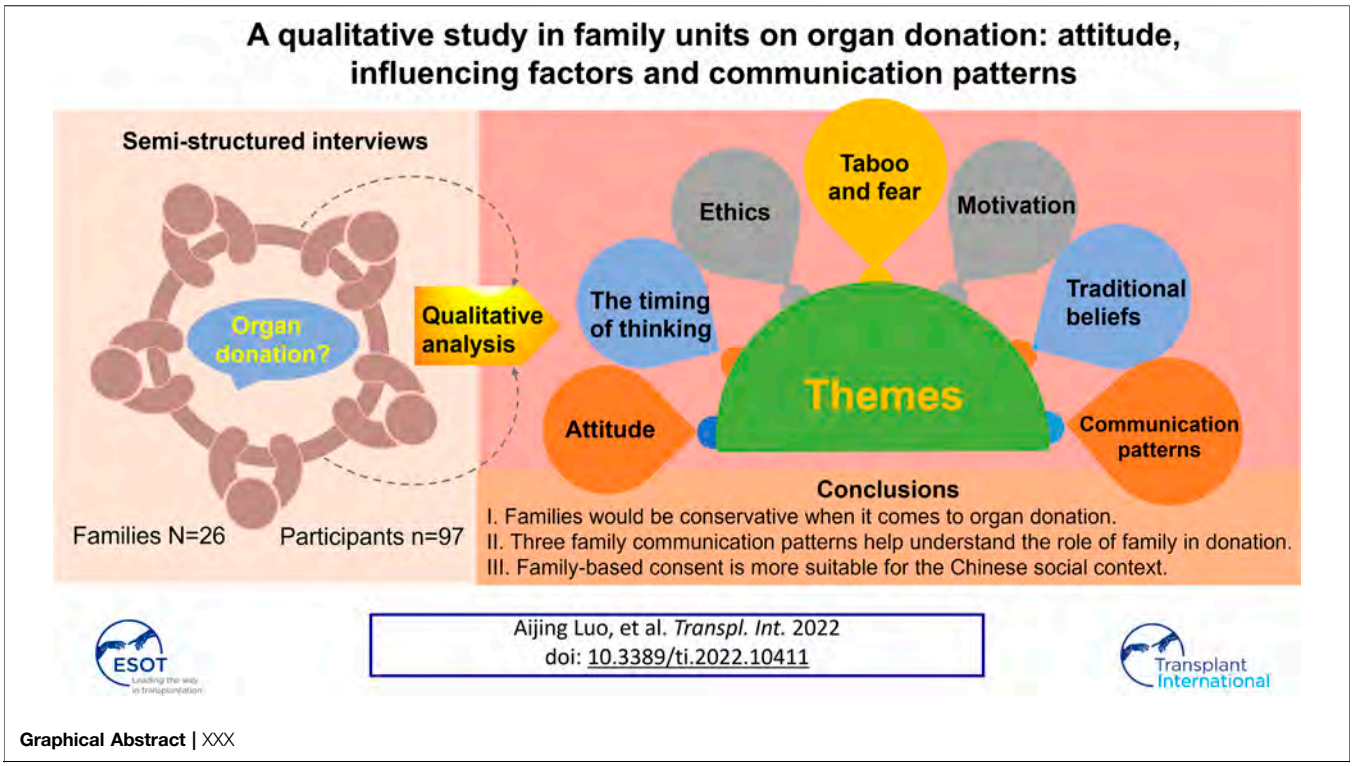
Received: *07 February 2022*
Accepted: *24 February 2022*
Published: *23 March 2022*

Citation:
Luo A, He H, Xu Z, Ouyang W, Chen Y, Li K and Xie W (2022) A Qualitative Study in Family Units on Organ Donation: Attitude, Influencing Factors and Communication Patterns. Transpl Int 35:10411. doi: 10.3389/ti.2022.10411

BACKGROUND

Organ transplantation has been regarded as a life-saving treatment for patients with organ failure (1). However, demand considerably exceeds the supply of suitable organs made shortage of organs a critical problem worldwide (2,3). For example, there are around 6,000 people on the United Kingdom Transplant Waiting List and over 350 people died while waiting for a transplant in 2019 (4). In the United States, more than 6,000 patients die every year while waiting for a transplant (5). Moreover, organ shortage is particularly serious in China (6), with a donation rate of 3.46 donors per million population (dpmp) compared with the United States (38.0 dpmp) and Spain (37.9 dpmp) (3).

Factors that influence organ donation rate are manifold: organ donation system, legal regulations, cultural beliefs, region, knowledge and attitude toward donation are important factors that affect donation rate worldwide (7–10). Demographic factors such as age, gender, education level,



Graphical Abstract | XXX

occupation and nationality are also associated with being a donor (11–13). Previous studies have shown that family played an essential role in organ donation (14,15). Researchers collected 1886 questionnaires on organ donation from 11 cities in China, they found that 69.9% of participants considered family consent necessary and 77.1% thought that the view of their family had a great, even decisive, influence on them to decide to become donors (16). By monitoring the public's discussion about organ donation on a Chinese social media platform (Weibo), among 1,755 posts related to organ donation, most positive posts were "saluting the organ donors" and most negative posts involved "fear of the family's passive medical decision" (17). A study in Taiwan showed that the factors contributing to an aversive preference of cancer patients included the necessity to consider the emotions of family members, traditional perceptions and religious reasons (18). Furthermore, families can overrule the known wishes of the deceased in some countries, such as India, Japan and Canada (19). In Switzerland, although patients had registered as a donor, over 40% of donations were stopped because of family refusal.(20) This phenomenon is particularly common in China. For example, next-to-kin, especially grown-up children, may refuse to carry out the patient's wish to donate organs after death, because they fear that agreeing to such a wish would not be filial and it would fail to keep the body intact (21). It is therefore of supreme importance to increase family consent on organ donation.

Previous research focused on family's attitude toward donation (22), family bereavement (23), influencing factors (24), ethical exploration (25) and motivation to donate (26). Many of these studies were conducted in the United States, Australia, Canada and the United Kingdom. Nevertheless, few researchers explored the interaction between family members in organ donation based on Chinese cultural background. Many Chinese were affected by the Confucian cultural norm which states that "It is a responsibility to maintain the physical integrity of the body after death." Although cremation is currently practiced in China, Chinese funeral custom is inhumation with a whole body. Compared with people who had Confucianism funeral belief, those without the belief were more willing to donate (27). Besides, consent from all immediate family members is required in the practice of organ donation in China. Family communication is important because the opinions of family members on organ donation need to be unified. This research aims to elicit the families' attitude toward organ donation and how family members interact with each other in China. Family discussion in private settings improves family experience (28). Hence, we explored the family attitude, influencing factors and communication patterns on organ donation by conducting interviews with family units.

METHODS

Research Design

Qualitative research can capture human emotions and perceptions hidden behind their experiences and offers complementary information to that uncovered by quantitative surveys (12). Therefore, we chose a qualitative method to facilitate an in-depth exploration of family attitude, influencing factors and communication patterns toward organ donation using a semi-structured interview schedule. Interview questions were

TABLE 1 | Interview guide.

Thoughts and attitude toward organ donation
Have you heard about organ donation?
Is talking about organ donation taboo for you? Why?
What do you think of organ donation?
Cremation is implemented in China. It is better to donate organs to save a life rather than being burned after death. What do you think?
Confidence in fair distribution of organs
What makes you want to be a donor/not to be a donor?
Do you believe that organs can be distributed fairly and justly? Why?
What do you think about brain death?
Family communication
What do you think about signing the donor card? For example, sign up as a donor when you get your driver's license?
Will you communicate with your next-to-kin before you make a decision?
Whose opinion would influence you most?
Will you agree with your family members (especially your children/ parents/ couples) if they would like to be a donor? Why?
Will you consider being a donor if donor families would have priority in organ distribution when needed?

TABLE 2 | Demographic data of participants.

Characteristic	***n* (%)**
Gender	
Male	34 (35)
Female	62 (65)
Age group	
18–30	37 (38)
31–60	56 (58)
61+	4 (4)
Education	
Junior high school and below	20 (21)
Trade school	2 (2)
High school diploma or equivalent	16 (16)
Some college	12 (12)
Bachelor's degree	18 (19)
Master's degree	29 (30)
Household income, *N* = 26	
<15,000	9 (35)
15,000–30,000	11 (42)
30,000–45,000	2 (8)
>45,000	4 (15)
Relationship with volunteers	
Volunteers	26 (27)
Parents	42 (43)
Spouse	4 (4)
Grandparents	4 (4)
Brothers/Sisters	10 (11)
Cousins	5 (5)
Uncles/Aunts	3 (3)
Parents-in-law	2 (2)
Other	1 (1)

categorized into three domains: 1) understand the thoughts and attitude to organ donation, 2) confidence in fair distribution of organs, 3) family communication (See **Table 1**).

Participants

We have released a recruitment notice on our laboratory's official website. The inclusion criteria for the volunteers were as follows: 1) above the age of 18, 2) good at communication, 3) would like to talk about organ donation with families, 4) agreed to participate in the interviews. The sampling period lasted from August 2018 to January 2020, a total of 52 volunteers would like to participate in this project. We trained each volunteer in qualitative interview skills to ensure the quality of the experiment. During this period, nine volunteers dropped out of the project for personal reasons, seven were unable to conduct qualitative interviews skillfully. Thus, 36 volunteers conducted semi-structured interviews according to the outline with all their families together face to face or through online video. Family members include but are not limited to parents, spouses, grandparents, uncles and aunts, etc.

We stopped recruiting volunteers and family interviews once we reached theoretical saturation, themes and trends were well developed, and no new concepts or structures emerged (29). Finally, twenty-six families, 97 participants completed the interview and each interview was audio recorded. Ten volunteers failed because family members were reluctant to talk about organ donation. **Table 2** shows the demographic characteristics of the participants.

Analysis

Interview recordings were transcribed verbatim by the researchers who searched for concepts, themes, and ideas. We performed a qualitative content analysis on these transcripts (30) and used NVivo software (QSR, Version 12) to catalog coded data. At least three coders met regularly to adjudicate differences in codes and discuss emerging themes for higher-level analysis with a focus on content related to family communication.

Ethics

All participants gave their written consent and were aware that they had the right to withdraw from the project without giving a reason. We promised that the interview content will only be used for scientific research and ensure the privacy of participants. The study was approved by the IRB of the Third Xiangya Hospital, Central South University.

RESULTS

Key findings on the seven main themes, "attitude to organ donation in family," "timing of thinking," "motivation," "fear

TABLE 3 | Themes and representative quotations.

Theme	Subtheme	Representative quotations
Attitude		"I would like to register as an organ donor. but I don't want my family member to donate. Em. . .I don't want them to lose any part of their body." (N10, mother, 46 y) "I can donate my organs when deceased. But I'm afraid if my next to kin missing a part of their body." (N18, volunteer, 24 y)
Timing of thinking	Seriously ill or facing death	"If I was ill and life is irretrievable, I will consider donation according to the actual situation." (N2, aunt, 42 y)
	The media	"I once saw a video about organ donation. I vaguely remembered that a baby was crying all the time. However, the baby stopped crying when a man who looked very rough held him. The reason seems to be that the heart of that man was donated by the baby's mother. The baby didn't cry when he heard the heartbeat just like his mother. Since I became a mother, I think if my organs can help people in need and continue in their lives, I feel as if I am still in this world and my children can still feel my existence." (N14, sister, 32 y)
	Sympathy	"You will know how painful they (people who on the waiting list) were when you came to the hospital. Especially seeing a child with organ failure lying on the bed. Just one organ can save and change their lives. I would like to donate my organ after death if someone needed." (N8, sister, 27 y)
Motivation	Altruism	"I think it's noble to donate organs and save people's lives. "(N11, volunteer, 24 y) "Organ donation is a good deed that benefits others and society. "(N26, father, 49 y)
	Usefulness	"If the donated organs can be used properly, that can yet be regarded as a continuation of life, make the best use of organs. "(N5, volunteer, 25 y) "It's a waste that being buried or burn after death. It is better to donate to save someone's life." (N21, father-in-law, 64 y)
	Good for Family	"I can sacrifice for my family." (N3, mother, 51 y) "If my family members need a transplant and have priority rights of organ, I will be the first one to sign it. "(N4, mother, 46 y) "I would not hesitate to do it if it is good for my family." (N12, cousin, 20 y)
Taboo and fear	Taboo	"It's inauspicious to talk about it (organ donation), a taboo, I do not like to hear it. "(N7, mother, 49 y) "When it comes to organ donation, I will think of death, which makes me sad." (N5, mother, 49 y)
	A bad omen	"I wouldn't sign it. It's like an omen. I don't like it. "(N5, mother, 49 y) "If I sign this thing, I always feel that I have to remind myself from time to time when I drive. That put an inexplicable pressure on me." (N10, sister, 28 y)
	Fear	"Organs are donated when people die unnaturally." (N11, father, 45 y) "I was so scared to have my organs cut off after death. "(N17, mother, 54 y)
Traditional beliefs	Filial piety	"Your hair and skin are received from your parents. Keeping the body intact is a form of filial piety." (N2, aunt, 42 y) "From ancient times to the present, even if a person died, he should have a complete body." (N12, father, 51 y)
	Metempsychosis	"I believe in reincarnation. For example, If I donate my cornea, I would be blind next life." (N1, mother, 49 y) "What if I had a heart or kidney problem? What if I lose my arm or leg the next life?" (N6, mother, 55 y)
Ethics	Fairness	"But I think the system and the supervision are not perfect. If I donate my organ, who will use the organ? How much is the charge? Is it reasonable? Anyway, at least now I don't think it's fair." (N14, sister, 32 y) "If the rich get sick, they don't have to wait at all. It seems that they can transplant as long as they are matched. It's obvious that so many people need organs, but the rich can change one after another. "(N18, volunteer, 24 y)
	Mistrust	"I've heard that someone was diagnosed to be brain-dead, and later came back to life. Doctor may misdiagnose" (N3, father, 54 y) "I don't trust the doctor's brain death diagnosis. What if there's a miracle?" (N7, volunteer, 24 y) "What if my organs are donated and the bad guys illegally make huge profits? Isn't that against my original intention? I am afraid." (N14, sister 32 y)
Three family communication patterns	The whole family participate actively	"Organ donation is not just an individual matter, but a whole family matter." (N7, father, 49 y) "You should ask the family for advice first! You cannot decide (organ donation) by yourself." (N24, brother, 24 y) "I'll ask the whole family for advice, and if they say no. I will not sign it." (N4, young brother, 19 y) "Because organ donation is too important to decide by yourself." I would ask my wife and parents for advice. (N9, brother, 30 y)
	Family makes the decision for me	"Whatever, I don't care. It depends on you (son or daughter). After we both die, you can do whatever you want." (N2, grandmother, 68 y) "It depends on my son and daughter. If they agree (donation), then I will agree. "(N4, mother, 46 y)
	Make my own decision	"If I learned more about organ donation, maybe I will sign the donor card. I don't need to ask my family for advice. I can decide on my own." (N16, sister, 26 y) "If something bad happened to me, I may willing to be a donor. I can make my own decision." (N16, volunteer, 34 y) "I don't have to ask my family for permission. It's just like donating blood." (N21, husband, 32 y)

TABLE 4 | The attitude of family members toward organ donation.

Consent to families donation	Personal attitude toward organ donation			
	Willing to donate *n* = 38	Unwilling to donate *n* = 57	Not applicable *n* = 2	Total *n* = 97
Yes	24	2	0	26
Respect theirs wishes	6	14	0	20
NO	8	37	0	45
Not applicable	0	4	2	6

and taboo," "body intact," "fair and trust," "family communication patterns" are shown in **Table 3**.

Attitude Toward Organ Donation in the Family

We enrolled 26 families, 97 family members in interviews based on family units. Twenty-six families were numbered as N1, N2, N3. . .. . .N25, N26. Respondents included grandparents, parents, spouses, brothers and sisters, cousins and uncles, etc. Most respondents have heard about organ donation. They learned about organ donation knowledge mainly through TV, the internet, school and friends. According to the interview, we counted the attitude of family members towards organ donation (See **Table 4**).

Of 97 participants, 38 expressed their willingness to donate when death is inevitable. However, majority of the participants refused to sign up as a donor. Moreover, there was a great possibility that they would prevent relatives from organ donation. We also found an interesting point, some participants would like to be a donor, but they cannot accept their next-to-kin (especially their parents and children) donate organs. For example, the mother of the N10 family would like to be a donor herself, while she did not want her children or husband to donate organs. The volunteer of the N18 family indicated that she could donate her organ, but she thought it's unacceptable to donate her parents' organ.

The Timing of Thinking

Many of our respondents expressed that they have heard about organ donation. However, few of them talked about it with family members or ever thought to be a donor. Many knew little about donation and felt that organ donation is far behind their lives.

Seriously Ill or Facing Death

Many participants indicated that they would consider organ donation when they suffered from an incurable disease or a traffic accident.

The Media

Media, which includes television, radio, magazine, and the internet, is important access that people know more about organ donation. Some participants have thought to be a donor because they were deeply touched by the relevant documentaries and public service advertising.

Sympathy

Some participants worked at the hospital, they often came into contact with patients with end-stage diseases. They knew how desperately these patients need transplants. Just one organ can save a life and a family. They seemed more likely to donate because of sympathy.

Motivation

Altruism

Altruism is the main factor that participants would like to be a donor. Being able to help someone else is a positive reason for participants to support organ donation. Some participants believed that organ donation is meaningful and can enrich their lives. They can save people who are seriously ill and contribute to society through organ donation.

Usefulness

Some agreed that people are turned to ashes after death, it is better to donate organs to help those who need them. Families declared that organs were precious and cherished, they had little worth to the donor but can prolong the lives of others.

Good for Family

The family occupies a very important place in Chinese. Many indicated that if families had priority in organ distribution when needed, this will promote their willingness to donate. While for those who had less willing to donate, they would consider registering as an organ donor for their families.

Taboo and Fear

Taboo

Ten volunteers failed to conduct the interview because family members refused to talk about organ donation or they felt uncomfortable during the deep discussion about family member's donation. They thought it was a taboo topic to them. When talking about organ donation, some expressed that they could not help thinking of bereavement, which made them feel anguished.

A Bad Omen

Signing the donor card when getting the driver's license makes many respondents feel uncomfortable. The participants were more or less superstitious, they would be anxious about something bad will happen to them and their families, and

this caused psychological stress on them. Moreover, they did not want their families to worry about them because of signing as a donor.

Fear

Including fear of mortality, fear of being separated after death, and fear of unnatural death. Some participants refused donation because they thought that only unnatural death would donate organs.

Traditional Beliefs

Filial Piety

Families who were affected by Confucian culture believed that one should keep the body intact even when they died. That is one of the reasons why people refused the donation. There is an old saying in china that goes "filial piety is the foundation of all virtues." Thus, some participants who support organ donation may refuse their parent's organ donation. Because they do not want to be unfilial.

Metempsychosis

In China's unique traditional cultural background, many believed in metempsychosis which may be influenced by Buddhism. They think that the soul will reincarnate after death. Therefore, some mentioned that if the body is incomplete, they would be disabled the next life.

Ethics

Fairness

Many worried about the fairness of the distribution of organs. They indicated that the poor may not be able to afford to transplant operation, so only the rich could have a transplant. Besides, they worried whether the regulatory systems can protect the donor's rights and interests.

Mistrust

Most believed in doctor's diagnosis of brain death because it is scientifically validated. However, some were skeptical. For one thing, they questioned the scientificity of the brain death diagnosis and wondered if brain-dead people were really dead. For another thing, they were afraid that doctors may not try their best to save them if they signed up as a donor.

Three Communication Patterns of Families

Pattern 1: The Whole Family Participates Actively

Families believed that organ donation is a big deal that everyone should participate to make the decision. In this situation, the willingness of organ donation was greatly affected by the family. If the elders in the family were positive about organ donation, it seems that children would be more likely to accept donation. If someone in the family disagreed, donation was unlikely to succeed. Because they have to consider the opinions and feelings of their families.

Pattern 2: Family Makes Decisions for Me

This situation usually happened to the elderly who relied on their sons and daughters. Few Chinese people made wills, although they knew they were responsible for their bodies, they indicated that donate or not is up to posterity.

Pattern 3: Make My Own Decision

Some who are not willing to donate may not communicate or ask for advice from family. Three people mentioned that he/she would not consult the family, he/she could make the decision themselves.

DISCUSSION

This study conducted in-depth qualitative research to analyze the attitude, influencing factors and communication patterns on donation in family units. Most families have never discussed this serious topic with next to kin before and their attitude varied. Limited knowledge, motivation, traditional beliefs, especially family attitude have a great influence on their decision. We constructed three family communication patterns according to the analysis.

There is an interesting point that has never been found in previous studies. Some would like to be a donor while he/she would not agree their next to kin to donate. In our study, thirty-eight indicated that they would like to a donor. However, eight of them couldn't accept their family donate organs. Besides, people who had little willingness to donate may sign up the donor card if their family could have priority to transplant when needed. All these showed that family comes first in most Chinese, sometimes one may sacrifice for the family. Besides, it seems that filial piety is not only an obstacle to organ donation but also promotes organ donation.

We found the factors from different aspects that influence the family's decision. Limited knowledge, lack of family discussion, some traditional beliefs and mistrust may discourage donation. Altruism and family support are likely to encourage donation. This is consistent with previous research results (7,31–33). Many refused to talk about organ donation because it is a serious topic that makes them uncomfortable. Some believed that their relatives would not be supportive, because of an excessive number of family members, consensus could not be achieved (18). Besides, in Chinese special culture, we also found some factors that have never been discovered before. For example, people thought that sign up the donor card when healthy may be a bad omen. Some affected by Buddhism believed in metempsychosis. Therefore, they insisted on keeping the body wholeness after death. Overwhelmingly, the imperfect regulatory systems and mistrust made many families refused to sign up the donor card even if they have a strong willingness to donate. People worried that donated organs may be used in improper trading (17). Our research showed that there are many potential donors in public. Thus, regulatory measures are needed to ensure that the rights and interests of donors would be well protected and everyone has an equal right to obtain organs.

Obviously, family opinion played a vital role in the successful donation (34). Previous studies often simply described the decision-making model as family centered (35). Ya-Ping Lin

had constructed 3 patterns of communication and decision-making processes in living donor liver transplantation among Tawanese (26). However, we established three family communication patterns that apply to the public in China mainland based on varying family structures, relationships, personal attitude and traditional beliefs. We encouraged communication pattern 1 that the whole family participates actively in the discussion. This could help understand the family's true will on organ donation and avoid the dilemma of disagreement when facing donation. The implementation of organ donation in China also requires the consensus of family members. Communication pattern 2 is not uncommon among the old. They have less willing to talk about organ donation with family and insist that their sons or daughters would decide for them. While some family with communication pattern 3 would make their own decision without discussion. They believed they do not need to talk about organ donation as they are responsible for their body. Understanding family communication patterns and influencing factors is vital for the policymaker to make perfect law. We suggest that donation regulations need to focus on families and formulate relevant preferential policies based on families.

The previous study also found that family discussion of organ donation was positively related to the attitude toward deceased organ donation (36). However, some family members refused to talk about this topic mainly because of some traditional beliefs and misunderstandings of organ donation. Thus, promoting the knowledge of organ donation and raising public awareness is necessary for improving family communication. Organ donation is regarded as the "gift-of-life" and an act of great love. Confucianism considers physical integrity as a form of filial piety, however, the core of Confucianism emphasizes "ren," which means benevolence (35). Therefore, organ donation can be promoted based on "ren." Social media, such as Weibo and WeChat, which were widely used in China (37, 38). OPO should make full use of the internet to share organ donation stories and awaken the heart of benevolence. Besides, publicize knowledge related to organ donation and transplantation would be helpful to dispel misunderstandings of donation. We also recommend that knowledge of organ donation can be added to school education to increase acceptance among the young.

This study has several limitations: a recruitment notice was issued through our laboratory website, we also repost it on our social platforms. In fact, organ donation is rarely talked about in daily life. Therefore, this may not have been a true census sample as we have only invited people who were interested in organ donation. Private discussions among family members enabled participants to better express their true thoughts on organ donation. Thus, we conducted qualitative interview training for volunteers. However, it is time-consuming to train the volunteers and to collect the data, that is the reason why our study lasted for so long. Besides, it is undeniable that controlling the quality of the family interview is not easy because researchers did not participate in the interview process.

CONCLUSION

Our study provided a deeper understanding of attitude, influencing factors and communication patterns in families on organ donation in China. We found that families would be conservative when it comes to organ donation. Limited knowledge, fear, some traditional beliefs and mistrust would discourage donation. Based on the analysis, this research provides insight into the family communication on donation. Family always comes first in Chinese society. We suggest that family-based consent and incentives are more suitable for the Chinese social context. Regulatory measures in the process of organ procurement and distribution should be strengthened to protect the interests of donors and increase public trust. Social media are recommended to dispel misunderstand of donation and improve the public's acceptance of organ donation.

DATA AVAILABILITY STATEMENT

The data analyzed during this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the IRB, Third Xiangya Hospital, Central South University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AL, WX, HH contributed to the conception and design of the study. HH, AL, ZX and WO collected the data. HH, WX, YC and KL performed the statistical analysis. HH and WX and wrote the first draft of the manuscript and contributed to the manuscript revision, read, and approved the submitted version.

FUNDING

This research was supported by the Social Science Foundation of Hunan Province (16YBQ074) and Natural science foundation of Changsha city (kq2014270).

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
published: 10 March 2022
doi: 10.3389/ti.2022.10176

Post-Transplant Extracorporeal Membrane Oxygenation for Severe Primary Graft Dysfunction to Support the Use of Marginal Donor Hearts

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Severe primary graft dysfunction (PGD) is the leading cause of early postoperative mortality following orthotopic heart transplantation (OHT). Veno-arterial extracorporeal membrane oxygenation (VA-ECMO) has been used as salvage therapy. This study aimed to evaluate the outcomes in adult OHT recipients who underwent VA-ECMO for severe PGD. We retrospectively reviewed 899 adult (≥18 years) patients who underwent primary OHT at our institution between 1997 and 2017. Recipients treated with VA-ECMO (19, 2.1%) exhibited a higher incidence of previous cardiac surgery (p = .0220), chronic obstructive pulmonary disease (p = .0352), and treatment with a calcium channel blocker (p = .0018) and amiodarone (p = .0148). Cardiopulmonary bypass (p = .0410) and aortic cross-clamp times (p = .0477) were longer in the VA-ECMO cohort and they were more likely to have received postoperative transfusion (p = .0013); intra-aortic balloon pump (IABP, p < .0001), and reoperation for bleeding or tamponade (p < .0001). The 30-day, 1-year, and overall survival after transplantation of non-ECMO patients were 95.9, 88.8, and 67.4%, respectively, compared to 73.7, 57.9, and 47.4%, respectively in the ECMO cohort. Fourteen (73.7%) of the ECMO patients were weaned after a median of 7 days following OHT (range: 1–12 days). Following OHT, VA-ECMO may be a useful salvage therapy for severe PGD and can potentially support the usage of marginal donor hearts.

Keywords: outcomes, heart transplantation, ECMO, primary graft dysfunction, marginal donor heart

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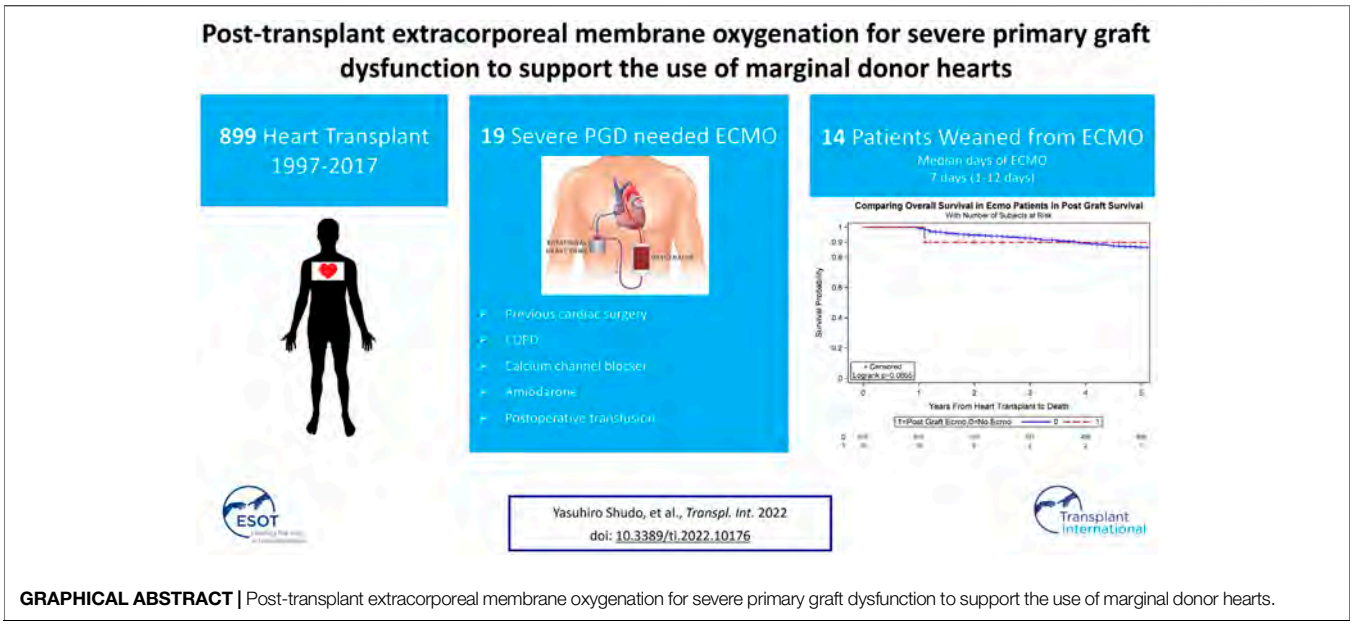
Received: *02 November 2021*
Accepted: *18 January 2022*
Published: *10 March 2022*

Citation:
Shudo Y, Alassar A, Wang H, Lingala B, He H, Zhu Y, Hiesinger W, MacArthur JW, Boyd JH, Lee AM, Currie M and Woo YJ (2022) Post-Transplant Extracorporeal Membrane Oxygenation for Severe Primary Graft Dysfunction to Support the Use of Marginal Donor Hearts. Transpl Int 35:10176. doi: 10.3389/ti.2022.10176

INTRODUCTION

Heart disease is the leading cause of death in the United States, and medically refractory heart failure represents end-stage heart disease (1). We are currently faced with a plethora of patients suffering from heart failure. Many treatments have been developed for patients with end-stage heart failure, among which orthotopic heart transplantation (OHT) remains the gold standard (2). However, primary graft dysfunction (PGD) is a devastating complication, and the associated 30-day mortality rate is as high as 30% (3–5). PGD is diagnosed within 24 h after OHT and is distinct from secondary graft dysfunction where there is a discernible cause such as hyperacute rejection, pulmonary hypertension, or known surgical complications (6). There are several possible treatment options for managing PGD, such as inotropes, intra-aortic balloon pump (IABP), and mechanical circulatory



GRAPHICAL ABSTRACT | Post-transplant extracorporeal membrane oxygenation for severe primary graft dysfunction to support the use of marginal donor hearts.

assist, among others. According to the International Society for Heart and Lung Transplantation (ISHLT) Registry consensus statement (6), the most severe form of PGD was defined as the requirement of mechanical circulatory assistance for treatment.

Although over 20,000 patients may benefit from OHT per year, only 3,000 will receive a new heart, with a waitlist mortality of 10.7 deaths per 100,000 waitlist-years (7). Due to the persistent and worsening shortage of available donor hearts, we have previously proposed alternative approaches to maximize organ allocation, including repairing the donor's valvular heart disease (8), harvesting donor hearts from more distant locations and accepting longer cold ischemic time (9), as well as utilizing hearts from obese donors (10). Despite growing evidence supporting the safety of using these marginal organs, there are concerns regarding PGD following OHT with marginal hearts.

Veno-arterial extracorporeal membrane oxygenation (VA-ECMO) is a versatile mechanical circulatory support technique that may be used as salvage therapy for patients with low-output post-cardiotomy syndrome. In the context of OHT, VA-ECMO represents an increasingly common therapeutic option for post-transplant recipients with severely depressed postoperative cardiac output and dysfunction (3–5). Therefore, this study aimed to review the outcomes of adult heart transplant recipients who underwent VA-ECMO for severe PGD.

METHODS

For confidentiality reasons, the data and study materials will not be made available to other researchers for purposes of reproducing the results.

Patient Selection

We retrospectively reviewed all patients who underwent OHT at Stanford University Hospital between January 1997 and December 2017 (n = 1,181).

The exposure of interest was postoperative VA-ECMO usage within 30 days of OHT due to severe PGD. Patients were excluded if they were below 18 years old (n = 261), or if there was incomplete post-OHT ECMO data (n = 21, **Figure 1**). The patients were assigned to two groups based on the requirement of VA-ECMO to manage severe PGD following OHT.

Information obtained from our institutional database included donor characteristics (age, sex, height, body weight, body mass index), past medical history (diabetes, hypertension, tobacco use, hepatitis C), donor's left ventricular ejection fraction, recipient baseline characteristics (age, sex, height, body weight, and body mass index), past medical history (diabetes, hypertension, hyperlipidemia, hemodialysis, chronic obstructive pulmonary disease, history of cerebrovascular accident), etiology of heart failure, total waiting time, and preoperative life support (hospitalization, inotropic support, ventilator support, IABP, ECMO, durable ventricular assist device [VAD]), preoperative medication, and allograft ischemic time.

The primary outcomes were 30-day, 1-year, and overall mortality, which were defined as patient death post transplantation. Studies involving this dataset have been exempted from review by the Institutional Review Board of Stanford University School of Medicine.

Statistical Analysis

In the descriptive analyses of the study, continuous variables were presented as means ± standard deviation and compared to the mean differences between groups by analysis of variance (ANOVA). The χ^2 test was used to evaluate the association

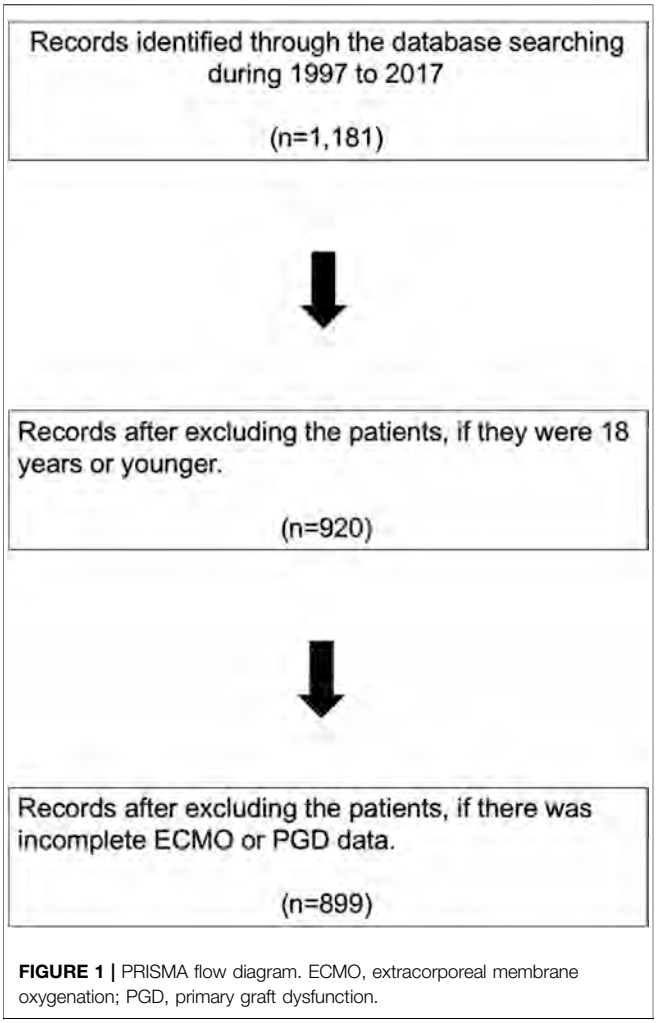


FIGURE 1 | PRISMA flow diagram. ECMO, extracorporeal membrane oxygenation; PGD, primary graft dysfunction.

between the categorical variables. Survival curves were constructed using the Kaplan-Meier method, stratified over post-transplant ECMO usage, and were tested using the log-rank test. Exact matching with risk adjustment for confounders was performed to identify patients who did not undergo ECMO after transplantation but who had similar essential characteristics as those who received post-transplant VA-ECMO support (4). The matching criteria for this study were: transplant year ±5 years, recipient age ±4 years, recipient gender, recipient history of prior cardiac surgery, and recipient preoperative life support (inotropic support). Matching criteria were applied sequentially to produce two matched cohorts containing all the possible pairings. The endpoints were then compared between the two matched cohorts. For all analyses, *p*-values <.05, were considered statistically significant. All analyses were performed using SAS version 9.4 (SAS Institute Inc. NC, United States).

RESULTS

A total of 899 adult (≥18 years) primary OHT patients who fulfilled the study entry criteria were identified. The cohorts differed in demographic and preoperative clinical characteristics, depending on the requirement for post-transplant VA-ECMO. Nineteen patients (2.1%) received VA-ECMO support in the very early post-transplant period due to severe PGD, and 880 patients (97.9%) did not receive VA-ECMO.

Recipient Characteristics

Recipient characteristics stratified by recipient post-transplant ECMO use are shown in **Table 1**.

The mean age of all recipients was 51.5 ± 12.9 years. A total of 73.9% of recipients were male, and the mean body mass index was 26.2 ± 5.0 kg/m^2. Overall, 31.7% of recipients were diabetic, 44.4% were hypertensive, 39.3% had a history of hyperlipidemia, 24.1% had a history of cigarette use, 10.1% had a history of COPD, and 4.1% were on hemodialysis.

The prevalence of COPD in recipients undergoing ECMO after OHT (26.3%) was significantly higher than that in recipients who did not undergo ECMO after OHT (9.8%), p = .0352. In addition, the prevalence of previous cardiac surgery was significantly greater among recipients in the post-transplant ECMO group (57.9%) than among recipients without post-transplant ECMO (31.1%), p = .022. The percentages of patients receiving a calcium channel blocker (31.3% vs. 5.5%, p = .0018) and amiodarone (75.0% vs. 38.6%, p = .0148) were also significantly higher in the ECMO cohort compared to the non-ECMO cohort.

Mechanical circulatory support usage before OHT was not significantly different between the two groups (IABP, ECMO, and durable VAD; p = .2003, 1, and .3855, respectively). Similarly, the proportion of patients admitted to the intensive care unit (ICU) prior to OHT was not significantly different between the two groups. These results suggest that post-transplant ECMO was utilized independently and was not associated with the recipient's preoperative clinical status.

Donor Characteristics

Donor characteristics stratified by post-transplant VA-ECMO use are shown in **Table 2**. The mean age of all donors was 33.0 ± 12.4 years. A total of 73.5% of donors were male, and the mean body mass index was 26.7 ± 5.5 kg/m^2. Overall, 2.4% of donors were diabetic, 13.0% were hypertensive, and 21.9% had a history of cigarette use. The incidence of hepatitis C positive donors was extremely low (1.0%). The left ventricular ejection fraction was excellent in both groups. There were no significant differences in the donor baseline characteristics between the two groups.

Operative Variables

Operative variables stratified by post-transplant VA ECMO use are shown in **Table 3**. Cardiopulmonary bypass (209.7 ± 59.1 vs. 167.2 ± 52.8 min, p = .041) and aortic cross clamp times (125.4 ± 44.9 vs. 102.2 ± 44.5 min, p = .0477) were longer in the post-transplant ECMO cohort. There were no significant differences between recipients with ECMO (232.1 ± 69.0 min) and those without ECMO (219.9 ± 56.6 min, p = .2444) regarding the allograft ischemic time.

TABLE 1 | Recipient characteristics stratified by recipient post-transplant ECMO usage.

	N[a]	Without ECMO	N[a]	With ECMO	N[a]	Total	p value
Age (y)	880	51.55 ± 12.92 [55 (45, 61)]	19	48.26 ± 12.45 [49 (40, 58)]	899	51.48 ± 12.92 [54 (45, 61)]	.1058
Gender, male, *n* (%)	879	649 (73.83%)	19	15 (78.95%)	898	664 (73.94%)	.7938
Height (cm)	846	166.14 ± 35.19 [172.7 (165.1, 180.3)]	19	139.96 ± 74 [172.7 (160, 182.9)]	865	165.56 ± 36.6 [172.7 (165.1, 180.3)]	.9809
Body weight (kg)	846	78.69 ± 17.04 [78 (66.4, 89.8)]	19	81.49 ± 30.91 [82.5 (62.59, 102.5)]	865	78.75 ± 17.43 [78 (66.4, 89.8)]	.325
Body mass index (kg/m^2)	835	26.19 ± 4.91 [25.6 (22.8, 29)]	18	27.89 ± 6.94 [26 (24.7, 34.3)]	853	26.22 ± 4.96 [25.6 (22.8, 29)]	.6293
Past medical history							
Diabetes mellitus, *n* (%)	880	278 (31.59%)	19	7 (36.84%)	899	285 (31.7%)	.624
Hypertension, *n* (%)	880	388 (44.09%)	19	11 (57.89%)	899	399 (44.38%)	.2508
Hyperlipidemia, *n* (%)	880	346 (39.32%)	19	7 (36.84%)	899	353 (39.27%)	1
On hemodialysis, *n* (%)	880	35 (3.98%)	19	2 (10.53%)	899	37 (4.12%)	.182
COPD, *n* (%)	880	86 (9.77%)	19	5 (26.32%)	899	91 (10.12%)	.0352
History of CVA, *n* (%)	880	36 (4.09%)	19	2 (10.53%)	899	38 (4.23%)	.1898
Tobacco usage, *n* (%)	880	209 (23.75%)	19	8 (42.11%)	899	217 (24.14%)	.0983
Etiology of heart failure							
Non-ischemic cardiomyopathy, *n* (%)	879	290 (32.99%)	19	2 (10.53%)	898	292 (32.52%)	.1872
Ischemic cardiomyopathy, *n* (%)	879	234 (26.62%)	19	6 (31.58%)	898	240 (26.73%)	
Congenital heart disease, *n* (%)	879	71 (8.08%)	19	4 (21.05%)	898	75 (8.35%)	
Restrictive heart disease, *n* (%)	879	62 (7.05%)	19	1 (5.26%)	898	63 (7.02%)	
Hypertrophic cardiomyopathy, *n* (%)	879	57 (6.48%)	19	1 (5.26%)	898	58 (6.46%)	
Valvular heart disease, *n* (%)	879	19 (2.16%)	19	0 (0%)	898	19 (2.12%)	
Familial cardiomyopathy, *n* (%)	879	40 (4.55%)	19	1 (5.26%)	898	41 (4.57%)	
Repeat heart transplantation, *n* (%)	879	39 (4.44%)	19	1 (5.26%)	898	40 (4.45%)	
Total waitlist time (years)	841	131.33 ± 250.52 [47 (16, 134)]	17	215.29 ± 211.36 [138 (43, 314)]	858	132.99 ± 249.97 [47 (16, 138)]	.086
Previous cardiac surgery, *n* (%)	880	274 (31.14%)	19	11 (57.89%)	899	285 (31.7%)	.022
Pre-operative life support, *n* (%)							
Hospitalization, *n* (%)	866	311 (35.91%)	18	6 (33.33%)	884	317 (35.86%)	1
Inotropic support, *n* (%)	842	368 (43.71%)	19	13 (68.42%)	861	381 (44.25%)	.0367
Ventilator support, *n* (%)	833	92 (11.04%)	17	3 (17.65%)	850	95 (11.18%)	.4243
IABP, *n* (%)	833	10 (1.2%)	17	1 (5.88%)	850	11 (1.29%)	.2003
ECMO, *n* (%)	833	1 (0.12%)	17	0 (0%)	850	1 (0.12%)	1
Durable VAD, *n* (%)	854	172 (20.14%)	18	5 (27.78%)	872	177 (20.3%)	.3855
Pre-operative medication, *n* (%)							
Beta blocker, *n* (%)	756	214 (28.31%)	16	7 (43.75%)	772	221 (28.63%)	.1746
Calcium channel blocker, *n* (%)	740	41 (5.54%)	16	5 (31.25%)	756	46 (6.08%)	.0018
Angiotensin receptor blocker, *n* (%)	763	181 (23.72%)	16	3 (18.75%)	779	184 (23.62%)	.7743
Angiotensin converting enzyme-inhibitor, *n* (%)	752	113 (15.03%)	16	2 (12.5%)	768	115 (14.97%)	1
Aspirin, *n* (%)	765	268 (35.03%)	16	6 (37.5%)	781	274 (35.08%)	.7979
Plavix, *n* (%)	495	35 (7.07%)	16	1 (6.25%)	511	36 (7.05%)	1
Anticoagulation (Warfarin, heparin), *n* (%)	767	387 (50.46%)	16	6 (37.5%)	783	393 (50.19%)	.3257
Lasix, *n* (%)	544	204 (37.5%)	16	5 (31.25%)	560	209 (37.32%)	.7945
Spironolactone, *n* (%)	750	234 (31.2%)	16	9 (56.25%)	766	243 (31.72%)	.0531
Amiodarone, *n* (%)	734	283 (38.56%)	12	9 (75%)	746	292 (39.14%)	.0148
Digoxin, *n* (%)	756	175 (23.15%)	16	4 (25%)	772	179 (23.19%)	.7721
Pre-operative data							
White blood cell count (×1,000/ml)	761	7.85 ± 2.88 [7.3 (5.9, 9.1)]	14	8.93 ± 3.28 [9.05 (6.4, 11.4)]	775	7.87 ± 2.89 [7.3 (5.9, 9.1)]	.2777
Hemoglobin (g/dl)	737	11.57 ± 2.11 [11.5 (10.1, 13)]	14	12.14 ± 2.62 [12.85 (10, 13.7)]	751	11.58 ± 2.12 [11.5 (10.1, 13)]	.2786
Platelet (×1,000/ml)	763	223.42 ± 89.1 [207 (165, 261)]	14	190.14 ± 73.76 [181.5 (144, 216)]	777	222.82 ± 88.92 [207 (165, 260)]	.1058
Sodium (mmol/L)	585	134.1 ± 4.92 [135 (131, 137)]	11	136.45 ± 5.11 [135 (134, 137)]	596	134.15 ± 4.93 [135 (131, 137)]	.6776
Blood urea nitrogen (mg/dl)	772	29.76 ± 18.41 [24.5 (18, 35)]	15	26.6 ± 12.77 [25 (14, 38)]	787	29.7 ± 18.32 [25 (18, 35)]	.804
Creatinine (mg/dl)	772	1.52 ± 0.96 [1.3 (1, 1.7)]	15	1.56 ± 0.66 [1.39 (1, 2.1)]	787	1.52 ± 0.96 [1.3 (1, 1.7)]	.7849
Total bilirubin (mg/dl)	539	1.26 ± 1.32 [1 (0.6, 1.5)]	10	1.1 ± 1.55 [0.6 (0.4, 1)]	549	1.26 ± 1.32 [1 (0.6, 1.5)]	.1949

(Continued on following page)

TABLE 1 | (*Continued*) Recipient characteristics stratified by recipient post-transplant ECMO usage.

	N^a	Without ECMO	N^a	With ECMO	N^a	Total	*p* value
Aspartate transaminase (U/L)	550	46.01 ± 67.33 [30 (23, 43)]	10	47.3 ± 47.32 [31.5 (21, 46)]	560	46.03 ± 67 [30 (23, 43)]	1
Alanine transaminase (U/L)	540	57.83 ± 148.8 [34 (24, 48)]	9	49.33 ± 35.48 [38 (31, 48)]	549	57.69 ± 147.64 [34 (24, 48)]	.7308
Albumin (g/dl)	548	3.42 ± 0.63 [3.4 (3, 3.9)]	10	3.52 ± 0.52 [3.6 (3, 3.8)]	558	3.42 ± 0.63 [3.45 (3, 3.9)]	.5237
INR	544	1.89 ± 0.89 [1.6 (1.2, 2.4)]	9	1.68 ± 0.64 [1.4 (1.1, 2.3)]	553	1.89 ± 0.89 [1.6 (1.2, 2.4)]	.7375

ECMO, extra corporealmembrane oxygenation. COPD, chronic obstructive pulmonary disease. CVA, cerebrovascular accident. IABP, Intra-aortic baloon pump. VAD, ventricular assist device. INR, international normalized ratio.
[a]N, *available number of patients.*

TABLE 2 | Donor characteristics stratified by recipient post-transplant ECMO usage.

Donors' characteristics	N^a	Without ECMO	N^a	With ECMO	N^a	Total	*p* value
Age (y)	880	32.98 ± 12.4 [31 (22, 43)]	19	35.84 ± 12.73 [40 (22, 45)]	899	33.04 ± 12.4 [32 (22, 43)]	.4829
Gender, male, *n* (%)	856	628 (73.36%)	18	14 (77.78%)	874	642 (73.46%)	.7931
Height (cm)	856	174.32 ± 9.7 [175 (168, 181)]	18	176.96 ± 8.78 [177 (171, 183)]	874	174.38 ± 9.69 [175 (168, 181)]	.244
Body weight (kg)	856	81.3 ± 18.47 [79 (69, 90.7)]	18	77.34 ± 22.62 [77.5 (61, 81.5)]	874	81.22 ± 18.56 [79 (69, 90.2)]	.5091
Body mass index (kg/m^2)	856	26.72 ± 5.48 [25.9 (22.7, 29.4)]	18	24.66 ± 7.14 [23.25 (19.9, 26.6)]	874	26.68 ± 5.52 [25.9 (22.7, 29.3)]	.1524
Donor's ejection fraction (%)	600	64.85 ± 11.08 [64.73 (60, 71.76)]	14	64.62 ± 11.47 [64.97 (59, 72.96)]	614	64.85 ± 11.08 [64.73 (60, 71.83)]	1
Past medical history							
Diabetes mellitus, *n* (%)	849	21 (2.47%)	18	0 (0%)	867	21 (2.42%)	1
Hypertension, *n* (%)	845	109 (12.9%)	17	3 (17.65%)	862	112 (12.99%)	.4751
Tobacco usage, *n* (%)	834	184 (22.06%)	17	2 (11.76%)	851	186 (21.86%)	.3902
Hepatitis C positive, n (%)	823	8 (0.97%)	19	0 (0%)	842	8 (0.95%)	1

ECMO, extracorporeal membrane oxygenation.
[a]N, *available number of patients.*

The percentage of postoperative transfusion was greater in the post-transplant ECMO group (93.8% vs. 53.8%, p = .0013). Similarly, the incidence of reoperation for bleeding or tamponade was greater in the post-transplant ECMO cohort (81.3% vs. 7.4%, p < .0001). These results suggest that significant postoperative transfusion and bleeding may cause hemodynamic instability, leading to the requirement for ECMO.

Interestingly, the distance of donor organ travel was similar between the groups (157.2 ± 203.9 miles for recipients with ECMO, compared to 140.9 ± 160.1 miles for those without ECMO, p = .8062). There were no multiorgan transplant recipients in the post-transplant ECMO cohort, whereas 6.5% of recipients in the non-ECMO cohort received multiorgan transplants.

Outcomes

The frequency of postoperative pneumonia (31.6% vs. 7.4%, p = .0023) and renal failure requiring dialysis (68.4% vs. 14.2%, p < .0001) were significantly higher in the ECMO cohort. Length of hospital stay (49.5 ± 57.8 vs. 20.8 ± 24.4 days, p = .0002) and ICU stay (37.1 ± 45.6 vs. 8.8 ± 12.7 days, p = .0001) were significantly longer in the post-transplant ECMO cohort.

In the entire cohort, the 30-day, 1-year, and overall survival rates after transplantation were 95.9, 88.8, and 67.4%, respectively. In the ECMO cohort, the 30-day, 1-year, and overall survival rates after transplantation were 73.7, 57.9, and 47.4%, respectively. To assess the effect of post-transplant ECMO usage on survival, time-to-event survival analyses were conducted. The p-value of the log-rank tests on the Kaplan-Meier survival estimations of the two groups was <.0001 for overall survival (**Figure 2**). The odds ratios of 1-year mortality were 5.737 for the unadjusted analysis and 5.544 for the adjusted analysis (p = .0002 and .0004, respectively). Unadjusted and adjusted odds ratios for overall survival were 2.295 and 2.269, respectively, although these differences did not reach statistical significance (p = .074 and .0784, respectively).

Interestingly, conditional survival, defined as survival for recipients who survived for at least 1 year after surgery, was 92.6% and 86.5% at 3 years and 5 years in the cohort with ECMO, and 90.0% and 90.0% at 3 years and 5 years in the cohort without ECMO (log-rank test, p = .0865; **Figure 3**).

Among the 19 patients with post-transplant ECMO, 14 (73.7%) were weaned from ECMO at a median duration of 7 days following OHT (range: 1–2 days).

Outcomes After Exact Matching Analysis

Of the 899 recipients in this study, 82 were successfully matched based on several important factors, using the exact matching algorithm previously described (without ECMO, n = 63; with ECMO, n = 19). In the matched cohort, the mean age for adult primary OHT was 49.1 years old. In total, 68 recipients (82.9%) were men. There were no significant differences in the recipient or donor baseline characteristics between the two matched cohorts.

TABLE 3 | Operative measures stratified by recipient post-transplant ECMO usage, before and after exact matching.

Operative Measure	Before matching					After matching[a]				
	Without ECMO		With ECMO		*p*-value	Without ECMO		With ECMO		*p*-value
	N[b]	Estimate	*N*[b]	Estimate		*N*[b]	Estimate	*N*[b]	Estimate	
Cardiopulmonary bypass time (minutes)										
Mean ± SD	768	167.15 ± 52.78	15	209.73 ± 59.14	.0041	57	179.58 ± 48.48	15	209.73 ± 59.14	.3873
Median (IQR)		157 (133, 189)		193 (173, 286)			173 (143, 215)		193 (173, 286)	
Aortic cross clamp time (minutes)										
Mean ± SD	599	102.21 ± 44.54	13	125.38 ± 44.92	.0477	43	112.02 ± 28.49	13	125.38 ± 44.92	.1168
Median (IQR)		95 (80, 115)		122 (107, 136)			103 (92, 138)		122 (107, 136)	
Allograft ischemic time (minutes)										
Mean ± SD	862	219.93 ± 56.61	19	232.1 ± 69	.2444	63	222.2 ± 52.67	19	232.1 ± 69	.9273
Median (IQR)		216 (186, 252)		228 (204, 282)			228 (198, 246)		228 (204, 282)	
Transfusion										
Intraoperative, *n* (%)	553	286 (51.72 %)	16	9 (56.25 %)	.8030	52	39 (75 %)	16	9 (56.25 %)	.2098
Postoperative, *n* (%)	413	222 (53.75 %)	16	15 (93.75 %)	.0013	52	30 (57.69 %)	16	15 (93.75 %)	.0071
Distance organ travelled (miles)										
Mean ± SD	769	140.87 ± 160.06	17	157.24 ± 203.91	.8062	58	120.41 ± 130.65	17	157.24 ± 203.91	.4474
Median (IQR)		81 (25, 168)		51 (31, 254)			119 (23, 147)		51 (31, 254)	
Transplant year										
Median (IQR)	880	2,008 (2,003, 2,014)	19	2,015 (2,012, 2,016)	.0020	63	2,014 (2,010, 2,016)	19	2,015 (2,012, 2,016)	.2365
Postoperative IABP										
n (%)	805	33 (4.1%)	16	9 (56.25%)	<.0001	57	4 (7.02%)	16	9 (56.25%)	<.0001
Postoperative VA ECMO										
n (%)	876	0 (0%)	19	19 (100%)	N/A[a]	63	0 (0%)	19	19 (100%)	N/A[a]
Postoperative VV ECMO										
n (%)	876	0 (0%)	19	4 (21.05%)	N/A[a]	63	0 (0%)	19	4 (21.05%)	N/A[a]
Reoperation for bleeding or tamponade										
n (%)	826	61 (7.38%)	16	13 (81.25%)	<.0001	59	8 (13.56%)	16	13 (81.25%)	.0022
Multiorgan transplant										
n (%)	813	53 (6.52%)	16	0 (0%)	.6167	0	0 (0%)	16	0 (0%)	N/A[a]

[a]*Patients were matched on Transplant Year (±5 years), Recipient's Age (±4 years old), Recipient's Gender, Recipient's History of Prior Cardiac Surgery, and Recipient's Preoperative Life Support (inotropic support) with those with ECMO.*

[b]*Available number of patients.*

[c]*Statistic is not applicable. ECMO, extracorporeal membrane oxygenation. IABP, intra-aortic baloon pump.*

For operative variables, the matched cohort without ECMO showed no significant difference compared to the ECMO cohort with regard to cardiopulmonary bypass time (p = .3873) and aortic cross-clamp time (p = .1168, **Tables 3**, **4**). In the ECMO cohort, 30-day, 1-year, and overall survival after transplant were 73.7%, 57.9%, and 47.4%, respectively, while in the matched cohort without ECMO, 30-day, 1-year, and overall survival after transplant was 93.7%, 87.3%, and 74.6 (log-rank test, p = .0006, **Figure 4**).

DISCUSSION

This comprehensive study investigated the impact of post-transplant VA-ECMO usage on the outcome of adult primary OHT recipients using the Stanford University heart transplant database. We stratified the cohort by disjoint categories of VA-ECMO usage in the early post-transplant period due to severe PGD. Severe PGD was defined as the requirement for mechanical circulatory assistance for treatment according to the ISHLT Registry consensus statement (6).

Historically, many treatments have been developed for patients with end-stage heart failure, among which OHT remains the gold standard (2). However, the persistent and worsening shortage of available donor organs has resulted in an ever-increasing waitlist of patients and longer waiting periods for heart transplants. Approximately 10% of all candidates on the waiting list for solid-organ transplantation die each year without receiving an organ (7). In order to address this challenge, we have previously proposed alternative approaches to maximize organ allocation by utilizing marginally acceptable organs (8), harvesting donor hearts from distant locations and accepting longer cold ischemic time (9), as well as utilizing obese donor hearts (10). Despite growing evidence supporting the safety of using these marginal organs, there may be concerns regarding the occurrence of PGD. Therefore, the utilization of VA-ECMO following OHT is expected to increase in the future and may become a common therapeutic option for post-transplant recipients with severely depressed postoperative cardiac output and dysfunction (3–5). Favorable outcomes of post-transplant ECMO utilization have been reported (4, 11–13). Together with improvements in technology and management of ECMO (14),

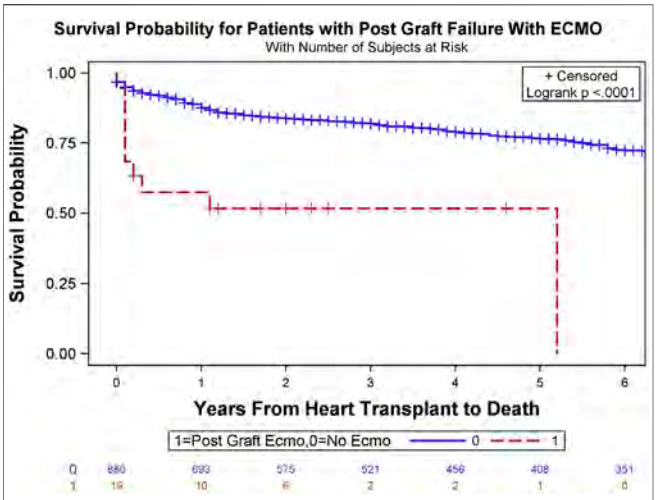


FIGURE 2 | Overall survival Kaplan-Meier estimates stratified based on the requirement of veno-arterial extracorporeal membrane oxygenation (VA-ECMO) to manage severe primary graft dysfunction (PGD) following orthotopic heart transplant (OHT) (log-rank test, $p < .0001$).

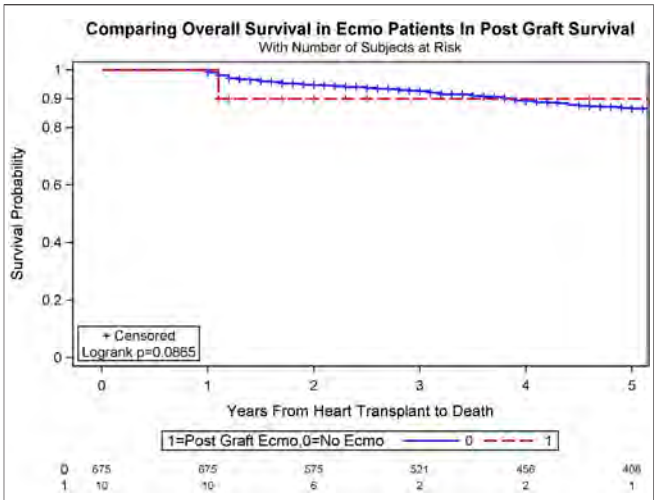


FIGURE 3 | Conditional survival, defined as survival for recipients who survive for at least 1 year after surgery, was 90.0% and 90.0% at 3 years and 5 years in the recipients who underwent veno-arterial extracorporeal membrane oxygenation (VA-ECMO), and 92.6% and 86.5% at 3 years and 5 years in the recipients who did not undergo VA-ECMO (log-rank test, $p = .0865$).

these positive outcomes may also be due in part to a new approach of placing recipients with global myocardial dysfunction on ECMO rather than introducing high doses of inotropes and vasopressors.

In the current study, our data revealed that the cohort with post-transplant ECMO usage had a higher incidence of previous cardiac surgery and diabetes mellitus. In addition, our data showed a higher percentage of preoperative amiodarone and calcium channel blocker use in the ECMO cohort. Together with the previous report that pre-transplant amiodarone use is independently associated with increased incidence of severe PGD (15), we speculate that preoperative amiodarone and calcium channel blocker use may induce temporary arrhythmogenic or vasoplegia-related hemodynamic instability leading to ECMO usage following OHT, due to the effects of long-term use or overdosing of these medications. VA-ECMO can be a good treatment option to stabilize the patient until recovering from hemodynamic instability that may be related to atrioventricular conduction or vascular tone issues. In addition, our data demonstrated that a higher incidence of postoperative blood transfusion and reoperation for bleeding or tamponade was observed in recipients receiving post-transplant ECMO. We speculate that patients with previous complicated cardiac surgery are likely to have a higher chance of reoperation for bleeding or tamponade, as well as increased postoperative blood transfusion requirements. It is also possible that ECMO itself can worsen coagulopathy and cause bleeding, which eventually may require blood products, and altogether these effects may have deleterious consequences, including hemodynamic instability and PGD. This possibility is supported by reports showing that post-transplant survival was negatively affected by complications after previous placement of a VAD (16). Moreover, our data revealed that recipients undergoing ECMO following transplant had longer aortic cross-clamp time in unmatched cohort, and a previous study suggested that aortic cross-clamp time was inversely related to post-transplant survival (9).

Equally important in this study was the identification of factors that were not significantly different in the recipients' baseline characteristics. These included the incidence of mechanical circulatory support usage, the incidence of pre-transplant hospitalization in the ICU, and donor characteristics such as age, sex, and medical history. Interestingly, our data also showed that the donor left ventricular ejection fraction was excellent in both groups. Although, in general, the perception was that heart grafts from marginal donors are of inferior quality, the incidence of post-ECMO usage due to severe PGD was observed equally regardless of recipient clinical status and donor graft quality.

Next, we discovered that the rate of severe PGD was as low as 2.1% in our cohort who underwent OHT over the last 20 years, ranking among the lowest incidences of severe PGD reported in previous studies (2–26%) (3–5, 17, 18). Although our sample size was small, we believe that the low rate of severe PGD may be attributed to our multidisciplinary patient management during the perioperative period. There may also be a number of mitigating factors related to operative techniques. Briefly, we routinely provide sufficient reperfusion time (30–240 min) together with maintaining mean arterial pressure at 75–90 mmHg on cardiopulmonary bypass, which can potentially enable the graft to recover from the stressful and edematous state and regain cardiac function following organ procurement and transplantation. This is a possible explanation for our data showing a significantly prolonged cardiopulmonary bypass time in the cohort with ECMO. We have several therapeutic options, such as leaving the chest open to remove potential mechanical stress, or aggressively introducing

TABLE 4 | Outcomes stratified by recipient post-transplant ECMO usage, before and after exact matching.

Outcome	Before matching					After matching[a]				
	Without ECMO		With ECMO		*p*-value	Without ECMO		With ECMO		*p*-value
	N[b]	Estimate	*N*[b]	Estimate		*N*[b]	Estimate	*N*[b]	Estimate	
Follow up duration (years)										
Mean ± SD	880	5.66 ± 5.19	19	1.31 ± 1.53	.0005	63	3.57 ± 4.29	19	1.31 ± 1.53	.1340
Median (IQR)		4.1 (1, 9.15)		1.1 (0.1, 2)			1.9(1, 4.2)		1.1 (0.1, 2)	
Length of hospital stay (days)										
Mean ± SD	669	20.76 ± 24.35	17	49.53 ± 57.82	.0002	52	23.48 ± 27.48	17	49.53 ± 57.82	.0018
Median (IQR)		13 (10, 20)		30 (25, 39)			15.5 (11, 26)		30 (25, 39)	
Length of ICU stay (days)										
Mean ± SD	435	8.77 ± 12.74	17	37.06 ± 45.57	.0001	48	11.63 ± 17.96	17	37.06 ± 45.57	<.0001
Median (IQR)		5 (4, 8)		21 (18, 28)			6 (4, 9.5)		21 (18, 28)	
Major morbidity										
Pneumonia, *n* (%)	880	65 (7.39%)	19	6 (31.58%)	.0023	63	6 (9.52%)	19	6 (31.58%)	.0271
Urinary tract infection, *n* (%)		40 (4.55%)		0 (0%)	N/A[c]		3 (4.76%)		0 (0%)	N/A[c]
Septicemia, *n* (%)		26 (2.95%)		2 (10.53%)	.1155		2 (3.17%)		2 (10.53%)	.2281
Sternal wound infection, *n* (%)		17 (1.93%)		1 (5.26%)	.3217		2 (3.17%)		1 (5.26%)	.5516
Renal failure requiring dialysis, *n* (%)		125 (14.2%)		13 (68.42%)	<.0001		8 (12.7%)		13 (68.42%)	<.0001
Stroke, *n* (%)		3 (0.34%)		0 (0%)	N/A[c]		0 (0%)		0 (0%)	N/A[c]
Rejection within 1-yr post transplant, *n* (%)		103 (11.7%)		1 (5.26%)	.7142		7 (11.11%)		1 (5.26%)	.6740
Mortality										
30-day, *n* (%)	880	36 (4.09%)	19	5 (26.32%)	.0011	63	4 (6.35%)	19	5 (26.32%)	.0277
1-year, *n* (%)		99 (11.25%)		8 (42.11%)	.0008		8 (12.7%)		8 (42.11%)	.0084
Overall, *n* (%)		287 (32.61%)		10 (52.63%)	.0836		16 (25.4%)		10 (52.63%)	.0465

ECMO, extracorporeal membrane oxygenation.

[a]*Patients were matched on Transplant Year (±5 years), Recipient's Age (±4 years old), Recipient's Gender, Recipient's History of Prior Cardiac Surgery, and Recipient's Preoperative Life Support (inotropic support) with those with ECMO.*

[b]*Available number of patients.*

[c]*Statistic is not applicable.*

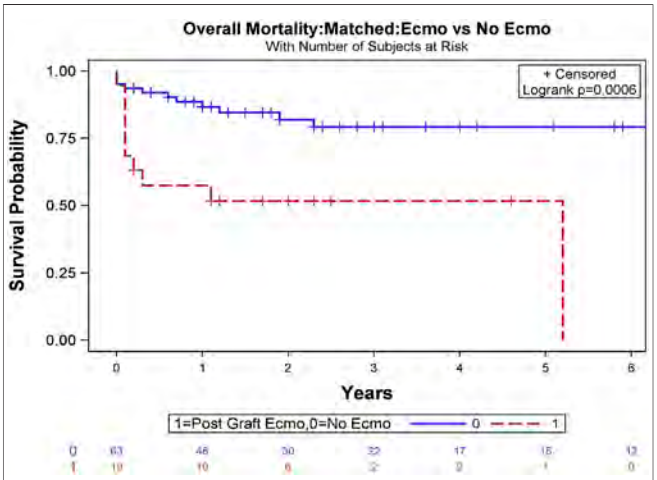


FIGURE 4 | Overall survival Kaplan-Meier estimates stratified based on the requirement of veno-arterial extracorporeal membrane oxygenation (VA-ECMO) to manage severe primary graft dysfunction (PGD) following orthotopic heart transplant (OHT) after exact matching analysis (log-rank test, $p = .0006$).

continuous renal replacement therapy to attenuate right ventricular dysfunction (which was reflected by our data indicating that 68.4% of the ECMO cohort required continuous renal replacement therapy). As a result of these interventions, only 2.1% required post-transplant ECMO therapy in our study cohort. Interestingly, our data did not show any statistical significance in the allograft ischemic time. This is likely because we have modified the sequence of anastomoses if the allograft ischemic time is expected to be prolonged (9).

Last, VA-ECMO can be administered using multiple techniques, including peripherally or centrally (19). Both techniques carry attendant risks of bleeding, and peripheral cannulation has an additional risk of limb ischemia. The peripheral cannulation technique, however, is minimally invasive, is immediately available, and allows rapid cannula insertion at the bedside. Femorally cannulated VA-ECMO can be discontinued without reopening the chest, which may reduce the risk of infection and re-bleeding. In the femorally cannulated VA-ECMO patients in this study, a reperfusion cannula was routinely used, and no instances of leg ischemia were observed. In the current study, two patients (10.5%) had septicemia and one patient (5.3%) had sternal wound infection in the post-transplant ECMO cohort. Given that the complications of VA-ECMO therapy increase with time, it is important to minimize the duration of VA-ECMO support. Our data showed that there were no ECMO-associated bleeding complications at the cannulation site, which is likely because our cohort had a median duration of only 7 days on ECMO support. We routinely combined IABP support for the treatment of severe PGD requiring VA-ECMO therapy. In our cohort, nine patients (56.3%) had IABP placement in addition to ECMO support. Combined IABP with ECMO therapy can additionally improve

coronary perfusion and provide peripheral pulsatility, reducing left ventricular afterload by slight venting, and thereby indirectly reducing pulmonary stasis and right ventricular afterload. No IABP-associated complications were observed in our cohort. Due to the short duration of ECMO support, these patients were left intubated. Importantly, the demonstration of equivalent graft outcomes in the cohort of post-transplant ECMO survivors in adults should lower the threshold for the utilization of ECMO for severe PGD.

Limitations of the Database

This study has limitations consistent with retrospective analyses and the use of a single-center database. The number of patients and events in each group was low, thus limiting its statistical power. The 100% follow-up and additional data, otherwise unavailable to national or international registries, are the two most important strengths of this study. The main focus of our current study is to determine the influence of post-transplant usage of ECMO on the outcome of recipients; however, specific donor or recipient characteristics may contribute to recipient mortality, and several of those have not been included in our analysis. The selection of a suitable donor is a complicated process. Clinicians need to consider multiple factors, including recipient urgency against donor characteristics, ischemic time, recipient sensitization, and donor/recipient size mismatch. Therefore, our findings may not be applicable to other centers. Only donors whose hearts were accepted for transplant were included in this study. To ascertain the real burden of marginal donors, it will be essential to distinguish donor hearts initially rejected by other centers for non-quality reasons or quality reasons (20). In addition, as this study addressed only mortality, further data are needed on the impact of post-transplant ECMO usage on morbidity in OHT. In the future, multicenter studies including larger cohorts are required.

CONCLUSION

Our data suggest that VA-ECMO may be a useful salvage therapy for adult heart transplant recipients with severe PGD, especially in the setting of prior cardiac surgery history or relatively suboptimal recipient selection. In particular, the improvement in conditional survival suggests that ECMO utilization following OHT can potentially increase the use of marginally acceptable donor grafts, thereby ameliorating the shortage of donor organs, reducing waitlist times for heart transplantation, and potentially decreasing mortality rates for patients on the waiting list.

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

Studies involving this dataset have been exempted from review by the Institutional Review Board of Stanford University School of Medicine.

AUTHOR CONTRIBUTIONS

YS contributed to the design of the research and took the lead in writing the manuscript; AA, HW, YZ, WH, JM, JB, AL, and MC helped determine data metrics and collected the data; BL performed statistical analysis and created figures; HH verified statistical analyses; YJW supervised the manuscript. All authors provided critical feedback and revisions to the analysis and writing 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.

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ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/ti.2022.10121

Association Between Cytomegalovirus Serostatus, Antiviral Therapy, and Allograft Survival in Pediatric Heart Transplantation

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Background: Cytomegalovirus (CMV) is an important complication of heart transplantation and has been associated with graft loss in adults. The data in pediatric transplantation, however, is limited and conflicting. We conducted a large-scale cohort study to better characterize the relationship between CMV serostatus, CMV antiviral use, and graft survival in pediatric heart transplantation.

Methods: 4,968 pediatric recipients of solitary heart transplants from the Scientific Registry of Transplant Recipients were stratified into three groups based on donor or recipient seropositivity and antiviral use: CMV seronegative (CMV-) transplants, CMV seropositive (CMV+) transplants without antiviral therapy, and CMV+ transplants with antiviral therapy. The primary endpoint was retransplantation or death.

Results: CMV+ transplants without antiviral therapy experienced worse graft survival than CMV+ transplants with antiviral therapy (10-year: 57 vs 65%). CMV+ transplants with antiviral therapy experienced similar survival as CMV- transplants. Compared to CMV seronegativity, CMV seropositivity without antiviral therapy had a hazard ratio of 1.21 (1.07–1.37 95% CI, *p*-value = .003). Amongst CMV+ transplants, antiviral therapy had a hazard ratio of .82 (0.74–.92 95% CI, *p*-value < .001). During the first year after transplantation, these hazard ratios were 1.32 (1.06–1.64 95% CI, *p*-value .014) and .59 (.48–.73 95% CI, *p*-value < .001), respectively.

Conclusions: CMV seropositivity is associated with an increased risk of graft loss in pediatric heart transplant recipients, which occurs early after transplantation and may be mitigated by antiviral therapy.

Keywords: heart transplantation, graft survival, infection, antiviral, cytomegalovirus, pediatrics

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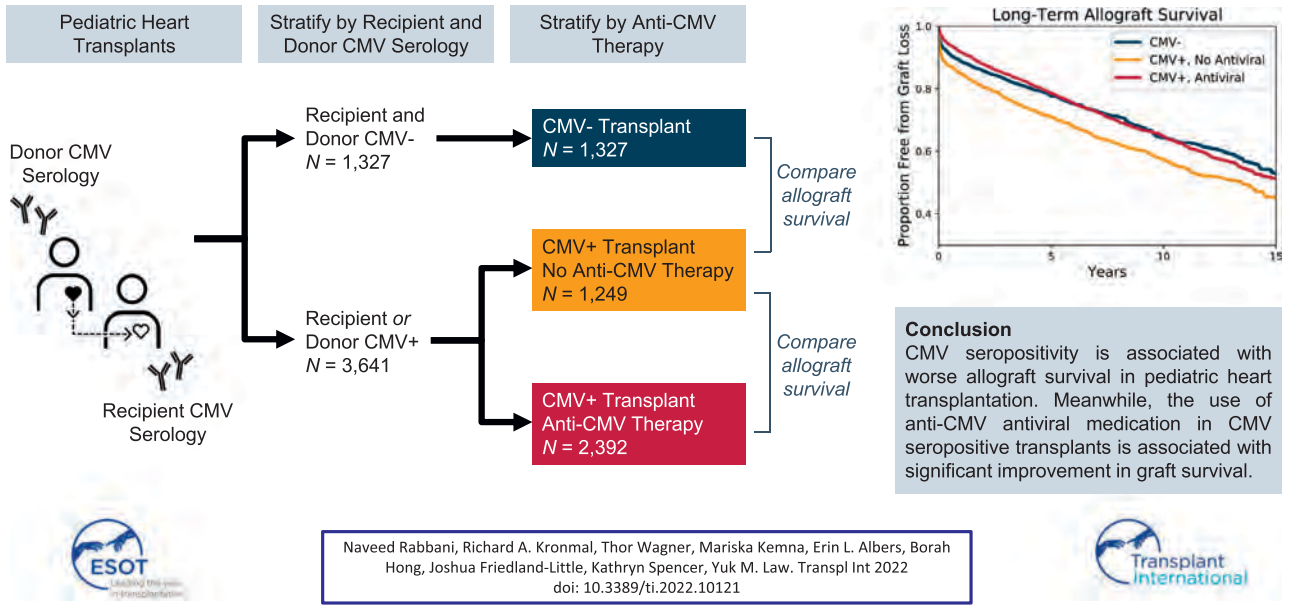
Received: *18 October 2021*
Accepted: *10 January 2022*
Published: *16 March 2022*

Citation:
Rabbani N, Kronmal RA, Wagner T, Kemna M, Albers EL, Hong B, Friedland-Little J, Spencer K and Law YM (2022) Association Between Cytomegalovirus Serostatus, Antiviral Therapy, and Allograft Survival in Pediatric Heart Transplantation. Transpl Int 35:10121. doi: 10.3389/ti.2022.10121

Abbreviations: CI, Confidence Interval; CMV, Cytomegalovirus; ECMO, Extracorporeal Membrane Oxygenation; SRTR, Scientific Registry of Transplant Recipients; SD, Standard Deviation; IQR, Interquartile Range.



GRAPHICAL ABSTRACT |

INTRODUCTION

Cytomegalovirus (CMV) infection is a common complication after heart transplantation (1). There is growing evidence that in addition to causing acute illness, CMV infection also contributes to cardiac allograft vasculopathy and long-term graft loss in adult heart transplant recipients (2–6). CMV infection may be associated with poor outcomes in pediatric recipients as well, but the data is limited and conflicting (7–9).

Large, high-quality studies from the 1990s established that anti-CMV treatment following transplantation reduces the risk for acute CMV illness (10) as well as cardiac allograft vasculopathy in adult recipients (11). This has been the main motivation for the use of CMV prophylaxis in heart transplant recipients. However, there is not yet a consensus, particularly in pediatric heart transplantation, regarding which patients should receive post-transplantation antiviral therapy.

Traditionally, risk for acute CMV infection is stratified by donor (D) and recipient (R) serostatus combination, with D+/R– considered to be the highest risk. Thus, these patients were the first to widely receive CMV prophylaxis. However, there is some evidence that anti-CMV therapy may be beneficial in all CMV seropositive transplants, regardless of whether the recipient or donor is positive (12–15).

In order to advance post-transplant antiviral practice in pediatric heart transplant, we sought to better characterize both the impact of CMV serostatus and CMV antiviral therapy on graft survival. We present the findings of a large-scale cohort study using the Scientific Registry of Transplant Recipients (SRTR) to answer these two questions.

MATERIAL AND METHODS

We performed a cohort study of pediatric heart transplants using de-identified data from the SRTR database. The SRTR data system includes data on all donor, wait-listed candidates, and transplant recipients in the United States, submitted by the members of the Organ Procurement and Transplantation Network (OPTN). The Health Resources and Services Administration (HRSA), U.S. Department of Health and Human Services provides oversight to the activities of the OPTN and SRTR contractors.

Our cohort included patients younger than 21 years of age at time of transplant who underwent solitary primary heart transplantation in the United States between 1987 and March 30, 2015. Follow-up information was available through August 2019 with a median follow-up time of 7 years. The primary outcome for analysis was graft loss, as defined by either death or retransplantation.

Transplants occurring on or after March 31, 2015, were excluded, since questions regarding CMV serology and CMV antiviral therapy were no longer included in the SRTR data collection from that point onward. Retransplantation and multi-organ transplants were excluded. Any transplants with missing CMV serology status for donor or recipient were excluded (**Figure 1**). A transplant was deemed to be CMV serostatus positive if either the donor and/or recipient had positive CMV serologies. A patient was considered to have received CMV antiviral therapy if the registry indicated that the patient received either ganciclovir or valganciclovir after transplantation. An age threshold of 21 years was chosen to

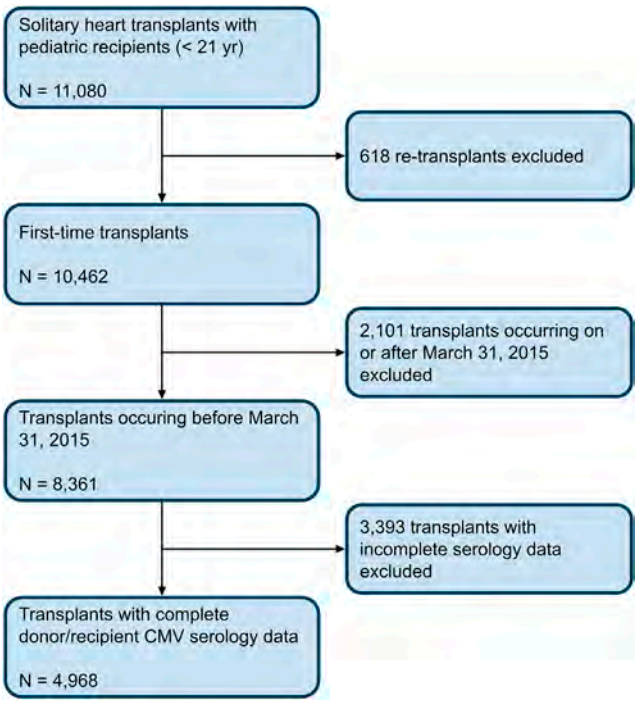


FIGURE 1 | Cohort Selection Criteria. Flowchart depicting the inclusion criteria used to select the cohort of transplants from the 2019 SRTR database.

match our own clinical practice. At our pediatric institution, we regularly perform heart transplants on young adult patients, many of whom are diagnosed with heart disease as children and cared for accordingly by our pediatric transplant team.

Several covariates, including recipient and donor demographic information and medical history were extracted from the database, and are summarized in the following section of this manuscript. Post-transplant dialysis was specifically included since renal failure after transplantation may be a relative contraindication to antiviral use. Year of transplant was also included to account for era effect. Candidates with adult listing status were converted to an equivalent pediatric status and all pediatric statuses were simplified to status 1 or 2. Recipients were deemed to have congenital heart disease if any of the following fields in the database were marked: valvular heart disease, congenital heart defect, hypoplastic left heart syndrome, congenital heart defect with surgery, or congenital heart defect without surgery. The field for anti-CMV immunoglobulin therapy was sparsely populated, and recipients were assumed to not have received this treatment unless explicitly indicated in the database.

Kaplan-Meier survival models were created to estimate overall graft survival in the entire cohort as well as by donor-recipient CMV serostatus combination groups (D+/R+, D+/R-, D-/R+, D-/R-). Amongst each of these four groups, additional survival models and pairwise log-rank tests were calculated comparing graft survival in recipients who received antiviral therapy to those who did not.

To better characterize the relationship between CMV serology status, antiviral therapy, and graft survival, recipients were then stratified into three groups: recipients of CMV serostatus negative transplants (CMV-, defined as D-/R- transplants), recipients of CMV serostatus positive transplants (CMV+, defined as D+/R+, D+/R-, and D-/R+ transplants) who did not receive antiviral therapy, and recipients of CMV+ transplants who received antiviral therapy. This stratification allows one to separate the effect of CMV positivity and antiviral therapy, which are often confounded. CMV- transplants were not further separated by antiviral use since antiviral therapy was not expected to have an effect on graft survival in these transplants. This assumption is later confirmed by the donor-recipient subgroup analysis and is discussed in further detail in the Results section.

Transplant characteristics and summary statistics were computed across these three groups. Kaplan-Meier survival curves were then calculated comparing graft survival. A multivariable Cox proportional hazards model was created using the trichotomous stratification above in addition to clinically relevant covariates, which were pared down by backwards elimination to include only statistically significant predictors. In order to estimate the effect of untreated CMV seropositivity on graft loss, a hazard ratio was calculated comparing CMV negativity to CMV positivity without antiviral therapy. Additionally, to estimate the effect of antiviral use on graft loss, a hazard ratio was calculated comparing CMV positivity without antiviral therapy to CMV positivity with antiviral therapy.

To further control for potential confounding, the multivariable model above was also recalculated with the addition of a propensity score estimating the probability of antiviral use amongst CMV+ transplants. The propensity score was computed using a logistic regression model whose components were selected from the same pool of clinical covariates above and pared down via backwards elimination to include only statistically significant predictors.

A second multivariable Cox proportional hazards model was created to estimate the risk of graft loss occurring within the first year after transplant. Covariates were again selected by backwards elimination and a model was created both with and without the antiviral use propensity score. Using the same methodology as above, a hazard ratio was calculated by contrasting CMV- transplants to CMV+ transplants without antiviral therapy and by contrasting CMV+ transplants that did not receive therapy to CMV+ transplants that did. In order to assess for the possibility of selection bias for those who survived the early post-operative period, this 1-year survival model was also recalculated excluding recipients who had a graft loss event within the first week after transplantation.

The dataset was prepared using Python 2.7 with the PANDAS library (version 0.24.2) (16). Summary statistics and Kaplan-Meier survival curves were created using Python 2.7 with the SciPy (version 1.2.0) and LifeLines (version 0.19.5) libraries (17,18). Cox proportional hazards models and propensity scores were computed using STATA 15. *p*-values less than 0.05 were considered statistically significant. The study was approved by Seattle Children's Institutional Review Board (approval number STUDY00002063, protocol HRP-503B).

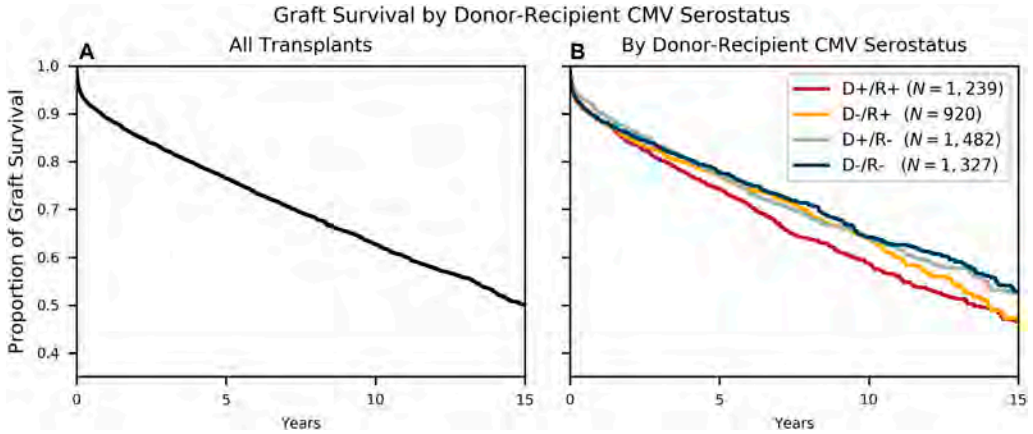


FIGURE 2 | Survival by Donor-Recipient Serostatus. Kaplan-Meier survival curves modeling freedom from allograft loss **(A)** for the entire cohort and **(B)** stratified by the four donor-recipient serostatus combinations.

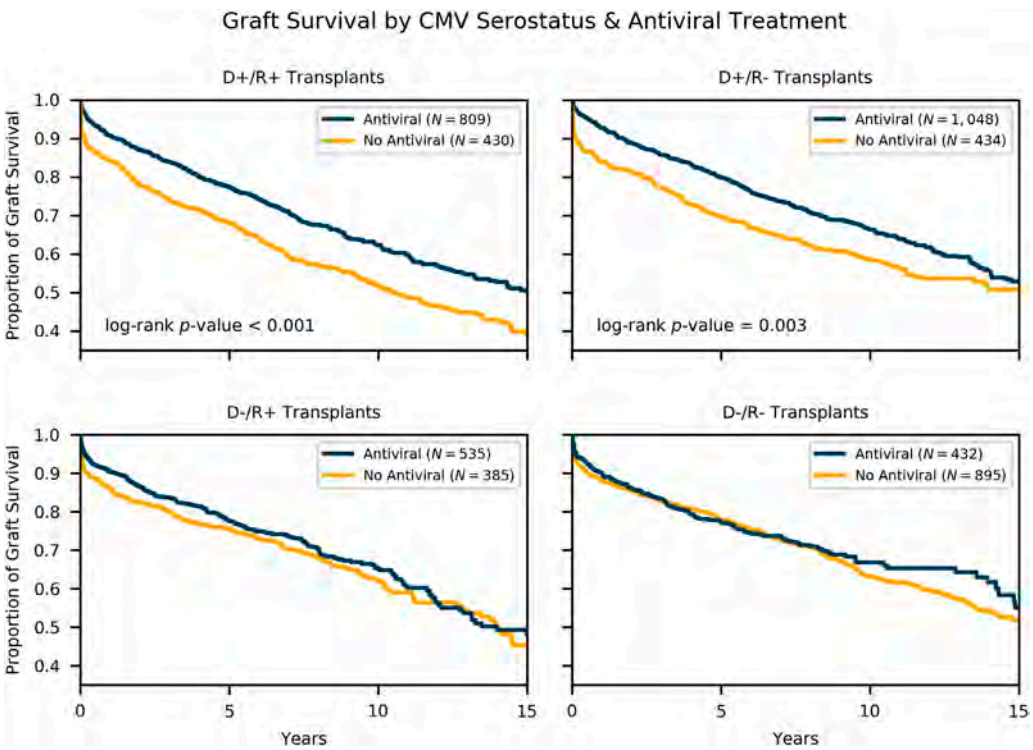


FIGURE 3 | Survival by Donor-Recipient Serostatus and Antiviral Therapy. Kaplan-Meier survival estimates of freedom from allograft loss comparing recipients who received antiviral therapy to those who did not, stratified by donor-recipient serology combination. In this subgroup analysis, antiviral therapy was associated with a statistically significant improved survival in D+/R+ and D+/R- subgroups.

RESULTS

A total of 8,361 patients younger than 21 years of age underwent primary, solitary heart transplantation in the United States between May 25, 1987 and March 30, 2015. Of these, 4,968 had complete CMV serology data available and were included in the final analysis. The median transplant year was 2008, with 4,755 transplants (96%) occurring during or after the year 2000. There were 1,239 D+/R+ transplants, 1,482 D+/R- transplants, 920 D-/R+ transplants, and 1,327 D-/R- transplants. Within these groups, the proportion of CMV antiviral use was 65, 71, 58, and 33% respectively. Of all included transplants, 350 (7%) ended in retransplant and 1,544 (31%) ended in death, for a total of 1,894 (38%) graft loss events.

TABLE 1 | Demographic and clinical characteristics stratified by CMV serostatus and antiviral use.

	CMV-	CMV+, No Antiviral	CMV+, Antiviral	All	N
	N = 1,327	*N = 1,249*	*N = 2,392*	*N = 4,968*	
Transplant Outcomes					
Retransplant	85 (6%)	95 (8%)	170 (7%)	350 (7%)	4,968
Death	379 (29%)	471 (38%)	694 (29%)	1,544 (31%)	4,968
Graft Loss (Retransplant or Deazh)	464 (35%)	566 (45%)	864 (36%)	1,894 (38%)	4,968
Transplant Year (median ± i.q.r.)	2009 ± 8 years	2007 ± 8 years	2008 ± 8 years	2008 ± 8 years	4,968
ABO Incompatibility	44 (3%)	33 (3%)	57 (2%)	134 (3%)	4,968
Ischemic Time (min) (mean ± s.d.)	218 ± 71	212 ± 72	217 ± 74	216 ± 73	4,787
Post-Transplant Dialysis	74 (6%)	89 (7%)	145 (6%)	308 (6%)	4,968
D:R Weight Ratio (mean ± s.d.)	1.4 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	4,967
D:R Height Ratio (mean ± s.d.)	1.1 ± 0.2	1.1 ± 0.4	1.1 ± 0.2	1.1 ± 0.3	4,944
Recipient Characteristics					
Age					4,968
<1 year	392 (30%)	373 (30%)	556 (23%)	1,321 (27%)	
1–3 years	229 (17%)	158 (13%)	256 (11%)	643 (13%)	
3–6 years	143 (11%)	78 (6%)	221 (9%)	442 (9%)	
6–12 years	191 (14%)	195 (16%)	383 (16%)	769 (15%)	
>12 years	372 (28%)	445 (36%)	976 (41%)	1,793 (36%)	
Gender (Male)	742 (56%)	677 (54%)	1,336 (56%)	2,755 (55%)	4,968
Race					4,968
White	1,005 (76%)	846 (68%)	1,742 (73%)	3,593 (72%)	
Black	247 (19%)	328 (26%)	476 (20%)	1,051 (21%)	
Other	75 (6%)	75 (6%)	174 (7%)	324 (7%)	
CMV+ Serology Status	0	815 (65%)	1,344 (56%)	2,159 (43%)	4,968
Antiviral Therapy	432 (33%)	0	2,392 (100%)	2,824 (57%)	4,968
Anti-CMV Ig Therapy	58 (4%)	73 (6%)	479 (20%)	610 (12%)	4,968
Listing Status					4,963
Status 1	1,193 (90%)	1,130 (90%)	2,149 (90%)	4,472 (90%)	
Status 2	134 (10%)	117 (9%)	240 (10%)	491 (10%)	
Congenital Heart Disease	632 (48%)	525 (42%)	958 (40%)	2,115 (43%)	4,968
Cardiothoracic Surgery	385 (29%)	307 (25%)	673 (28%)	1,365 (27%)	4,968
Pre-Transplant Dialysis	21 (2%)	41 (3%)	64 (3%)	126 (3%)	4,943
ECMO	59 (4%)	102 (8%)	116 (5%)	277 (6%)	4,968
Donor Characteristics					
Age					4,968
<1 years	317 (24%)	315 (25%)	424 (18%)	1,056 (21%)	
1–3 years	252 (19%)	173 (14%)	347 (15%)	772 (16%)	
3–6 years	148 (11%)	111 (9%)	199 (8%)	458 (9%)	
6–12 years	201 (15%)	172 (14%)	317 (13%)	690 (14%)	
>12 years	409 (31%)	478 (38%)	1,105 (46%)	1,992 (40%)	
Gender (Male)	799 (60%)	738 (59%)	1,381 (58%)	2,918 (59%)	4,968
Race					4,965
White	1,019 (77%)	936 (75%)	1,832 (77%)	3,787 (76%)	
Black	270 (20%)	277 (22%)	486 (20%)	1,033 (21%)	
Other	36 (3%)	36 (3%)	73 (3%)	145 (3%)	
CMV+ Serology Status	0	864 (69%)	1,857 (78%)	2,721 (55%)	4,968
Diabetes	17 (1%)	4 (0%)	17 (1%)	38 (1%)	4,955
Hypertension	21 (2%)	22 (2%)	55 (2%)	98 (2%)	4,953

The overall estimated 10-year cohort graft survival rate was 63%. Subsequent Kaplan-Meier survival models stratified by donor-recipient CMV serostatus showed a 10-year graft survival of 59% for D+/R+ transplants and 64% 10-year survival for the other three groups (**Figure 2**).

For each of the four CMV serology groups, Kaplan-Meier survival curves were calculated comparing graft survival in those recipients who received CMV antiviral therapy to those who did not (**Figure 3**). Antiviral therapy was associated with improved freedom from graft loss in D+/R+ transplants (10-year survival of 62 vs 52%, log-rank p-value < .001) and D+/R- transplants (10-year survival of 66 vs 59%, log-rank p-value = 0.003). The difference in survival was observed early after transplantation and holds throughout the follow up period. For D-/R+ transplants, there is early separation between the curves, however, the log-rank test is not significant. As expected, the D-/R- survival plots showed no appreciable difference between the two treatment groups. Importantly, these survival curves demonstrate that amongst D+ transplants, recipients who received antiviral therapy achieved similar overall graft survival compared to recipients of CMV- transplants.

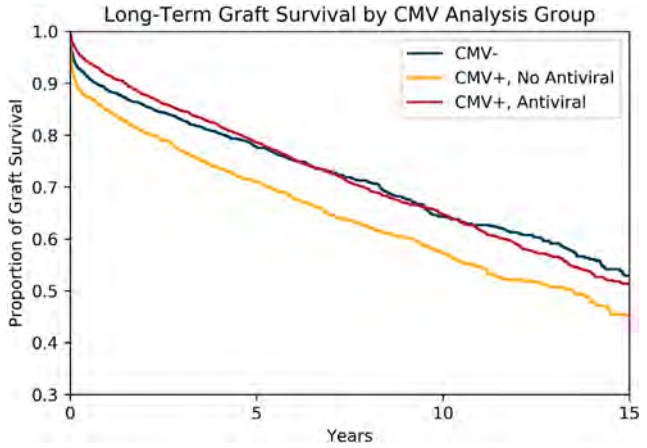


FIGURE 4 | Survival of CMV- Transplants Compared to CMV+ Transplants with and without Antiviral Therapy. Kaplan-Meier survival curves modeling freedom from allograft loss amongst CMV- transplants, CMV+ transplants without antiviral therapy, and CMV+ transplants with antiviral therapy. CMV+ transplants with antiviral therapy demonstrated similar overall graft survival when compared to CMV- transplants.

Further analysis was done comparing CMV- transplants to CMV+ transplants without antiviral therapy and subsequently CMV+ transplants without antiviral therapy to CMV+ transplants with antiviral therapy. This method allows one to separate the effects of CMV serostatus positivity and antiviral therapy in a multivariable risk regression model. These are exposures that are otherwise strongly correlated and confounded. When all eight donor-recipient-antiviral combinations were included in this multivariable model, no additional predictive value was achieved, which is further evidence that the three-group analysis is sufficient to describe the association between CMV serostatus, CMV antiviral therapy, and graft loss.

Table 1 summarizes the donor, recipient, and transplant characteristics that were used to create the adjusted multivariable risk models. There were 1,327 CMV- transplants, 1,249 CMV+ transplants without antiviral therapy, and 2,392 CMV+ transplants with antiviral therapy.

Kaplan-Meier curves (**Figure 4**) comparing graft survival across the three groups showed that amongst recipients of CMV+ transplants, those who received antiviral therapy had significantly improved graft survival compared to those who did not (at 10-year 65 vs 57%, log-rank *p*-value < .001). The difference in graft survival between the two groups was observed early after transplantation. Recipients of CMV+ transplants who received antiviral therapy achieved similar rates of long-term graft survival as recipients of CMV- transplants.

In the unadjusted Cox proportional hazards model, when compared to CMV- transplants, CMV positivity without antiviral therapy had a hazard ratio of 1.34 (*p*-value < .001, 95% CI 1.18–1.51). In a fully-adjusted multivariable model, this hazard ratio was 1.21 (*p*-value = 0.003, 95% CI 1.07–1.37). Meanwhile, in the unadjusted model, antiviral use amongst CMV+ transplants had a hazard ratio of .77 (*p*-value < .001, 95% CI 0.69–0.86) when compared to CMV+ transplants that did not receive antiviral therapy. In the fully-adjusted model, this hazard ratio was 0.82 (*p*-value < .001, 95% CI .74–.92). These hazard ratios changed minimally with the addition of an antiviral use propensity score to the model (**Table 2**).

Other significant risk factors from the multivariable model included post-transplant dialysis, donor age, donor male gender, recipient congenital heart disease, recipient ECMO, recipient prior cardiothoracic surgery, and SRTR-reported recipient race of Black. Factors associated with improved allograft survival included later transplant year, recipient male gender, higher donor-recipient weight ratio, and donor history of hypertension. Anti-CMV immunoglobulin was not statistically significantly associated with graft survival. The complete results of the fully-adjusted multivariable model are summarized in **Supplementary Table 1**.

As noted in the Kaplan-Meier survival curves, the difference in graft survival between the three groups was observed early after transplantation. Furthermore, across the entire observation period, the test for deviation from proportional hazards was highly significant (*p*-value < .001).

Therefore, subsequent analysis focused on the first year after transplantation. Within that time period, the proportion of graft loss was 11% in CMV- transplants, 15% in CMV+ transplants without antiviral therapy, and 8.4% in CMV+ transplants with antiviral therapy. This translates to an absolute difference in graft loss within the first year of +4% for CMV seropositivity without antiviral use (compared to CMV negativity) and −6.6% for antiviral use in CMV+ transplants.

In the unadjusted Cox proportional hazards model estimating graft loss within the first year, when compared to CMV- transplants, CMV positivity without antiviral use had a hazard ratio of 1.40 (*p*-value = .002, 95% CI 1.13–1.74). Antiviral use amongst CMV+ transplants had a hazard ratio of 0.52 (*p*-value < .001, 95% CI .43–.64). In the fully-adjusted model, these hazard ratios were 1.32 (*p*-value = .014, 95% CI 1.06–1.64) and .59 (*p*-value < .001, 95% CI 0.48–0.73), respectively. These hazard ratios changed minimally with the addition of an antiviral use propensity score to the model (**Table 3**). In this model, factors

TABLE 2 | Hazard ratios for CMV seropositivity and antiviral therapy.

	Unadjusted			Adjusted			Adjusted model with propensity score		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
CMV positivity without antiviral therapy	1.34	1.18–1.51	<0.001	1.21	1.07–1.37	0.003	1.25	1.10–1.42	0.001
Antiviral therapy in CMV+ transplants	0.77	0.69–0.86	<0.001	0.82	0.74–0.92	<0.001	0.82	0.73–0.92	<0.001

TABLE 3 | Hazard ratios of graft loss within the first year after transplantation for CMV seropositivity and antiviral therapy.

	Unadjusted			Adjusted			Adjusted model with propensity score		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value	HR	95% CI	*p*-value
CMV positivity without antiviral therapy	1.40	1.13–1.74	0.002	1.32	1.06–1.64	0.014	1.39	1.10–1.74	0.005
Antiviral therapy in CMV+ transplants	0.52	0.43–0.64	<0.001	0.59	0.48–0.73	<0.001	0.61	0.49–0.75	<0.001

significantly associated with graft loss included post-transplant dialysis, recipient congenital heart disease, recipient history of cardiothoracic surgery, recipient ECMO, and SRTR-reported recipient race of Black. Factors associated with improved graft survival included later transplant year, recipient age, and recipient male gender. Complete results of this fully-adjusted multivariable model are summarized in **Supplementary Table 2**. Finally, when excluding recipients who experienced graft loss within the first week of transplant from the model, the hazard ratios remained significant at 1.33 (p-value = .024, 95% CI 1.04–1.71) and .68 (p-value < 0.001, 95% CI 0.54–0.85), respectively.

DISCUSSION

This longitudinal cohort study of a large, national database of pediatric heart transplants demonstrates that CMV seropositivity (recipient or donor) is associated with decreased graft survival time in recipients who did not receive CMV antiviral therapy after transplant. Furthermore, it demonstrates that the use of CMV antiviral medication with either ganciclovir or valganciclovir in CMV seropositive transplants is associated with a significant improvement in graft survival. This relationship is observed early after transplant.

CMV is a herpesvirus that leads to persistent latent infection after resolution of acute illness. History of CMV infection is common in the general population, and in immunocompetent hosts is usually clinically insignificant (19). However, CMV infection can cause serious morbidity in immunocompromised persons and is of particular importance in transplant recipients. Acute illness can occur through first-time exposure to the virus or reactivation of latent infection. There is also growing evidence that in addition to acute illness, CMV also contributes to graft loss in transplant recipients through longer-term effects such as the development of cardiac allograft vasculopathy (2–6).

Traditionally, the risk for acute CMV infection is stratified by donor (D) and recipient (R) serostatus combination, with D+/R- considered to be the highest risk. Thus, these patients are more likely to receive anti-CMV medication. Although this pattern of risk has been observed in several studies (9,20), other studies have casted doubt on this conventional wisdom. For example, one transplant center observed that D+/R+ transplants actually had the highest risk for CMV infection in their prospective cohort of pediatric heart transplant recipients (21).

There have been only a few attempts at estimating the relationship between CMV seropositivity and CMV antiviral therapy on graft survival. Such studies in pediatric transplantation have yielded conflicting results. For example, a study of pediatric heart transplant recipients at a single institution by Hussain *et al.* found that recipient CMV seropositivity was significantly associated with the development of cardiac allograft vasculopathy and decreased graft survival (22). In this cohort, CMV antiviral use was too infrequent to adequately analyze. On the other hand, analysis of an earlier version of the SRTR database by Snydman *et al.* demonstrated a positive association between CMV antiviral therapy and graft survival (13). However, this study was unable to demonstrate a statistically significant association between CMV serostatus and graft survival.

Meanwhile, a large study of the Pediatric Heart Transplant Society (PHTS) database by Mahle *et al.* failed to show any association between CMV serostatus and graft survival or between CMV antiviral therapy and survival (9), and an analysis of pediatric recipients in the Registry of the International Society for Heart and Lung Transplantation (ISHLT) also found no association between donor-recipient CMV serology mismatch and 1-year mortality (23).

One explanation for such inconsistent results is that the use of antiviral therapy is naturally associated with CMV seropositivity. Therefore, it is possible that a potentially detrimental effect of CMV seropositivity and a potentially favorable effect of CMV antiviral therapy may negatively confound each other, making the true underlying impact of these exposures difficult to detect. This is the reason for our three-group analysis, which allows one to statistically quantify the relationship between CMV seropositivity (without antiviral treatment) and graft survival as well as the relationship between antiviral therapy amongst CMV+ transplants and graft survival. Furthermore, considering all CMV+ transplants together, regardless of whether the recipient or the donor is positive, also reflects the growing practice of treating all donor or recipient seropositive transplants with CMV prophylaxis (24,25).

Our analysis reveals that CMV serostatus positivity without antiviral therapy has a significant association with decreased graft survival when compared to CMV seronegative transplants. The separation in the survival curves between the groups is observed early after transplant. An adjusted model estimating the risk of graft loss in the first year after transplant shows that CMV positivity without antiviral therapy has a hazard ratio of 1.32 when compared to CMV- transplants.

The hazard ratio of graft loss during that same time period for antiviral therapy amongst CMV+ transplants was .59. Meanwhile, the unadjusted absolute difference in graft loss between treated and untreated CMV seropositive transplants was −6.6% after 1 year, a substantial difference for the field of pediatric heart transplantation.

These findings seem to indicate that CMV serostatus positivity in either the donor or recipient is a significant risk factor for post-

transplant graft loss, with a survival difference that is observed unexpectedly early after transplantation. These are important and novel observations from this multicenter cohort study of pediatric heart transplant recipients. Perhaps more importantly, this study also demonstrates that the risk of CMV serostatus positivity appears to be mitigated by antiviral therapy.

Additional subgroup analysis of all four donor-recipient CMV serostatus combinations showed the largest effect of antiviral therapy was observed in donor seropositive transplants (for both seropositive and seronegative recipients). These findings suggest that recipient CMV serostatus positivity may not be as protective as previously believed. Altogether this evidence supports the more widespread use of CMV prophylaxis beyond the traditionally high-risk D+/R- mismatched transplants.

Although a cohort study cannot determine the mechanism underlying the observed relationships, one theory to explain both the magnitude and early timing of graft loss is that a cardiovascular-tropic virus such as CMV may promote early graft failure in the setting of procurement injury and intense immunosuppression. For example, latent CMV infection residing in the graft or recipient endothelium may potentiate procurement and reperfusion injury leading to additional ischemia, graft dysfunction, or rejection in the already pro-inflammatory post-transplant state. Regardless, the results of this study support the need for future investigation into the biological mechanisms of CMV-mediated graft loss and additional studies aimed at the optimization of post-transplantation antiviral regimens.

This registry-based cohort study has inherent limitations. Importantly, the details of dosing, timing, and duration of post-transplant CMV prophylaxis, which varies between centers, is not captured by the binary fields of the SRTR registry. There is also no data on CMV viral load to assess for viremia. Furthermore, the database contains some fields with incomplete data and the questionnaire-based data submission process itself can be prone to errors or oversimplification of clinical details. Another important limitation is that due to incomplete cause of death data in this registry, we were unable to further investigate the relationship between CMV serostatus, antiviral therapy, and specific causes of graft loss, such as rejection, infection, primary graft failure, multiorgan failure, or cardiac allograft vasculopathy, which could have provided more information as to the etiology of CMV-associated morbidity. The interpretation of CMV serology status also has its own limitations. CMV serology status may be falsely positive from exposure to blood products, which are commonly used in heart failure patients. Infant serology status is also limited by the possibility of positivity from passively-acquired maternal antibodies (15).

Finally, like all observational studies, there may be unmeasured confounders that could explain the observed associations. However, thorough analysis was done to address potential sources of bias by considering demographic information, era (through inclusion of transplant year), and conventional clinical characteristics in our models. Multivariable models were also adjusted by a propensity score estimating the use of antiviral medication as well as the occurrence of post-transplant dialysis, since renal failure may delay or limit the use of antiviral medication. In order to minimize potential selection bias (e.g., the antiviral use variable may be inadvertently selecting those recipients who survived long enough to receive treatment) an additional model was calculated excluding those recipients with allograft loss within the first week after transplant. This analysis demonstrated that these additional factors had little effect on the strength of the association between CMV serostatus, CMV antiviral therapy, and risk of graft loss.

CONCLUSION

This large-scale analysis of a multi-institutional national database of pediatric heart transplant recipients demonstrates that CMV serostatus positivity, as defined by either donor or recipient positivity, is associated with an increased risk of graft loss that is largely observed early after transplantation in recipients who are not treated with CMV antiviral therapy. Additionally, this study shows that the use of CMV antiviral therapy amongst CMV seropositive transplants is associated with a significant improvement in graft survival. When treated with CMV antiviral therapy, recipients of CMV seropositive transplants experienced similar graft survival times as recipients of seronegative transplants. These findings suggest that patients involved in a CMV serostatus positive transplant with either the donor *or* recipient being CMV+ may benefit from CMV antiviral medication after transplantation. Of course it is important to recognize that these findings are limited by the observational and registry-based nature of the study and do not embody all of the complexity of the medical management of heart transplant patients. However, this serves as strong motivation for future studies into the mechanisms behind CMV-mediated allograft loss and prospective studies aimed at optimizing post-transplant antiviral regimens.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.srtr.org/about-the-data/the-srtr-database.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Seattle Children's Hospital IRB. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NR, RK, and YL conceived and designed the study. NR prepared the data. NR, RK, and YL performed the analysis and interpretation of results. NR prepared the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content and provided final approval for its submission.

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

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ORIGINAL RESEARCH
published: 21 March 2022
doi: 10.3389/ti.2022.10122

Donor-Derived Cell-Free DNA for the Detection of Heart Allograft Injury: The Impact of the Timing of the Liquid Biopsy

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Background: In heart transplant recipients, donor-derived cell-free DNA (ddcfDNA) is a potential biomarker for acute rejection (AR), in that increased values may indicate rejection. For the assessment of ddcfDNA as new biomarker for rejection, blood plasma sampling around the endomyocardial biopsy (EMB) seems a practical approach. To evaluate the effect of the EMB procedure on ddcfDNA values, ddcfDNA values before the EMB were pairwise compared to ddcfDNA values after the EMB. We aimed at evaluating whether it matters whether the ddcfDNA sampling is done before or after the EMB-procedure.

Methods: Plasma samples from heart transplant recipients were obtained pre-EMB and post-EMB. A droplet digital PCR method was used for measuring ddcfDNA, making use of single-nucleotide polymorphisms that allowed both relative quantification, as well as absolute quantification of ddcfDNA.

Results: Pairwise comparison of ddcfDNA values pre-EMB with post-EMB samples (n = 113) showed significantly increased ddcfDNA concentrations and ddcfDNA% in post-EMB samples: an average 1.28-fold increase in ddcfDNA concentrations and a 1.31-fold increase in ddcfDNA% was observed (p = 0.007 and p = 0.03, respectively).

Conclusion: The EMB procedure causes iatrogenic injury to the allograft that results in an increase in ddcfDNA% and ddcfDNA concentrations. For the assessment of ddcfDNA as marker for AR, collection of plasma samples before the EMB procedure is therefore essential.

Keywords: liquid biopsy, ddcfDNA, cfDNA, heart transplantation, endomyocardial biopsy

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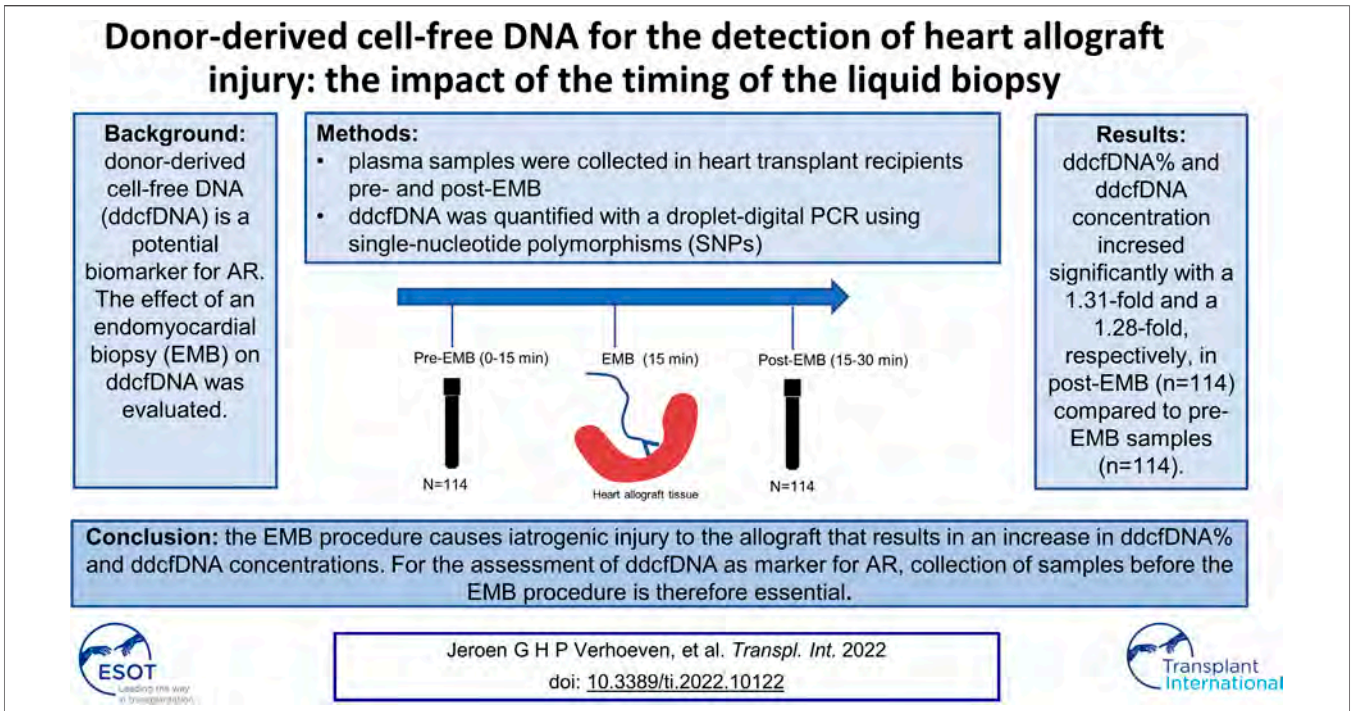
Received: *18 October 2021*
Accepted: *16 February 2022*
Published: *21 March 2022*

Citation:
Verhoeven JGHP, Hesselink DA, Peeters AMA, de Jonge E, von der Thüsen JH, van Schaik RHN, Matic M, Baan CC, Manintveld OC and Boer K (2022) Donor-Derived Cell-Free DNA for the Detection of Heart Allograft Injury: The Impact of the Timing of the Liquid Biopsy. Transpl Int 35:10122. doi: 10.3389/ti.2022.10122



GRAPHICAL ABSTRACT |

INTRODUCTION

Heart transplant recipients are monitored for acute rejection (AR) by a strict endomyocardial biopsy (EMB) surveillance scheme. Histopathological examination of an EMB is currently the gold standard for diagnosing AR. However, this procedure is invasive, costly and can result in several complications, including coronary artery fistula formation and tricuspid regurgitation (1). Moreover, the diagnosis of AR may be missed as a result of sampling error. Finally, considerable variability exists in the interpretation of an EMB between pathologists (2). There is thus an unmet need for minimally-invasive biomarkers to timely diagnose heart transplant rejection.

Donor-derived cell-free DNA (ddcfDNA) is a promising biomarker that could improve AR monitoring in heart transplant recipients (3–7). ddcfDNA is highly fragmented DNA derived from apoptotic and necrotic cells (8). Based on genetic differences between the donor and recipient, such as single nucleotide polymorphisms (SNPs) or insertion and deletion variations of DNA sequences, it is possible to specifically detect donor cfDNA in blood plasma in a background of recipient cfDNA. The release of ddcfDNA especially occurs at times of allograft injury, including AR. Increased values of ddcfDNA were observed during high-grade heart transplant rejection (3, 4, 6, 7).

An EMB procedure itself also causes allograft injury that may result in an increase in ddcfDNA. Therefore, it is important to establish whether the timing of sampling is important for the interpretation of the ddcfDNA values.

DdcfDNA can be quantified as fraction (% ddcfDNA of total cfDNA) or as absolute concentration (copies/ml plasma). So far, in heart transplant recipients, studies mainly focussed on ddcfDNA% and not on concentration. An important limitation of ddcfDNA% is that values may be affected by fluctuations in recipient cfDNA, the denominator in the calculation of ddcfDNA%. These fluctuations in recipient cfDNA occur both during physiological conditions (9, 10), as well as pathological conditions, including infection and cancer (11, 12), that occur frequently in heart transplant recipients (13). For this reason, using ddcfDNA concentration might be more accurate to avoid the variability of ddcfDNA% (14). Additionally, the EMB procedure might not only affect the level of donor cfDNA but also of the recipient cfDNA. This implies that a potential effect of an EMB procedure on ddcfDNA% might be different in magnitude than for ddcfDNA concentration. Therefore, it is important to assess both values.

This present study aims 1) to determine the effect of the EMB procedure on plasma ddcfDNA and; 2) to assess both ddcfDNA% and ddcfDNA concentration (not subject to fluctuations in recipient cfDNA).

MATERIALS AND METHODS

Study Design

Adult heart transplant recipients who were scheduled for an EMB were eligible for participation in this clinical study that was performed at the Erasmus MC, University Medical Center,

TABLE 1 | Baseline characteristics.

Baseline characteristics	Study population (*n* = 15)
Patients	15
Age (years)	49 (18–63)
Female/Male	6 (40.0%)/9 (60.0%)

Continues variables are described as mean (range). Categorical variables as number of cases (%).

TABLE 2 | Biopsy results.

Biopsy result and classification	Biopsies (*n* = 113)
ACR 0, ACR 1, AMR 0	111
ACR 2	2
AMR 2	0

Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection.

Rotterdam, the Netherlands. The study was approved by the institutional review board of the Erasmus MC (Medical MEC-Review Board number 2017-196) and recipients gave written informed consent prior to participation. The study was conducted in accordance with the principles of the Declaration of Helsinki, consistent with the Good Clinical Practice guidelines of the International Conference on Harmonization.

Clinical Sample Collection and Processing

Blood samples were collected from heart transplant recipients who underwent routine surveillance EMB. Samples were collected immediately before (<15 min pre-biopsy) and immediately after the biopsy procedure (<15 min post-biopsy). The EMB was performed via the jugular vein with a bioptome size of 7 French. In the early post-transplant phase, routine EMB was performed weekly for the first 2 months, monthly for the next 4 months, and then every 3 months.

Ten milliliters of blood was collected in anti-coagulated CellSave blood collection tubes (Menarini, Florence, Italy). Samples were stored at 4°C within 3 h after collection. The plasma was separated by centrifugation at 1,600 × g for 20 min within 24 h after collection, and stored at −30°C.

DNA Isolation and Single Nucleotide Polymorphism Genotyping

Genomic DNA from recipients was obtained from peripheral blood mononuclear cells, and DNA from their corresponding donor was obtained from either spleen cells or heart transplant tissue (collected with routine surveillance of transplant rejection from an EMB) by automated purification (Maxwell, Promega, Leiden, Netherlands). According to Dutch law, spleen cells are considered as left over material. Therefore, for the use of these spleen cells, no informed consent of donors was necessary. Recipients and donors were genotyped by using an in house designed panel of 10 preselected SNPs by a quantitative PCR (Applied Biosystems™ QuantStudio™, Foster City, CA,

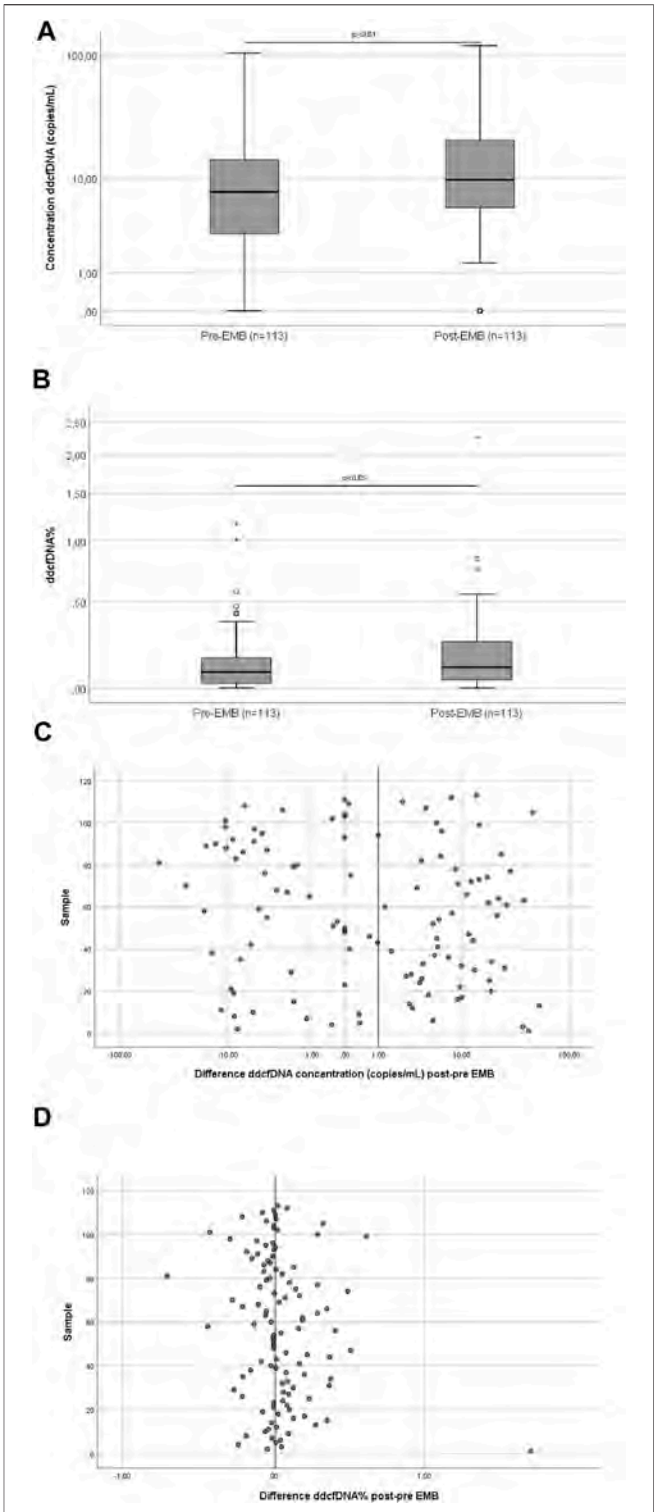


FIGURE 1 | Pairwise comparison of ddcfDNA concentration **(A)** and ddcfDNA % **(B)** and absolute differences in ddcfDNA concentration **(C)** and ddcfDNA% **(D)** of samples taken before and after the EMB procedure. The middle line of the box represents the median and the upper and lower borders of the box represent the 25 and 75% percentile. Whiskers represent the 5th–95th percentile and the small circles represent outliers **(A,B)**. The vertical line on the x-axis represents the median differences **(C,D)**. Abbreviations: EMB, endomyocardial biopsy.

United States). Per patient, one to three discriminative SNPs were selected for ddcfDNA quantification.

cfDNA Isolation and Donor-Derived Cell-Free DNA Measurement

cfDNA was isolated from 3 ml of anti-coagulated blood plasma by using the Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The QX100 droplet digital PCR (ddPCR) system (Bio-Rad Laboratories, CA, United States) was used for the quantification of (dd)cfDNA. Samples of 20 µl were prepared for PCR reactions by making a mixture containing purified cfDNA, water, a donor specific target assay (discriminative SNP) and ddPCR Supermix for Probes (Bio-Rad). Droplets were generated with a QX100 droplet generator (Bio-Rad) according to the manufacturer's instructions. The ddPCR was performed using the T100^{TM} Thermal Cycler (Bio-Rad) with the following amplification protocol: 95°C for 10 min, 40× (94° for 30 s, 55° for 1 min), then 98°C for 10 min. The quantified droplets were analyzed through a QX100 droplet reader (Bio-Rad) using Quantasoft software version 1.0.596 (Bio-Rad). ddcfDNA values were quantified either as fraction (%) (donor-specific SNP signal/ total SNP signal (donor-specific SNP signal + non-donor-specific SNP signal)) or as concentration (copies/ml plasma). In samples were ddcfDNA was quantified with two or three SNPs, the ddcfDNA values were averaged.

Biopsy Examination

All biopsies were examined and scored according to the ISHLT grading system by an experienced transplant pathologist (JvT) (15, 16). Biopsies were classified as acute cellular rejection (ACR) grade 0R–2R and as antibody-mediated rejection (pAMR) grade 0–2.

Statistical Analysis

The primary objective of this study was to assess the effect of the EMB procedure on ddcfDNA values. IBM SPSS version 25 (Armonk, NY, United States) was used for statistical analysis of the data and for making the figures. Continuous variables are presented as median with interquartile range (first and third, IQR) for non-normally distributed data. Nonparametric data of paired samples before and after biopsy were compared pairwise using the Wilcoxon matched-pairs signed rank test. Results were considered statistically significant for two sided *p*-values below 0.05.

RESULTS

Patients and Samples

A total of 226 paired samples from 15 patients (aged 18–63 years) was collected both pre-EMB (n = 113) and post-EMB (n = 113) between November 2019 and August 2020. The paired samples was collected between day 7 and day 509 post-transplant. Patient characteristics and an overview of the biopsy results are depicted in **Tables 1**, **2**. An overview of the timing of the EMB biopsies with the ddcfDNA values and biopsy results is presented in **Supplementary Figures 1A,B**.

Effect of Endomyocardial Biopsy Procedure on Donor-Derived Cell-Free DNA

In order to assess the effect of the EMB procedure on ddcfDNA values, pre-EMB ddcfDNA values were compared with post-EMB values in the paired samples (n = 113). The median (IQR) pre-EMB ddcfDNA concentration was 7.5 (3.0–14.5) copies/ml. This concentration increased to 9.6 (5.4–20.8) copies/ml post-EMB, corresponding to a 1.28-fold increase (**Figure 1A**; p = 0.007). ddcfDNA% increased significantly from 0.08% (0.00–0.14) pre-EMB to 0.10% (0.02–0.20) post-EMB, corresponding to a 1.31-fold increase in ddcfDNA% (**Figure 1B**; p = 0.03). The absolute differences in ddcfDNA concentration and ddcfDNA% between pre- and post-EMB samples are represented in **Figures 1C,D**. There was no correlation between age (18–63 years) and fold change in both ddcfDNA% (n = 113; Spearman's correlation coefficient r = −0.02, p = 0.74) and ddcfDNA concentration (r = −0.04, p = 0.64).

DISCUSSION

The present study was performed to assess the effect of the EMB procedure on plasma ddcfDNA values. We observed an increase in ddcfDNA concentration (1.28-fold) and ddcfDNA% (1.31-fold) in post-EMB samples, compared to pre-EMB samples. This illustrates that the EMB procedure causes iatrogenic injury to the allograft.

The EMB-related effect is mild in comparison with the effect of allograft rejection on ddcfDNA values as the reported differences in ddcfDNA values between acute rejection and non-rejection seem to be more pronounced; 0.17% during acute rejection and 0.07% during non-rejection, indicating a more than 2-fold increase in ddcfDNA% which is more than the 1.31-fold increase in ddcfDNA % in post-EMB samples (4).

The use of ddcfDNA as minimally invasive biomarker for acute rejection is meant to help clinicians determine whether it is necessary to perform an invasive EMB or not. This should reduce the amount of unnecessary EMBs in heart transplant recipients. However, despite the fact that the EMB procedure slightly increases ddcfDNA values in post-EMB samples, this effect could potentially still affect the evaluation of ddcfDNA as biomarker for allograft rejection in studies.

The currently published studies for acute rejection monitoring suggest threshold values for ddcfDNA% ranging from 0.15% to 2.0% (5). For example, a previous study suggested a threshold of 0.2%, with a corresponding sensitivity of 44% and a negative predictive value (NPV) of 97% for the detection of heart allograft rejection (4).

For the determination of a certain threshold value, the use of post-EMB samples could lead to inappropriately high suggested thresholds. An inappropriately high threshold means that the sensitivity of the assay decreases; more rejection episodes would be missed as the ddcfDNA values during these episodes are below the threshold that triggers for the performance of an EMB. In order to rule out such a potential effect of timing of sample

collection on threshold values, samples thus need to be collected before an EMB procedure.

Another potential clinical application of ddcfDNA is to monitor the response of anti-rejection therapy within heart transplant recipients. A previous study showed that ddcfDNA % decreases after the start of anti-rejection therapy (17). To reliably examine a response of anti-rejection therapy, it is important that the ddcfDNA values are not affected by the EMB procedure. This is also a reason why samples need to be collected before an EMB procedure.

To the best of our knowledge, this is the first study that examined the effect of an EMB on ddcfDNA values in an adult heart transplant population. A previous publication of the effect of the EMB on ddcfDNA values in young heart transplant recipients observed a stronger EMB related increase in ddcfDNA which seemed to be age-dependent (18); a 35.1-fold increase in ddcfDNA concentration in pediatric patients and a 4.4 fold increase in young adults (aged 18–22 years) was observed (18). With respect to this age-dependent effect, the lower increase in the present study might be explained by a higher average age of the study population. Another explanation for the discrepancy between the results of these studies might be that both studies used different ddcfDNA quantification methods; the present used ddPCR, whereas ddcfDNA quantification in the previous study was performed by using quantitative real-time PCR. The time between the EMB and sample collection in both studies was similar and could therefore not be a reason for the observed discrepancy. This study had a limited amount of rejection episodes. Therefore, it was not possible to analyze ddcfDNA during rejection and non-rejection in these samples. In addition, there is no evidence that confounders such as rejection, infection, immunosuppressive therapy and time after transplantation influence the fold change induced by the EMB procedure. For a more robust analysis of these confounders, a larger cohort than that presented here, needs to be investigated.

The present study found that the EMB procedure affects both ddcfDNA% and ddcfDNA concentration alike as the fold increases in both were comparable (1.28-fold vs 1.31-fold). This illustrates that the EMB procedure itself does not cause fluctuations in recipient cfDNA.

To conclude, we observed an increase in ddcfDNA concentration and ddcfDNA% caused by iatrogenic injury occurring as a result of the EMB procedure. If ddcfDNA is to be a promising biomarker to detect allograft rejection in transplantation patients, it is important that this biopsy-related effect is taken into account. Collection of blood sampling before the EMB procedure is essential to prevent ddcfDNA values being affected by this procedure. The value of ddcfDNA concentration for rejection monitoring should be addressed in a future cohort with more rejection episodes.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Erasmus MC (Medical MEC-Review Board). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JV participated in research design, laboratory experiments, statistical analysis and writing of the paper. AP and EJ participated in laboratory experiments. JT participated in revision of biopsies. DH and KB participated in research design, data interpretation, and writing of the paper. CB, OM and RS participated in research design and data interpretation. All authors reviewed 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.2022.10122/full#supplementary-material

Supplementary Figure 1 | Timing of the biopsies post-transplant with the corresponding pre-EMB ddcfDNA values and biopsy result. **Figure 1A** shows ddcfDNA concentration and **Figure 1B** shows ddcfDNA%. Abbreviations: ACR, acute cellular rejection.

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ORIGINAL RESEARCH
published: 21 March 2022
doi: 10.3389/ti.2022.10197

Epidemiological Study of Tricuspid Regurgitation After Cardiac Transplantation. Does it Influence Survival?

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Background: Tricuspid valve disease is the most frequent valvulopathy after heart transplantation (HTx). Evidence for the negative effect of post-transplant tricuspid regurgitation (TR) on survival is contradictory. The aim of this study was to analyze the causes of post-transplant TR and its effect on overall mortality.

Methods: This is a retrospective observational study of all transplants performed in two Spanish centers (1009 patients) between 2000 and 2019. Of the total number of patients, 809 had no TR or mild TR and 200 had moderate or severe TR. The etiology of TR was analyzed in all cases.

Results: The prevalence of moderate and severe TR was 19.8%. The risk of mortality was greater when TR was caused by early primary graft failure (PGF) or rejection ($p < 0.05$). TR incidence was related to etiology: incidence of PGF-induced TR was higher in the first period, while TR due to rejection and undefined causes occurred more frequently in three periods: in the first year, in the 10–14-year period following HTx, and in the long term (16–18 years). In the multivariable analysis, TR was significantly associated with mortality/retransplantation (HR:1.04, 95% CI:1.01–1.07, p:0.02).

Conclusion: The development of TR after HTx is relatively frequent. The annual incidence depends on TR severity and etiology. The risk of mortality is greater in severe TR due to PGF or rejection.

Keywords: heart transplantation, survival, prognosis, tricuspid regurgitation, aetiology

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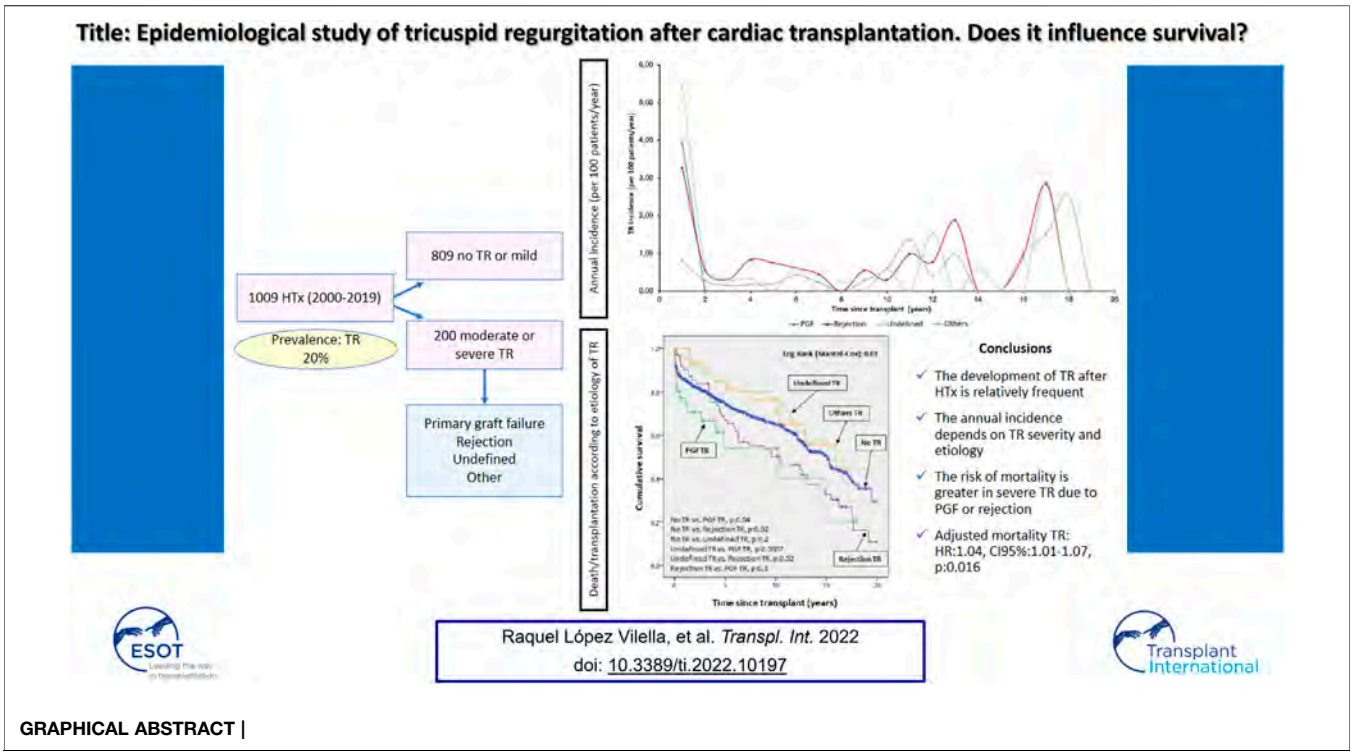
Received: *07 November 2021*
Accepted: *17 February 2022*
Published: *21 March 2022*

Citation:
López-Vilella R, Paniagua-Martín MJ, González-Vílchez F, Donoso Trenado V, Barge-Caballero E, Sánchez-Lázaro I, Aller Fernández AV, Martínez-Dolz L, Crespo-Leiro MG and Almenar-Bonet L (2022) Epidemiological Study of Tricuspid Regurgitation After Cardiac Transplantation. Does it Influence Survival? Transpl Int 35:10197. doi: 10.3389/ti.2022.10197

Abbreviations: HF, heart failure; IQR, interquartile range; HTx, heart transplantation; PGF, primary graft failure; TR, tricuspid regurgitation.



GRAPHICAL ABSTRACT |

INTRODUCTION

Heart transplantation (HTx) remains the treatment of choice for end-stage heart failure (HF) (1). Overall, outcomes of HTx have improved in recent decades (2); however, a series of short, medium, and long-term complications continue to have an impact on prognosis. Tricuspid regurgitation (TR) is the most frequent valve disease after orthotopic HTx in both the short and long-term, and has a prevalence ranging from 19% to 84%, depending on the series (3). In most cases, TR is mild and asymptomatic, but some cases of moderate or severe TR are associated with increased morbidity and mortality (3–7). However, the prognostic implications of TR after heart transplantation is not clearly defined. Some authors associate post-transplant TR with adverse outcomes, while according to others most cases of significant TR resolve within 1 year of transplant (8, 9). Identifying patients with significant TR who will develop such complications remains challenging, and warrants further clinical investigation. It has been suggested that the development and prognostic impact of TR depends not only on its severity, but also on its etiology. Thus, there is a type of early post-operative TR caused by primary graft failure (PGF) with or without pulmonary hypertension (7, 10, 11), and another later type of TR associated with rejection or other causes (9). In any event, TR, its causes, and its prognostic implications have not hitherto been studied in detail.

We hypothesized that not all causes of TR have the same effect on mortality or the same evolution in transplant patients. Studying the evolution of TR after heart transplantation and both its cause-specific and general impact would improve the characterization of this valvular disease, and help identify the therapeutic approach and follow-up that would be most beneficial in these patients.

We performed an epidemiological study in a large series of heart transplant patients to determine the prevalence of TR and its influence on long-term mortality. The secondary objective was to perform a subanalysis of the most common etiologies of TR, its differential characteristics, and its etiology-specific impact on survival after transplantation.

Patients and Methods

We performed a retrospective observational study that included all patients who had undergone HTx in two Spanish centers between 1 January, 2000 and 31 December, 2019. Multi-organ transplants, retransplants, patients under 16 years at the time of transplantation, and patients who died during the first 72 h of transplantation were excluded (**Figure 1**).

TR was grouped according to etiology: PGF, acute rejection, undefined causes, and others. TR due to undefined cause was defined as functional TR with no identifiable cause. All variables included in the Spanish Registry of Cardiac Transplants, defined elsewhere, were evaluated (12). The donor-recipient body size match was analyzed using the predicted right, left, and total ventricular mass, which has proven to be the body metric with the greatest prognostic value (13). Glomerular filtration rate was estimated using the formula recommended by the Chronic Kidney Disease Epidemiology Collaboration (14). The presence of TR, time of appearance after transplantation, severity of ventricular dysfunction, evolution of valve disease, clinical

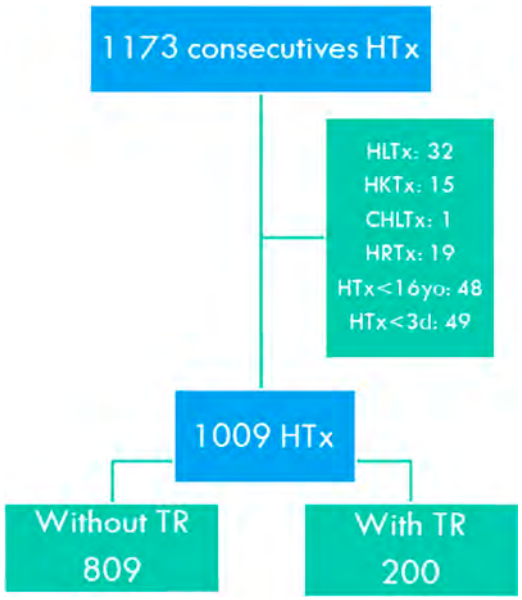


FIGURE 1 | Study flow chart. HTx, Heart transplant; HLTx, Combined heart-lung transplant; HKTx, Combined heart-kidney transplant; CHLTx, Combined heart-liver transplant; HRTx, Heart retransplant; HTx <16yo, Pediatric heart transplant; HTx <3d, Death within the first 3 days of transplantation. TR, Tricuspid regurgitation.

course, treatment, and patient status at the end of follow-up were also analyzed.

Information on TR grade was obtained from echocardiography reports following the recommendations in force in each time period. Four grades (absent, mild, moderate, or severe) were analyzed. The TR group comprised exclusively moderate and severe grade regurgitations. The cardiologists used semi-quantitative or qualitative parameters to evaluate tricuspid regurgitation, depending on the protocol in place in each center and the clinical status of the patient. Reduced ventricular function and chamber dilation were also diagnosed (1). Right ventricular dysfunction and right ventricular dilatation were evaluated both qualitatively and quantitatively by measurement of the basal diameter of the cavity, S wave, and systolic excursion of the tricuspid annulus (TAPSE). Echocardiography is a technique that is used systematically at any time during the follow-up of the cardiac transplant patient. In this series, the echocardiography protocol consisted of a transthoracic echocardiogram performed almost daily in the early phase of the transplant, during scheduled biopsies (7–9 during the first year), and during follow-up (every 4–6 months), according to the protocol implemented in each center. Echocardiography was also performed whenever instability, clinical deterioration, or valve involvement was suspected. It is important to emphasize that clinical guidelines on the measurement and quantification of tricuspid regurgitation did not change significantly over the course of the study. Both study centers followed the same measurement guidelines.

The study was approved by the Biomedical Research Ethics Committee of both participating centers, and the ethical principles for medical research in human subjects defined by the Declaration of Helsinki were followed.

Statistical Analysis

Continuous variables are summarized as median (interquartile range [IQR]), as all of them showed a non-normal distribution (Kolmogorov-Smirnov test). Categorical variables are summarized as frequency (percentage). Group variables were compared using the Mann-Whitney test in the case of continuous variables and using Fisher's exact test or the Chi-square test in the case of categorical variables.

A multivariate Cox proportional hazard regression model was used to determine independent predictors of TR, introducing as predictors the variables that showed a p value <0.10 in the univariable analysis.

The main outcome was a composite of all-cause mortality or cardiac retransplant. The association between the occurrence of TR and mortality/transplantation was first analyzed by the Kaplan-Meier procedure and the differences between groups by the Log-Rank test. As both graphs and the Schoenfeld residual test showed that TR violated the proportional hazard assumption, it was considered a time-dependent variable for the purpose of univariate and multivariate analysis. Association with the outcome was analyzed by means of Cox proportional hazards regression. In the multivariate analysis, variables that showed a significance level $p < 0.10$ in the univariable analysis were introduced as independent variables, including TR as a time-dependent variable.

All tests were two-tailed, establishing statistical significance for a p value <0.05. The analyses were performed with IBM SPSS Statistics Version 27 ®.

RESULTS

A total of 200 (19.8%) of the 1009 patients included developed moderate or severe TR during follow-up. TR was graded as moderate in 133 recipients (13.2%) and severe in 67 (6.6%).

Baseline Characteristics

Pre-transplant clinical characteristics for the entire population and by TR group are summarized in **Table 1**. Patients who developed TR were younger, had higher pre-transplant bilirubin, and were transplanted from older and female donors more frequently. Donor-recipient sex mismatch was more frequent (higher percentage of female donor to male recipient grafts), and the donor-recipient-predicted right ventricular mass ratio was lower in patients developing TR.

Etiological Characteristics of Tricuspid Regurgitation

Differences in TR characteristics according to etiology are summarized in **Table 2**. The most frequent etiology was undefined causes, followed by acute rejection. PGF-induced TR resulted more frequently in dilation and dysfunction of the right ventricle. All types of TR improved over time and subsided on echocardiography. In the group of other etiologies, TR was

TABLE 1 | Baseline characteristics.

	No TR (*n*: 809)	TR (*n*: 200)	*p* value	Total population (*n*: 1009)
Recipient				
Age (years)	56.0 (48.5–63.0)	55.0 (45.0–61.0)	0.017	56.0 (48.0–62.0)
Female sex, n (%)	143 (17.7)	42 (21.1)	0.22	185 (18.4)
Etiology, n (%)			0.33	
Ischemic	335 (41.4)	72 (36.0)		407 (40.3)
Dilated	329 (40.7)	86 (43.0)		415 (41.1)
Other	145 (17.9)	42 (21.0)		187 (18.5)
Body mass index (Kg/m^2)	25.4 (23.1–28.4)	24.8 (22.5–27.8)	0.058	25.2 (23.0–28.3)
Creatinine (mg/dl)	1.1 (0.9–1.4)	1.2 (0.9–1.4)	0.31	1.13 (0.9–1.4)
Glomerular filtration rate (mL/min/1.73 m^2)	69.6 (51.7–90.6)	67.8 (49.6–90.7)	0.45	69.5 (51.5–90.6)
Bilirubin (mg/dl)	1.0 (0.6–1.6)	1.2 (0.7–1.9)	0.024	1.0 (0.6–1.7)
PVR (Wood U.)	2.1 (1.3–3.0)	2.2 (1.5–3.1)	0.19	2.1 (1.3–3.0)
Pretransplant infection, n (%)	70 (8.7)	15 (7.5)	0.67	85 (8.4)
Diabetes Mellitus, n (%)	111 (13.7)	35 (17.5)	0.18	146 (14.5)
COPD, n (%)	83 (12.2)	23 (13.2)	0.70	106 (12.4)
Positive CMV serology, n (%)	643 (81.4)	167 (85.2)	0.25	810 (82.2)
Peripheral vascular disease, n (%)	37 (4.6)	10 (5.0)	0.85	47 (4.7)
Mechanical ventilation, n (%)	122 (15.1)	24 (12.1)	0.31	146 (14.5)
Circulatory support, n (%)			0.29	
No	603 (74.9)	150 (75.0)		753 (74.9)
IABP	88 (10.9)	26 (13.0)		114 (11.3)
ECMO	69 (8.6)	10 (5.0)		79 (7.9)
VAD	45 (5.6)	14 (7.0)		59 (5.9)
Previous sternotomy	144 (17.8)	41 (20.5)	0.36	185 (18.4)
Pretransplant neoplasy, n (%)	27 (3.4)	4 (2.1)	0.49	31 (3.1)
Donor				
Age (years)	44.0 (31.0–51.0)	47.0 (38.0–55.0)	<0.001	44 (32–52)
Female sex, n (%)	238 (29.5)	95 (47.5)	<0.001	333 (33.0)
Body mass index (Kg/m^2)	25.4 (23.9–27.7)	25.6 (23.9–27.8)	0.71	25.4 (23.9–27.7)
Positive CMV serology, n (%)	592 (76.4)	155 (81.2)	0.18	747 (77.3)
Predonation cardiac arrest, n (%)	56 (7.1)	18 (9.2)	0.36	74 (7.5)
Cause of death, n (%)			0.059	
Trauma	273 (33.7)	50 (25.0)		323 (32.0)
Cerebrovascular accident	364 (45.0)	103 (51.5)		467 (46.3)
Other	172 (21.3)	47 (23.5)		219 (21.7)
Donor-recipient interaction				
Sex mismatch, n (%)			<0.001	
No mismatch	573 (70.9)	112 (56.0)		685 (68.0)
Donor male/Recipient female	70 (8.7)	18 (9.0)		88 (8.7)
Donor female/Recipient male	165 (20.4)	70 (35.0)		235 (23.3)
CMV serology mismatch, n (%)			0.41	
No mismatch	506 (66.8)	135 (71.8)		641 (67.8)
Donor (-)/Recipient (+)	145 (19.1)	30 (16.0)		175 (18.5)
Donor (+)/Recicipient (-)	107 (14.1)	23 (12.2)		130 (13.7)
Donor-recipient PRVM ratio	1.12 (1.00–1.27)	1.06 (0.94–1.17)	<0.001	1.11 (0.99–1.25)
Donor-recipient PHM ratio	1.0 (1.0–1.1)	1.0 (1.0–1.1)	0.27	1.0 (1.0–1.2)
Surgical procedure				
Urgent code, n (%)[a]	264 (32.6)	68 (34.0)	0.74	332 (32.9)
Cold ischemia duration (min)	180 (115–222)	194 (114–248)	0.08	180.0 (115–227)
Bicaval technique, n (%)	660 (88.8)	161 (85.2)	0.17	821 (88.1)
Follow up				
Time (years)	5.8 (1.8–12.0)	6.3 (2.4–11.8)	0.27	5.9 (1.9–11.9)
Status, n (%)			0.15	
Alive	497 (61.4)	109 (54.5)		606 (60.1)
Dead	305 (37.3)	88 (44.0)		393 (38.9)
Retransplanted	7 (0.9)	3 (1.5)		10 (1.0)

[a]*Urgent Code transplantation was performed in severe cardiogenic shock.*

CMV, cytomegalovirus; COPD, chronic obstructive pulmonary Disease; ECMO, extracorporeal membrane oxygenation; IABP, Intra-Aortic Balloon Pump; PHM, predicted heart mass; PRVM, predicted right ventricular mass; PVR, pulmonary vascular resistance; TR, tricuspid regurgitation; VAD, ventricular assist device.

TABLE 2 | Characteristics of tricuspid regurgitation in transplanted patients according to the etiological types.

	Primary graft failure (*n*: 35)	Acute rejection (*n*: 64)	Undefined (*n*: 72)	Other (*n*: 29)	*p* value
Chronology	Very early	Late and very late	Very late	Very late	
Time of appearance	First year	1–18 years	11–18 years	10–18 years	
Prevalence, n (%)	35 (17.5)	64 (32.0)	72 (36.0)	29 (14.5)	0.008
Grading of TR					0.01
Moderate	19 (54.3)	40 (62.5)	58 (80.6)	16 (55.2)	
Severe	16 (45.7)	24 (37.5)	14 (19.4)	13 (44.8)	
Right ventricular dilatation	20 (57.1)	15 (23.4)	11 (15.3)	13 (41.8)	<0.001
Right ventricular dysfunction	31 (88.6)	32 (50.0)	9 (12.5)	14 (48.3)	<0.001
Left ventricular dysfunction	8 (22.9)	21 (32.8)	1 (1.4)	4 (13.8)	<0.001
Echocardiography time course					0.01
Improvement	29 (82.9)	46 (71.9)	58 (82.9)	16 (55.2)	
Stable	6 (17.1)	12 (18.8)	12 (17.1)	8 (27.6)	
Deterioration	0 (0.0)	6 (9.5)	0 (0.0)	5 (16.2)	
Congestive signs	14 (40.0)	42 (65.6)	22 (30.6)	12 (41.4)	0.001
Clinical course of congestive signs[a]					0.005
Improvement	9 (64.3)	23 (54.8)	15 (68.2)	5 (41.7)	
Stable	1 (5.0)	14 (33.3)	6 (27.3)	1 (8.3)	
Deterioration	0 (0.0)	5 (11.9)	1 (4.5)	6 (50.0)	
Number of diuretics[b]					<0.001
0	17 (48.6)	23 (35.9)	47 (65.3)	13 (44.8)	
1	18 (51.4)	29 (45.3)	21 (29.2)	10 (34.5)	
2	0 (0.0)	11 (17.2)	4 (5.6)	3 (10.3)	
3	0 (0.0)	1 (1.6)	0 (0.0)	3 (10.3)	
Treatment					<0.001
No/symptomatic	0 (0.0)	2 (3.1)	71 (98.6)	16 (55.2)	
Etiological	35 (100.0)	61 (95.3)	0 (0.0)	11 (37.9)	
Retransplantation	0 (0.0)	1 (1.6)	0 (0.0)	1 (3.4)	
Coronary stent	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.4)	
Annuloplasty	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	

[a]*Right-sided congestive signs that can be attributed to tricuspid regurgitation have been analyzed.*
[b]*Including any type of diuretic that each patient was prescribed (loop diuretics, thiazides, acetazolamide and/or tolvaptan).*
TR, tricuspid regurgitation.

TABLE 3 | Causes of post-transplant tricuspid regurgitation in the group "Other".

	N	%
Pulmonary hypertension	9	31.0
Cardiac allograft vasculopathy	7	24.1
Pacemaker Electrode	4	13.8
Biopsy complication	3	10.3
Chronic renal insufficiency	2	6.9
Severe pericardial effusion[a]	1	3.4
Valve prolapse	1	3.4
Atrial tachycardia	1	3.4
Massive Pulmonary Embolism	1	3.4

[a]*Severe pericardial effusion with distortion of the geometry of the right ventricular cavity and the valve annulus.*

more frequently associated with pulmonary hypertension, coronary allograft vasculopathy, pacemaker implantation, and biopsy complications (**Table 3**).

Incidence of Tricuspid Regurgitation

The incidence of TR (per 100 patient-years) over the 20 years of post-transplant follow-up according to the degree of regurgitation is shown in **Figure 2**. Median time to overall TR was 0.57 years (IQR, 0.06–5.60 years); this was significantly lower in moderate TR (median: 0.12 years [0.04–1.78 years]) compared to severe TR (median: 5.24 years [1.30–10.90 years]; $p < 0.001$).

The incidence of moderate TR was highest in the first period after HTx, while severe TR generally appeared later. **Figure 3** shows the temporal distribution of the appearance of TR according to etiology. The incidence of PGF-induced TR was highest in the first period while TR due to rejection and undefined causes occurred more frequently in three periods: in the first year, in the 10–14-year period after HTx, and in the long term (16–18 years), showing a triphasic distribution.

Independent Predictors of Post-Transplant Tricuspid Regurgitation

Univariate associations with the development of moderate-severe TR are summarized in **Supplementary Table S1**. Diabetes, ventricular assist device prior to heart transplantation, higher donor age, female donors, donor cause of death other than trauma, and donor-recipient sex mismatch (female donor for male recipient) were risk factors. Higher recipient body mass index and higher donor-recipient-predicted right ventricular mass ratio were protective factors. In the multivariate analysis, only diabetes, donor age, and donor-recipient sex mismatch (female

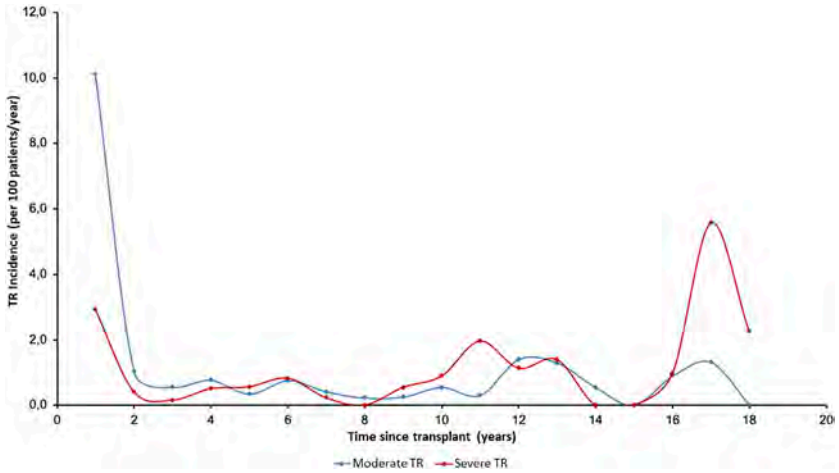


FIGURE 2 | Annual incidence (per 100 patients/year) of tricuspid valve disease in follow-up according to severity. TR, Tricuspid regurgitation.

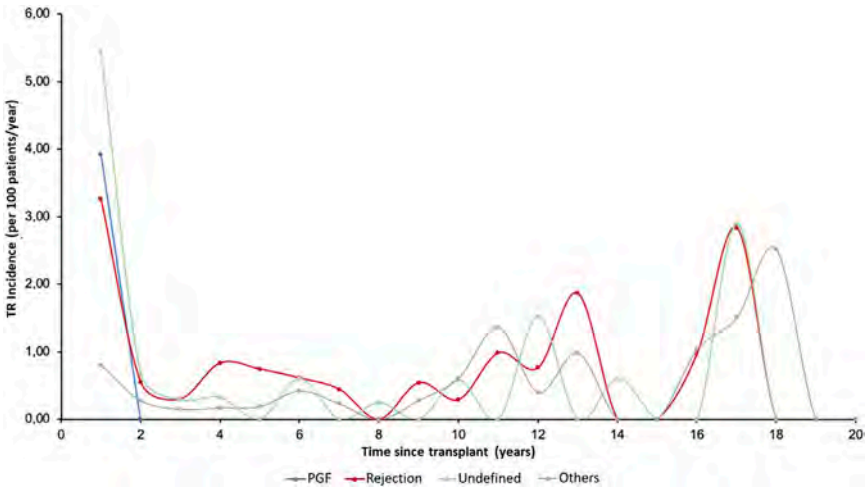


FIGURE 3 | Annual incidence (per 100 patients/year) of tricuspid valve disease in follow-up according to etiology. PGF, Primary graft failure; TR, Tricuspid regurgitation.

donor for male recipient) were independently associated with development of TR (**Figure 4**).

Survival

There were 393 deaths and 10 retransplants during a median follow-up of 5.9 years (IQR, 1.9–11.9). Survival analysis according to TR severity showed a higher rate of mortality (p:0.05) for severe TR compared to moderate TR and no TR (**Figure 5**).

The survival curves for mortality/transplantation showed a significantly worse prognosis when TR was due to PGF and rejection compared to other causes (p 0.04 and 0.02, respectively, **Figure 6**).

Prognostic Impact of Post-transplant Tricuspid Regurgitation

In the univariate analysis (**Supplementary Table S2**), post-transplantation TR was associated with higher mortality/retransplantation (HR: 1.04; 95% CI: 1.01–1.07; p: 0.02). The variables significantly associated with a higher risk for mortality/transplantation in the multivariable analysis were the presence of moderate to severe TR, recipient age at transplant, pre-transplant diabetes, and peripheral vascular disease. Protective factors were bicaval technique (versus biatrial technique), use of intra-aortic balloon pump (versus no pump), and a higher donor-recipient heart mass ratio. These results are shown in **Figure 7**.

DISCUSSION

TR is the most prevalent valve disease after HTx3. Its causes vary and each may have different prognostic implications. Some studies have analyzed the prevalence this valve disease and its implication in survival. However, the incidence, time of appearance, and TR etiology-specific prognostic implications have never been fully defined. In this study, we sought to

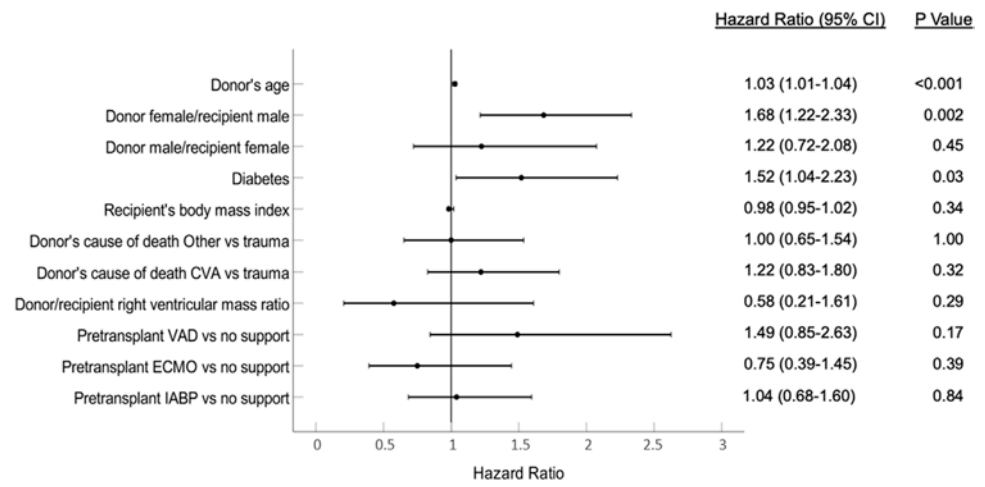


FIGURE 4 | Variables associated with the development of post-transplantation tricuspid regurgitation. CVA, Cerebrovascular accident; ECMO, Extracorporeal membrane oxygenation; IABP, Intra-aortic balloon pump; VAD, Ventricular assist device.

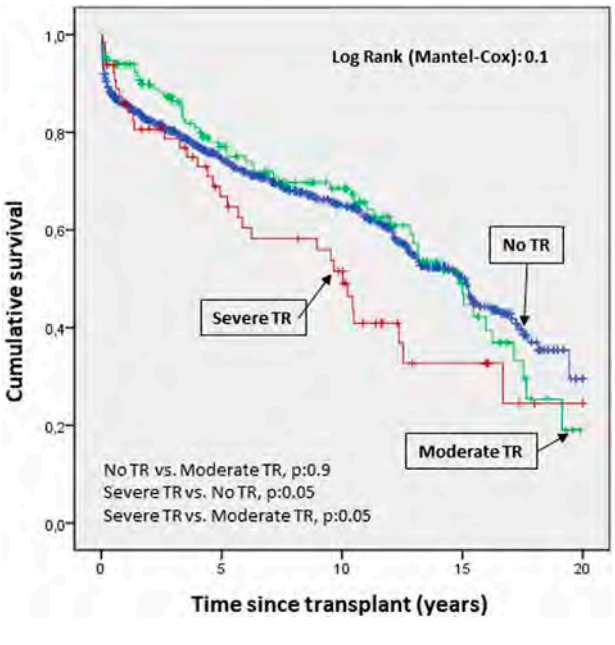


FIGURE 5 | Cumulative probability of mortality/transplantation according to severity of tricuspid valve disease. The probability of survival according to the severity of regurgitation showed a clear trend toward higher mortality in severe versus moderate tricuspid regurgitation and no regurgitation.

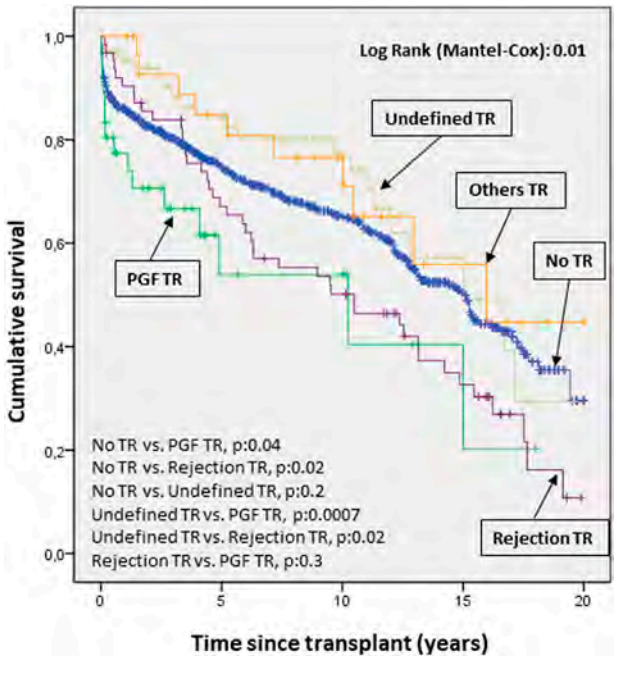


FIGURE 6 | Cumulative probability of death/transplantation according to etiology of tricuspid valve disease. The survival curves for mortality/transplantation show a significantly worse prognosis ($p < 0.05$) when tricuspid disease was due to primary graft failure and rejection compared to other causes. PGF, Primary graft failure; TR, Tricuspid regurgitation.

clarify these questions by analyzing a large series of heart transplant patients from two Spanish centers with high transplant activity. We found that the prevalence of post-transplant moderate or severe TR was nearly 20%, and that the most frequent cause was functional, i.e., no organic valve alteration and no specific cause for TR. TR appearing in the early stages of PGF and during acute rejection had the highest risk for mortality. TR was also found to be an independent predictor of mortality, and its appearance was related to donor age and donor-recipient sex mismatch (specifically, female donor for male recipient).

A total of 1,009 cardiac transplants were included in this study, constituting the largest cohort so far. A total of 200 patients in our series presented at least moderate TR during their evolution–a prevalence of 19.8%, similar to that found in previous

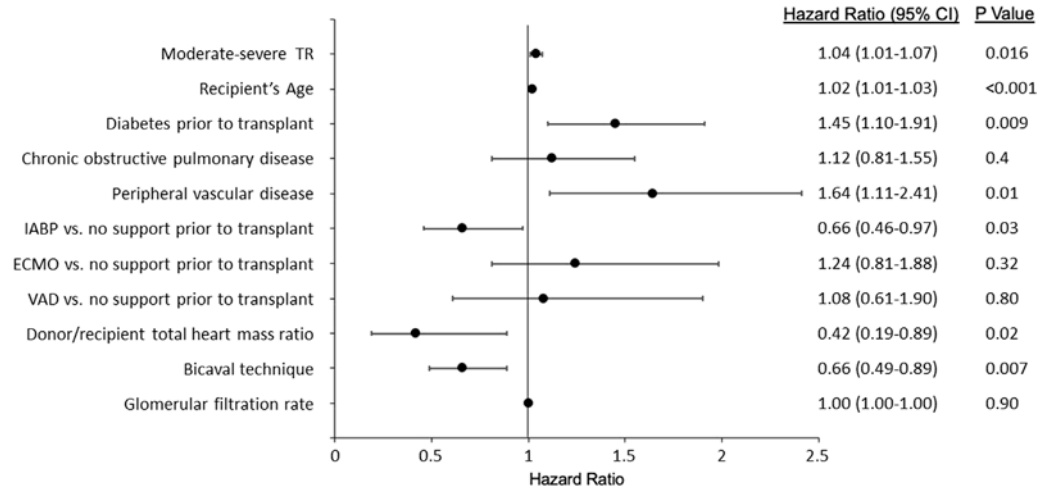


FIGURE 7 | Variables associated with mortality/transplantation. IABP, Intra-aortic balloon pump; ECMO, Extracorporeal membrane oxygenation; VAD, Ventricular assist device; TR, Tricuspid regurgitation.

publications (15). In this study, the pre-transplant clinical characteristics of patients in both groups were basically similar. Patients who developed TR were younger and had higher bilirubin; however, these differences were not clinically relevant. In addition, patients who developed TR had a lower donor-recipient-predicted right ventricular mass ratio, as observed in the univariate analysis. This index has predictive value for mortality after heart transplantation, but it has not hitherto been associated with the appearance of TR (13). Nevertheless, our multivariate analysis showed that the development of TR was independently associated with diabetes, donor age, and donor-recipient sex mismatch (female donor for male recipient). Previous studies have described a correlation between the appearance of TR and donor heart size - recipient pericardial cavity mismatch (16, 17), and female recipient has been shown to be an independent predictor of rapid progression of TR (18). However, the correlation between TR and sex mismatch, and between diabetes in the recipient and post-transplant TR have not hitherto been described in the literature. These findings could help optimize donor/recipient selection and reduce the risk of post-transplantation TR.

The etiology of TR after HTx has not been completely clarified. This is the first study to address both this issue and the timing time of TR onset according to etiology. Thus, the undefined etiology was the most frequent (functional TR with no identifiable cause) followed by acute rejection. PGR-induced TR showed the closest correlation with right ventricle dilation and presence of biventricular dysfunction, followed by rejection-induced TR, which was also associated with ventricular dysfunction. The timing of TR onset is also related to its etiology. Our findings show that TR associated with early primary graft failure is the first to appear, while TR due to rejection and undefined cause is triphasic, with an initial incidence (first year), another incidence in the medium term (10–14 years post-HTx) and finally, a long-term incidence (16–18 years). Few studies have analyzed the predictors of early vs late TR. Williams et al. reported a significant increase in TR on echocardiography performed at week 1 compared with the same study performed at 2.4 ± 1.3 years after HTx, with incidence rates of 63% and 71%, respectively (19). In another study, the incidence of severe TR increased from 5% at 1 year up to 50% 4 years after transplantation (17, 20). A previous study reported that the development of early TR was correlated with allograft rejection, high transpulmonary gradient, and high pulmonary vascular resistance, while the risk factors for late TR were biatrial surgical technique, the number of rejections, and the total number of endomyocardial biopsies performed (21). All these findings confirm that TR is a complication that can appear either very early after HTx or many years after the intervention. In fact, it appears to be a dynamic condition; severe early TR has been shown to subside 1 year after transplantation in more than 91% of recipients (22). For this reason, the reported incidence of TR is higher in the first post-transplant year, although there continues to be a risk of developing TR thereafter. This late risk can be aggravated by repeated endomyocardial biopsies (6, 21). In our study, echocardiographic study of most cases of TR showed improvement over time.

Regarding the impact of post-transplantation TR on mortality, the mortality/transplantation survival curves showed a clear trend towards higher mortality in severe TR compared with moderate TR and no TR. In previous studies, TR has been associated with decreased long-term survival after heart transplantation. However, although these data are contradictory (8, 9, 22, 23), in general, most authors agree that this valve disease is predictive of mortality (6). In some studies, even intraoperative TR was associated with increased mortality in HTx patients (8). In this study, the variables significantly associated with an increased risk for mortality/transplantation in the multivariable analysis were presence of moderate-severe TR, recipient age, pre-transplant diabetes, and peripheral vascular disease. Protective factors were bicaval technique (versus biatrial

technique), use of an intraaortic balloon pump (versus no pump), and a higher donor/recipient-predicted heart mass ratio. Previous studies have reported that the likelihood of developing TR was greater if HTx is performed using the biatrial technique compared to the bicaval technique. This may be due to the fact that the traditional technique (biatrial) significantly alters atrial geometry, resulting in deterioration of valve integrity (24–26). Regarding the finding of diabetes mellitus as a risk factor for the development of post-transplant TR, this is a finding that has not been described in the literature. One possible explanation could be that the vascular and microvascular involvement of these patients has an impact on ventricular morphology. However, this is only a hypothesis; it is possible that this is a clinically irrelevant finding, as it is not associated with the other independent predictors of the development of TR, which mainly refer to the donor.

Finally, the survival curves for mortality/transplantation showed a significantly worse prognosis when the etiology of TR was due to PGF and rejection compared to other causes. TR patients have similar long-term prognosis compared to patients without TR.

These data are consistent with the known prognosis for both conditions. Currently, PGF is one of the most frequent causes of mortality, especially in the first month after transplantation, while rejection is the second most frequent cause of death between the first and fifth year after transplantation (2).

This study has some limitations, especially due to its retrospective nature. The protocols for performing the echocardiographic study varied slightly, as they were performed in two different centers. Moreover, patients who died within the first 3 days of transplantation had to be ruled out because in these cases echocardiographic studies, especially in the presence of severe PGF, were focused primarily on assessing the degree of ventricular dysfunction, not the presence of tricuspid valve disease, and there were no data on TR in these echocardiography reports. Nevertheless, the major strength of the study is the large sample size and the detailed description of causes, time-related characteristics, and the prognostic impact of TR. The size of our series - 200 cases of tricuspid valve disease collected over 20 years of transplant activity in two centers with a high number of annual implants–supports the reliability of our findings. Furthermore, we have not found any previous studies with such a detailed description of the incidence of valve disease, its prognostic importance, and its influence on mortality. For all these reasons, we believe our conclusions can safely be extrapolated to other settings.

Based on our findings, we can conclude that the prevalence of moderate and severe tricuspid regurgitation is close to 20%, with a variable annual incidence depending on the severity and etiology of the valve disease. This valvulopathy, especially in its severe manifestation, is associated with a high risk of mortality, particularly when it is due to rejection and primary graft failure. The multivariate analysis shows a significant association between mortality/transplantation and TR.

DATA AVAILABILITY STATEMENT

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

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.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10197/full#supplementary-material

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ORIGINAL RESEARCH
published: 23 March 2022
doi: 10.3389/ti.2022.10320

Extracorporeal Photopheresis With Low-Dose Immunosuppression in High-Risk Heart Transplant Patients—A Pilot Study

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In severely ill patients undergoing urgent heart transplant (HTX), immunosuppression carries high risks of infection, malignancy, and death. Low-dose immunosuppressive protocols have higher rejection rates. We combined extracorporeal photopheresis (ECP), an established therapy for acute rejection, with reduced-intensity immunosuppression. Twenty-eight high-risk patients (13 with high risk of infection due to infection at the time of transplant, 7 bridging to transplant via extracorporeal membrane oxygenation, 8 with high risk of malignancy) were treated, without induction therapy. Prophylactic ECP for 6 months (24 procedures) was initiated immediately postoperatively. Immunosuppression consisted of low-dose tacrolimus (8–10 ng/ml, months 1–6; 5–8 ng/ml, >6 months) with delayed start; mycophenolate mofetil (MMF); and low maintenance steroid with delayed start (POD 7) and tapering in the first year. One-year survival was 88.5%. Three patients died from infection (POD 12, 51, 351), and one from recurrence of cancer (POD 400). Incidence of severe infection was 17.9% (n = 5, respiratory tract). Within the first year, antibody-mediated rejection was detected in one patient (3.6%) and acute cellular rejection in four (14.3%). ECP with reduced-intensity immunosuppression is safe and effective in avoiding allograft rejection in HTX recipients with risk of severe infection or cancer recurrence.

Keywords: extracorporeal photopheresis, heart transplantation, immunosuppression, CNI delay, induction therapy

OPEN ACCESS

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Received: *22 December 2021*
Accepted: *11 February 2022*
Published: *23 March 2022*

Citation:
Gökler J, Aliabadi-Zuckermann A, Zuckermann A, Osorio E, Knobler R, Moayedifar R, Angleitner P, Leitner G, Laufer G and Worel N (2022) Extracorporeal Photopheresis With Low-Dose Immunosuppression in High-Risk Heart Transplant Patients—A Pilot Study. Transpl Int 35:10320. doi: 10.3389/ti.2022.10320

Abbreviations: AMR, antibody-mediated rejection; ACR, acute cellular rejection; CDC, complement-dependent cytotoxicity; CMP, cardiomyopathy; CNI, calcineurin inhibitor; CTCAE, common terminology criteria for adverse events; DSA, donor-specific antibody; ECMO, extracorporeal membrane oxygenation; ECP, extracorporeal photopheresis; HLA, human leucocyte antigen; HOCM, hypertrophic obstructive cardiomyopathy; HTX, heart transplantation; ICU, intensive care unit; INTERMACS, interagency registry for mechanically assisted circulatory support; IMPACT, index for mortality prediction after cardiac transplantation; IQR, interquartile range; ISHLT, international society of heart and lung transplantation; MMF, mycophenolate mofetil; MNC, mononuclear cells; MOF, multiorgan failure; PCR, polymerase chain reaction; POD, postoperative day; PRAs, panel-reactive antibodies; UNOS, united network for organ sharing; VAC, vacuum-assisted closer; VAD, ventricular assist device.

INTRODUCTION

Cardiac transplantation remains the best option for patients with end-stage heart failure. In recent decades, the number of patients referred to transplantation has increased significantly. Many patients are at high risk for early postoperative infection, and patients with previous malignant disease are more often seen as potential transplant candidates (1, 2). Current immunosuppressive protocols are associated with risk of infectious complications and cancer (3, 4). Earlier attempts to use low-level immunosuppressive protocols to reduce these risks resulted in higher organ rejection rates (5, 6). Extracorporeal photopheresis (ECP) is a successful supportive therapy for the treatment of severe and/or recurrent rejection episodes in solid organ transplantation, including heart transplantation (7). ECP is an apheresis involving ultraviolet A irradiation of peripheral blood mononuclear cells with prior exposure to 8-methoxypsoralen. The mode of action is not fully understood, but ECP is believed to have immunostimulatory and immunosuppressive effects and it reduces T-cell-mediated immune responses (8). In 1998, Barr *et al.* published a landmark prospective randomized study that documented the benefit of ECP as adjunct to standard immunosuppression to prevent acute rejection (14). However, the applied immunosuppressive protocol (cyclosporine A, azathioprine) differs from the protocols used today.

The aim of our pilot study was to evaluate a novel approach consisting of 6-month ECP together with a reduced-intensity immunosuppressive protocol to treat challenging heart transplant recipients at high risk for either early postoperative infection or cancer recurrence.

MATERIALS AND METHODS

Study Design

The primary outcomes of this pilot study were 1-year and overall survival. Secondary outcomes were the safety of ECP, incidence of early postoperative infection (in-hospital and in the first 6 months of ECP therapy), number of rejection episodes according to the International Society of Heart and Lung Transplantation (ISHLT) criteria in the first year, and recurrence of malignancy. Approval for the study was obtained from the institutional review board of the Medical University of Vienna (EK 1107/2020). In accordance with local regulations, all use of patients' clinical research data required their consent.

Patients

Between September 2016 and January 2021, 200 heart transplant procedures were performed at the Medical University of Vienna. Twenty-eight patients (25% female, $n = 7$) were included in this study and treated according to our reduced-intensity immunosuppressive protocol without induction but combined with ECP. There was no adequate control group to compare with this heterogeneous pilot group of challenging HTX patients. Most patients (85.7%) had highly urgent status. Inclusion criteria for this protocol consisted of patients with a recent or current history of infection (patients with sepsis or systemic inflammatory response syndrome were excluded, as these are absolute contraindications for transplantation in our center), high risk for early postoperative infection (ECMO bridging to transplant), or high neoplastic risk (i.e., cardiac tumor as indication for transplantation, history of malignancy more than 5 years prior to transplantation, malignancy found in the donor after organ procurement).

Patient demographics and baseline characteristics are listed in **Tables 1**, **2**. Detailed information on indication for inclusion in the study protocol is presented in **Table 3**.

Outcome Parameters

Postoperative severe infection was defined as clinically relevant infection in the early postoperative phase. CMV disease was based on international classification (9).

Graft function was examined by transthoracic echocardiography, which was performed on a routine basis during the first year (weekly in month 1, monthly in months 2–12). Endomyocardial biopsies were performed at weeks 2, 3, and 4, and at months 2, 3, 6, and 12, and in case of clinical signs of rejection. Acute cellular rejection (ACR) as well as antibody-mediated rejection were defined according to the ISHLT nomenclature (10, 11).

Patients with a history of malignancy underwent close follow-up including CT, MRI, or PET scan where appropriate, on a regular basis.

Adjusted Immunosuppressive Protocol

There was no induction therapy (see **Figure 1**). For immunosuppression, the calcineurin inhibitor (CNI) tacrolimus was first administered after a CNI delay of at least 3 days in patients with normal renal function and up to 10 days in patients with reduced kidney function. The target range of tacrolimus was 8–10 ng/ml in months 1–6, and 5–8 ng/ml thereafter. Mycophenolate mofetil was started on postoperative day 0 with 1 g/day and increased to 2 g/day at the time of CNI start, in case of normal leukocyte counts (>4000 per microliter). After postoperative wound healing, MMF was switched to everolimus (starting dose 1.5 mg/d; through level 8 ng/ml) in the patients of the malignancy group due to its potential antineoplastic effects (12). Steroid was applied intraoperatively (500 mg methylprednisolone prior to opening the aortic clamp) and in the first 24 h (125 mg methylprednisolone every 8 h). Maintenance steroid (0.2 mg/kg/day prednisolone) was started on POD 7 and tapered by 2.5 mg every 3 months in the absence of rejection.

Prophylaxis of Infection

All patients without evidence of infection at the time of HTX received empiric broad-spectrum antibiotics for at least 5 days after transplantation, and all patients with infection at the time of transplantation were treated with targeted antimicrobial therapy adjusted to the antibiogram. Prophylaxis against *Pneumocystis jerovecii* with oral trimethoprim-sulfamethoxazole (160 mg trimethoprim and 800 mg of sulfamethoxazole, two tablets per

TABLE 1 | Patient demographics and baseline characteristics I.

	Total, *n* = 28	Infection, *n* = 13	ECMO, *n* = 7	Malignancy, *n* = 8
Age, years, med (IQR)	51.9 (42.2–57.6)	55.7 (52.5–63.4)	43 (37.2–51.8)	43.8 (39.5–51.4)
Gender, female, n (%)	7 (25)	2 (15.4)	1 (14.3)	4 (50)
Indication for HTX, n				
Ischemic CMP	5	3	2	0
Dilative CMP	10	5	0	5
Congenital disease	1	1	0	0
Bail out after cardiac surgery	6	1	5	0
Cardiac tumor	2	0	0	2
Other (CAV, HOCM)	4	3	0	1
HKTX, n (%)	2 (7.1)	1 (7.7)	0	1 (12.5)
Previous cardiac surgery, n (%)	18 (64.3)	7 (53.8)	5 (71.4)	6 (75)

CAV, cardiac allograft vasculopathy; CMP, cardiomyopathy; HKTX, combined heart-kidney transplant; HTX, heart transplantation; HOCM, hypertrophic obstructive cardiomyopathy; med (IQR), median and interquartile range.

TABLE 2 | Patient demographics and baseline characteristics II.

	Total, *n* = 28	Infection, *n* = 13	ECMO, *n* = 7	Malignancy, *n* = 8
High urgency status, n (%)	24 (85.7)	12 (92.3)	7 (100)	5 (62.5)
IMPACT score, med (IQR)	8 (5.8–13)	7 (6–10)	14 (12.5–16.5)	4.0 (2.5–7.8)
ICU, n (%)	14 (50)	6 (46.2)	7 (100)	1 (12.5)
Intubated, n (%)	3 (10.7)	0	3 (42.9)	0
Infection, n (%)	20 (71.4)	13 (100)	7 (100)	0
ECMO support, n (%)	7 (25)	0	7 (100)	0
VAD, n (%)	7 (25)	5 (38.5)	0	2 (25)
eGFR, med (IQR)	84.7 (36.9–104.2)	93.4 (35–100.9)	120 (69.8–174.1)	67.3 (29.6–84.7)
Creatinine, mg/dl, med (IQR)	1.1 (0.8–1.8)	1.2 (0.9–1.9)	0.6 (0.5–1)	1.2 (1–2.2)
RRT, n (%)	6 (21.4)	3 (30)	2 (28.6)	1 (12.5)
Bilirubin, (mg/dl), med (IQR)	0.8 (0.5–1.1)	0.8 (0.5–1.2)	1 (0.8–2)	0.5 (0.4–0.8)
Diabetes (IDDM), n (%)	4 (14.3)	3 (23.1)	0	1 (12.5)

ECMO, extracorporeal membrane oxygenation; eGFR, estimated glomerular filtration rate; ICU, intensive care unit; IDDM, insulin-dependent diabetes mellitus; IMPACT, index for mortality prediction after cardiac transplantation; med (IQR), median and interquartile range; RRT, renal replacement therapy; VAD, ventricular assist device.

day, 3 times per week) was given for 6 months. CMV prophylaxis consisted of 100 ml of anti-CMV hyper-immunoglobulin (Cytotect; Biotest Pharmaceuticals Corporation, Boca Raton, Florida) on POD 1, 7, 14, and 28, and patients at high risk for CMV infection (recipient CMV antibody negative and donor CMV antibody positive) received oral valganciclovir (900 mg/day) for 3 months. CMV infection was monitored using PCR for CMV DNA, and patients with CMV DNA >1000 copies/mL on any PCR test were treated pre-emptively with valganciclovir adjusted according to their renal function.

ECP Protocol

ECP therapy was based on the previously published protocol by Barr et al. (13), consisting of a total of 24 ECP procedures during a 6-month period starting immediately after transplantation as follows: on POD 1 and 2, 5 and 6, 10 and 11, 17 and 18, 27 and 28, on two consecutive days every other week in months 2 and 3, and on two consecutive days once a month in months 4–6 (13). ECP was performed using the Cellex Photopheresis System (Therakos Ltd.; Mallinckrodt Pharmaceuticals) with either double- or single-needle access. Briefly, during an ECP session, 1500 ml of whole blood was processed, and peripheral blood mononuclear cells (MNCs) were separated by centrifugation (14). After MNC collection, the photosensitizer 8-methoxypsoralen (Uvadex) at a dose of 20 μg/ml was added to the MNC collection bag and cells were irradiated with ultraviolet A light (1.5 J/m^2) before being returned to the patient. For anticoagulation, acid citrate dextrose A was used at a ratio of 1:10 to avoid bleeding complications.

Statistical Analyses

Data including demographic and transplant variables were obtained from the Medical University of Vienna Heart Transplant Database. The statistical analyses were performed using the Statistical Program of Social Sciences 22.0 (SPSS Inc., Chicago, IL United States). Categorical variables are described by absolute and relative frequencies, and continuous variables by median and interquartile range (IQR). The Kaplan-Meier estimate was used for survival analysis. P-values below 0.05 were considered statistically significant.

RESULTS

Survival

One-year survival in these high-risk recipients was 88.5% by Kaplan-Meier estimate (25/28 patients). Infectious complications leading to septic multiorgan failure (MOF) were the cause of death in three

TABLE 3 | Indication for ECP.

Infection *n* **= 13 (46%)**	**Microbiological result**	**Site of infection at time of HTX**	
1	*Staph. haemolyticus/epidermidis*	Blood culture, postop sternal VAC and ECMO	
2	*E. faecalis*	Site of kidney transplant with postop local VAC therapy	
3	*Staph. epidermidis*	Blood culture	
4	*Klebsiella pn.*, *Proteus mirabilis*	Ascites	
5	*Staph. aureus*	Blood culture	
6	Hepatitis B PCR +	Blood culture; HTX in deep hypothermia with circulatory arrest	
7	*E. coli*	Recurrent endocarditis, BAL	
8	*Staph. aureus*	Blood culture	
9	*P. aeruginosa* 4MRGN	Blood culture, driveline, mediastinum	
10	*Staph. aureus*	Blood culture, mediastinum	
11	*P. aeruginosa*	Blood culture, driveline, mediastinum	
12	*Citrobacter koseri* ESBL, *Aspergillus fumigatus*	Fungal sinusitis	
13	*Staph. lugdunensis*	Blood culture, driveline	
ECMO *n* **= 7 (25%)**	**Cause of ECMO**	**Detail**	**Days on ECMO before HTX**
1	Post cardiotomy	Mech Bentall procedure; LVAD; LVAD explant	5
2	Myocardial infarction	STEMI with PCI, ischemic ventricular rupture	14
3	Post cardiotomy	MV-repair and AVR	25
4	Post cardiotomy	STEMI, CABG	27
5	Post cardiotomy (endocarditis)	Mitral and aortic valve replacement, CABG (CX)	17
6	Post cardiotomy	Type A dissection (mech Bentall)	23
7	Right heart failure	CMP with decompensation	1
Malignancy *n* **= 8 (29%)**	**Histology**	**Interval between diagnosis and HTX**	**Complete remission**
1	Myxofibrosarcoma heart	12 months	no
2	Synovial sarcoma heart	6 months	no
3	Osteosarcoma; breast cancer (recurrence)	30 years; 12 years (8 years)	yes
4	PTLD (HTX)	10 years	yes
5	Renal cell carcinoma	10 years	yes
6	ALL; cerebral recurrence	13 years; 5 years	yes
7	Non-Hodgkin's lymphoma	42 years	yes
8	Adenocarcinoma in donor lung	0	yes

ALL, acute lymphoblastic leukemia; AVR, aortic valve replacement; BAL, bronchoalveolar lavage; CABG, coronary artery bypass graft; CX, circumflex artery; CMP, cardiomyopathy; ECMO, extracorporeal membrane oxygenation; ECP, extracorporeal photopheresis; E. coli, Escherichia coli*;* E. faecalis, Enterococcus faecalis*; HTX, heart transplantation; IQR, interquartile range; LVAD, left ventricular assist device;* Klebsiella pn.*,* klebsiella pneumoniae*; mech, mechanical; MV, mitral valve; PCI, percutaneous coronary intervention; PCR, polymerase chain reaction;* P. aeruginosa, Pseudomonas aeruginosa*; postop, postoperative; PTLD, post-transplant lymphoproliferative disorder;* Staph., Staphylococcus *STEMI, ST-elevation myocardial infarction; VAC, vacuum assisted closure.*

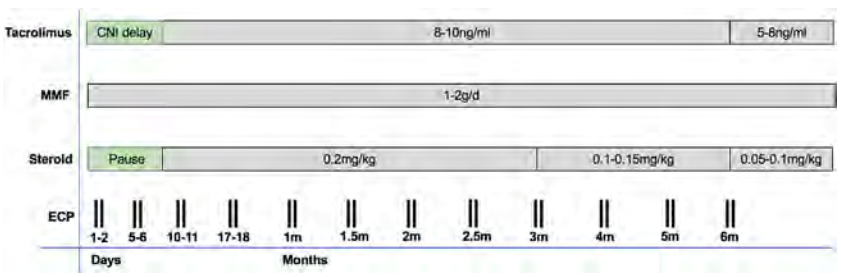

FIGURE 1 | An overview on our immunosuppressive protocol including ECP.

patients on POD 12, 51, and 351, respectively. One patient with a malignant tumor of the heart as transplant indication died due to recurrence of malignancy 400 days after HTX. Therefore, overall survival in our cohort was 84.0% ($n = 24$) with a median follow-up of 23.7 months (IQR 12.7–33.4). Considering the different indications for ECP, patients with pre-transplant infection had the highest mortality rate of 23% (3/13), patients with malignancy 12.5% (1/8), and there were no deaths in patients bridged to HTX with ECMO (see **Table 4**). The non-ECP cohort transplanted during the study period ($n = 172$) had an estimated 1-year survival rate of 93%.

Immunosuppressive Protocol

CNI delay was achieved in all patients with a median start time of tacrolimus on POD 3 (IQR 2–4), and the longest CNI delay was

TABLE 4 | Outcome variables.

	Total, *n* = 28	Infection, *n* = 13	ECMO, *n* = 7	Malignancy, *n* = 8
1-year survival, n (%)	25 (88.5)	10 (75)	7 (100)	8 (100)
Overall survival, n (%)	24 (84.0)	10 (75)	7 (100)	7 (87.5)
Follow-up, m, med (IQR)	23.7 (12.7–33.4)	23.6 (8.4–32.3)	30.7 (18.9–38.8)	24.1 (13.8–43.0)
ICU stay, d, med (IQR)	17.5 (10.8–31.8)	17.5 (10.5–29.5)	30 (15–32.5)	17.5 (10.5–29.5)
In hospital stay, d, med (IQR)	43 (32–55)	39.5 (32.5–54.3)	43.5 (35.5–54.5)	39.5 (32.5–54.3)
RRT, n (%)	13 (46.4)	8 (61.5)	2 (28.6)	3 (37.5)
Pneumonia, n (%)	5 (17.9)	3 (23.1)	0	2 (25)
Sepsis, n (%)	2 (7.1)	2 (15.4)	0	0
ACR≥2R in the first year, n (%)	4 (14.3)	1 (7.7)	1 (14.3)	2 (25)
AMR	1 (3.6)	1 (7.7)	0	0
PGD grade 3, n (%)	2 (7.1)	2 (15.4)	0	0

ACR, acute cellular rejection; AMR, antibody-mediated rejection; d, days; ICU, intensive care unit; med (IQR), median and interquartile range; m, months; PGD, primary graft dysfunction with grading according to the ISHLT, consensus 2014; RRT, renal replacement therapy.

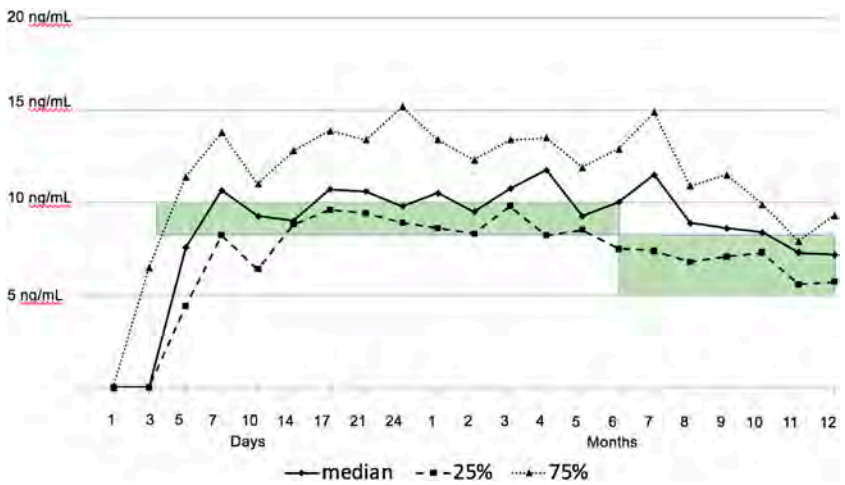

FIGURE 2 | Tacrolimus trough levels. The green bar highlights the intended target range of tacrolimus (8–10 ng/ml in months 1–6, and 5–8 ng/ml thereafter).

9 days in one patient. Target tacrolimus trough levels were attained for the whole patient cohort (see **Figure 2**). MMF was started on POD 0 in all patients. MMF was switched to everolimus in five patients of the malignancy group (62.5%). Steroids were given as described above. In our first two patients, a single dose of induction therapy with 100 mg of rabbit anti-thymocyte globulin (ATG) was given on POD 1.

All patients who survived the first 6 months received ECP according to the protocol. Overall, ECP was tolerated well. In one patient, elevated potassium levels occurred during the third ECP treatment and could not be attributed to ECP. Most likely, intravenous amphotericin A was administered too quickly, causing a shift of potassium (15, 16). Due to invasive fungal infection, maintenance immunosuppression (CNI, MMF, and steroid) and ECP were paused in two patients who died of sepsis leading to MOF on POD 12 and 51, respectively (see below).

Postoperative Infections

Severe bacterial ($n = 3$) and fungal ($n = 2$) infections emerged in five patients (17.9%) in the immediate postoperative period (see **Table 4**). All were lower respiratory tract infections necessitating either prolonged ventilation ($n = 3$) or reintubation ($n = 2$). The three patients with bacterial infections were successfully weaned from ventilation after targeted antimicrobial therapy. The two with invasive fungal infections died due to sepsis and MOF on POD 12 and 51. The identified pathogens in bronchoalveolar lavage and blood cultures were *Aspergillus niger* and *Candida albicans*, respectively. In both cases, the fungal strains were resistant to empirically administered antifung4al therapy. CMV disease with enteritis occurred in one CMV high-risk (D+/R-) patient 2 months after HTX, after prophylaxis with valganciclovir had been discontinued. However, the patient was successfully treated with valganciclovir in therapeutic dosage for 2 weeks. No other CMV infection was detected.

Sensitization and Rejection

Three patients showed sensitization prior to transplantation, with calculated panel-reactive antibodies of 23%, 51% and 67%, and were transplanted *via* negative virtual crossmatch, which was

confirmed by negative complement-dependent cytotoxicity (CDC) crossmatch after transplantation.

Donor-specific antibodies (DSA) were detected in five patients early after transplantation but disappeared or decreased significantly within the first 6 months after HTX. In one of these patients, histological findings revealed antibody-mediated rejection (1H) without increase of DSA in the first two biopsies of one patient. In addition to steroid therapy (500 mg methylprednisolone i.v. for 3 consecutive days), immunoadsorption treatment was started due to reduced biventricular function, which resolved after seven courses. All consecutive biopsies were negative.

During the first year, the incidence of ACR according to ISHLT criteria (≥2R) was 14.3% (n = 4), all occurring within the first month post transplantation. None showed hemodynamic compromise. All were treated successfully with i. v. steroid (500 mg methylprednisolone for 3 consecutive days). No patient showed recurrent rejection, nor rebound of ACR, after the end of ECP therapy. In one patient, immunosuppression was switched from tacrolimus to cyclosporine due to suspected tacrolimus-associated hyponatremia, 18 months post transplant. The patient consecutively developed ACR (ISHLT 2R) 3 months post conversion.

Recurrence of Malignancy

After a median follow-up of 24.1 months (13.8–43.0), all patients are free of cancer without cancer recurrence, except the two patients with malignant cardiac tumor as indication for HTX: one patient died due to disease progression 13.3 months after transplant, and the other is in good clinical condition after post-transplant hepatic metastasectomy and chemotherapy 59.5 months after HTX.

DISCUSSION

In this hypothesis-generating study including 28 selected high-risk HTX patients, an ECP protocol first described by Barr et al. (14) and accompanied by a reduced-intensity immunosuppressive protocol was successfully applied. The safety and efficacy of this protocol in challenging HTX patients were confirmed.

Due to constant improvements in the results of HTX, the number of high-risk patients eligible for transplantation has increased significantly (17). Recent changes in allocation policies benefit patients in more unstable pre-transplant conditions partly bridged with temporary mechanical assist devices or ventricular assist device (VAD) complications (infection) (18–20). Moreover, patients with a history of cancer, even cardiac cancers, are considered potential candidates for transplantation in many centers (2, 21, 22). However, the preoperative condition of a patient has been shown to be directly associated with risk of severe infection and mortality (1, 12). Several scores have been established to predict post-transplant survival based on the preoperative condition (23–25). The Index for Mortality Prediction After Cardiac Transplantation (IMPACT) score has been validated with United Network for Organ Sharing (UNOS) data and includes pre-transplant risk factors like infection, short term mechanical assist devices, and others (23). Data of the Spanish National Heart Transplant Registry revealed an association between preoperative Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) profiles and in-hospital mortality post-transplantation (1). Both reports have found infectious complications as one of the major causes of death post-transplantation (1, 17).

In our cohort, risk of early mortality was high, as 50% were already admitted to an intensive care unit before HTX and 25% were supported with temporary mechanical assist devices. This observation is supported by their high median IMPACT score of 8 (IQR 5.8–13).

Furthermore, patients with history of cancer might have a higher risk of developing malignancies after transplantation (2, 4). Overall immunosuppressive burden, time interval between pre-transplant cancer and transplantation, and cancer type seem to promote cancer development post-transplant (2, 4).

There is a general consensus that higher levels of immunosuppression are associated with a higher risk of infectious complications (26, 27). Moreover, critically ill patients seem to be immunocompromised (28). Therefore, it might be reasonable to aim for lower levels of immunosuppression after transplantation in patients at risk (5). However, strategies that avoid, delay, or minimize CNI use early after transplantation have shown higher rejection rates and the need for cytolytic antibody therapy, which bears a risk of infectious complications (5, 6, 29–31). On the other hand, immune monitoring of transplant patients has shown promising results, but never has reached routine clinical use (32).

ECP is an established therapy for the treatment of acute and chronic graft-versus-host disease after hematopoietic cell transplantation and rejection of solid organ transplantation and has been used for at least 25–30 years for these indications (33, 34). Nevertheless, the complete mode of action has not yet been elucidated. An increase in regulatory T cells and plasmacytoid dendritic cells has been observed during the use of ECP, which might have an immunomodulatory effect that leads to a more tolerogenic state of the immune system (35). Urbani et al. showed improved survival in high-risk liver transplant patients treated with ECP in combination with a CNI-sparing protocol, in comparison with a historical control group receiving standard triple immunosuppression (36). Although they partly failed in their main purpose of reducing CNI-induced toxicity, they did observe low infection rates and no deaths due to infection, compared with 16.5% in the control group. CNI was delayed by an average of 12.9 days. Acute rejection rates were numerically increased and rejection showed up earlier, which might have been due to the shorter duration of ECP therapy in combination with CNI delay. Only one prospective study has examined ECP early after HTX, comparing triple-drug immunosuppression with or without 6 months of ECP therapy (13). Barr et al. observed significantly lower rejection rates, similar overall infection, and lower CMV infection rates in the ECP group compared with the control group. No increase in rejection was detected after the end of ECP treatment. In both

studies, ECP was tolerated very well and not associated with adverse events.

Based on the experience of those two studies, we developed our alternative protocol to test in three high-risk groups (infection, bridge to transplant *via* ECMO, history of cancer). We aimed to analyze the safety and efficacy of this protocol before starting a prospective randomized trial comparing this protocol with standard immunosuppression in control groups. We decided to combine the ECP protocol of Barr et al. with a reduced-intensity immunosuppressive protocol consisting of CNI delay (median of 3 days), lower target levels of tacrolimus (8–10 mg/ml instead of 12–15 ng/ml) and delayed steroid therapy at a lower dose (start: day 7 with 0.2 mg/kg instead of 1 mg/kg). MMF use was similar to that in our routine protocol. We applied no ATG induction therapy in all but the two of our first patients (100mg ATG once on POD1), assuming this would lower the risk of severe infections early after transplantation without risking higher rates of rejection. We trusted that a combination of tacrolimus/MMF would be more effective than cyclosporine/azathioprine, even at lower tacrolimus target levels and with delayed start of tacrolimus and oral steroid. Therefore, we defined as a secondary outcome an acceptable rate of acute rejections in the first year after transplantation as a rate 1.3–1.5-fold higher than the observed rejection rate with our conventional immunosuppressive protocol (15–20%) (37). This target rejection rate was similar to rejection rates in several other published studies over the last 10–15 years (20–25% rejection) (17, 38, 39, 45). Moreover, we assumed that lower-intensity immunosuppression without induction therapy with ATG might have a protective effect against cancer recurrence.

One-year survival in our high-risk patient cohort was slightly lower than in the overall patient cohort transplanted in the same time period (88.5% vs 93%). Nevertheless, risk-adjusted patient survival calculated using the IMPACT score was better than expected (88.5% vs 84.6%). Surprisingly, our patients with the highest predicted mortality (ECMO bridging to HTX) had 100% survival, compared with 71% expected survival. Patients with pre-transplant infections did worse than expected (75% vs 86% survival) but two patients died in the immediate postoperative period from fungal infection with strains resistant to empirically administered antifungal therapy. Both had developed grade III primary graft dysfunction (40). Whether the complicated postoperative course with primary graft dysfunction and ECMO additionally increased the risk of infection is an open question.

The incidence of severe infections in our cohort was 17.9% (n = 5), and they were lower respiratory tract infections necessitating prolonged ventilation or reintubation. Three of them were in the pre-transplant infection cohort. The lower overall rate of severe infections was surprising, as 66.7% of our patients had elevated risk due to infection and/or ECMO support pre-transplant. Nevertheless, our data are in accord with earlier reports showing that ECP after HTX is not associated with higher rates of infection despite earlier concerns about ECP leading to potential T-cell damage with subsequent reduced immune defense (14, 41, 42).

An unexpected finding was the low rate of ACR (14.3%) in the first year, all occurring in the first month. ACR episodes were without hemodynamic compromise. ACR was not associated with lower tacrolimus levels. Tacrolimus was delayed until a median of 3 days after transplantation, and the target range was reached at the end of the first week. Median achieved tacrolimus levels were in the upper target range over the first year, and this might have contributed to the low rejection rates. Nevertheless, maintenance steroids were started on day 7, at a lower dosage as recommended by guidelines, and were tapered until the end of the first year (34).

Most prospective randomized immunosuppressive trials in heart transplantation have reported an acute rejection rate of 15–25% during the first year (39, 43, 44). Based on previous reports, we assume that our ECP protocol had an impact on the low rejection rates (7, 14). Barr et al. showed a reduction from 82% to 61% of patients with at least one rejection episode when ECP was added to an immunosuppressive protocol consisting of cyclosporine and azathioprine (14). Similarly, we did not observe any rebound of acute rejection after the end of ECP therapy (14). Only one patient had a rejection episode during long-term follow-up, after switching from tacrolimus to cyclosporine for immunosuppression, on day 660. We can only speculate whether ECP induction treatment would allow even further decrease in overall immunosuppression early after transplantation. There is not enough evidence to proof that this protocol is safe in immunological high-risk patients.

Our eight transplant patients with a high neoplastic risk were heterogeneous: five had a prior history of cancer (three hematologic, one renal cancer, one with osteosarcoma and breast cancer), two had cardiac sarcoma at the time of transplantation, and one received a heart from a donor with lung cancer detected after procurement. Those with a history of cancer were cancer free for at least 5 years pre-transplant. In a retrospective analysis of 111 thoracic transplant patients from northern European centers, time from cancer detection to transplantation had an impact on cancer-free post-transplant outcomes and survival (21). Shorter time between cancer and transplant was associated with higher post-transplant cancer rates and worse outcome (21). Our patient cohort showed a similar pattern, with no post-transplant recurrence in all patients with ≥5 years after cancer detection, whereas both patients with sarcoma of the heart showed re-emergence of cancer within 1 year, leading to death in one of them. In a UNOS registry analysis, Yoosabai et al. reported a higher risk of post-transplant cancer and a median time of 3.2 years until cancer development in patients with pre-transplant cancer history (45). The low cancer rate we observed in this study might have been influenced by the short median follow-up of 20.7 months.

Limitations

This is a hypothesis-generating study describing the outcome of a heterogeneous pilot group. Longer follow-up is needed to evaluate the incidence of cancer recurrence in patients with history of cancer. There is a strong need to compare our approach in a prospective randomized study with control groups for each indication.

Conclusion

To our knowledge, this is the first description of the use of prophylactic ECP as an additional immunomodulatory therapy

combined with reduced-intensity immunosuppressive maintenance therapy. There are no published data on a comparable protocol in HTX patients. In our heterogeneous pilot group of high-risk HTX patients, this innovative approach was safe, with low overall risk of rejection, and an effective strategy to address their high risk of infection or malignancy. Based on our data, future studies should be undertaken in a prospective randomized setting.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review board of the Medical University of Vienna (EK 1107/2020). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JG, AZ, AA-Z, and NW collected and analyzed the data, performed all statistical procedures and developed the manuscript. All authors managed patients during the study period and critically reviewed the manuscript before publication.

CONFLICT OF INTEREST

JG, AZ, RK, and NW have been part of Mallinckrodt's Speaker bureau.

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

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ORIGINAL RESEARCH
published: 21 March 2022
doi: 10.3389/ti.2022.10185

Impact of Pretransplant Renal Replacement Therapy on Clinical Outcome After Isolated Heart Transplantation

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End stage renal disease (ESRD) is a contraindication to isolated heart transplantation (HT). However, heart candidates with cardiogenic shock may experience acute kidney injury and require renal replacement therapy (RRT) and isolated HT as a life-saving operation. The outcomes, including survival and renal function, are rarely reported. We enrolled 569 patients undergoing isolated HT from 1989 to 2018. Among them, 66 patients required RRT before HT (34 transient and 32 persistent). The survival was worse in patients with RRT than those without (65.2% vs 84.7%; 27.3% vs 51.1% at 1- and 10-year, $p < 0.001$ and $p = 0.012$, respectively). Multivariate Cox analysis identified pre-transplant hyperbilirubinemia (Hazard ratio (HR) 2.534, 95% confidence interval (CI) 1.098–5.853, $p = 0.029$), post-transplant RRT (HR 5.551, 95%CI 1.280–24.068, $p = 0.022$) and post-transplant early bloodstream infection (HR 3.014, 95%CI 1.270–7.152, $p = 0.012$) as independent risk factors of 1-year mortality. The majority of operative survivors (98%) displayed renal recovery after HT. Although patients with persistent or transient RRT before HT had a similar long-term survival, patients with persistent RRT developed a high incidence (49.2%) of dialysis-dependent ESRD at 10 years. In transplant candidates with pretransplant RRT, hyperbilirubinemia should be carefully re-evaluated for the eligibility of HT whereas prevention and management of bloodstream infection after HT improve survival.

Keywords: acute kidney injury, renal replacement therapy, heart transplant, long term survival, renal failure

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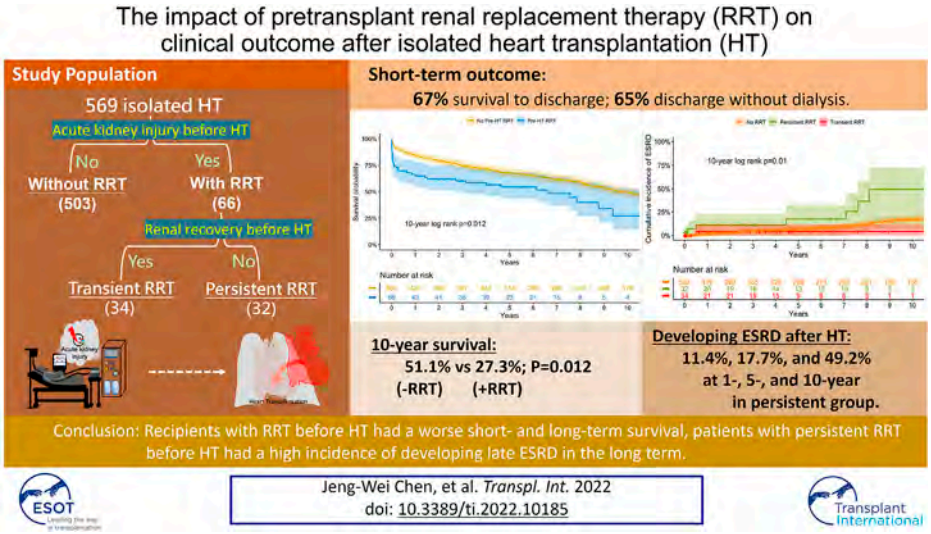
Received: *04 November 2021*
Accepted: *27 January 2022*
Published: *21 March 2022*

Citation:
Chen J-W, Chou N-K, Wang C-H, Chi N-H, Huang S-C, Yu H-Y, Chen Y-S and Hsu R-B (2022) Impact of Pretransplant Renal Replacement Therapy on Clinical Outcome After Isolated Heart Transplantation. Transpl Int 35:10185. doi: 10.3389/ti.2022.10185

INTRODUCTION

Heart transplant candidates with acute decompensated heart failure and complicating acute kidney injury (AKI) experience increased fluid overload and subsequent heart function deterioration before heart transplantation (HT) (1). With the advancement of mechanical circulatory support (MCS) and renal replacement therapy (RRT), more end-stage heart failure patients could wait for HT with a

Abbreviations: AKI, acute kidney injury; ECMO, extracorporeal membrane oxygenation; ESRD, end stage renal disease; HT, heart transplantation; MCS, mechanical circulatory support; RRT, renal replacement therapy; UNOS, united network for organ stage; VAD, ventricular assisted device.



GRAPHICAL ABSTRACT |

bridge using MCS devices such as an extracorporeal membrane oxygenator (ECMO) or a ventricular assisted device (VAD) (2, 3). More than 30% of patients with MCS devices develop AKI and require RRT (4), The requirement of RRT before HT is one of the major risk factors of 1-year mortality after HT (5).

The renal outcome in patients with AKI requiring RRT before HT varies widely. Some patients have improved renal function quickly after achieving stable hemodynamics, while others require persistent RRT even after HT (6). Persistent RRT can increase the long-term mortality after HT (5).

Combined heart and kidney transplantation (HKT) has been recommended in heart candidates with comorbid renal dysfunctions (7, 8). However, candidates with AKI requiring RRT might receive isolated HT as a life-saving operation. As the clinical outcomes, including survival and renal function, in these patients, are rarely reported, we sought to investigate the impact of pretransplant RRT on clinical outcomes after isolated HT (5).

PATIENTS AND METHODS

The study protocol was approved by the Ethics Review Board of the National Taiwan University Hospital (202006034RINC). The requirement for informed consent was waived. This retrospective cohort study included patients who underwent HT in this hospital between January 1989 and December 2018 (**Figure 1**).

For heart candidates with stage 4 or 5 chronic kidney disease (CKD) or dialysis-dependent end stage renal disease (ESRD), transplant nephrologists were consulted for evaluation of combined HKT. Seventeen patients with combined HKT were excluded. However, heart candidates with AKI requiring RRT and who had the potential for renal recovery may have received isolated HT.

Indications for RRT included metabolic acidosis (pH < 7.2), electrolyte imbalance (potassium > 6.5 mmol/L), severe pulmonary edema, and fluid overload that was unresponsive to intravenous diuretics. All our patients received the less stressful continuous veno-venous hemofiltration as the first-choice modality of RRT until termination of RRT or HT. Two patients required intermittent hemodialysis for inadequate urine output after stopping continuous veno-venous hemofiltration with stable hemodynamics. A nephrologist was regularly consulted for renal function evaluation. Each patient's urine output, fluid status, and biochemical data were evaluated daily to assess renal recovery and facilitate the eventual RRT discontinuation. Patients with RRT before HT were divided into two subgroups according to whether they could be weaned from RRT before HT. We defined transient RRT as weaned from RRT before HT and persistent RRT as requiring RRT until HT.

Mechanical Support Before HT

Policies regarding MCS before HT have been reported previously (9). In patients suffering from profound cardiogenic shock and who were potential candidates for HT, ECMO was applied to identify any complication that may have arisen as a result of resuscitation that would be a contraindication for HT. The relative and absolute contraindications of HT were followed according to the International Society for Heart and Lung Transplantation (ISHLT) listing criteria (9). After 4–5 days, VAD implantation was considered as a bridge to HT for patients who could not be weaned from ECMO.

Immunosuppression After HT

All patients received triple-drug immunosuppressive therapy according to previously reported protocols (10,11, 12). Briefly, rabbit anti-thymocyte globulin was administered post-transplantation for 3–5 days. Cyclosporine was administered orally within 5 days of transplantation or after renal function recovery. To reduce nephrotoxicity, the cyclosporine dose was

decreased to maintain a serum trough level of 250–350 ng/ml during the first 3 months and 150–250 ng/ml at 1 year. Azathioprine was administered post-transplantation, and the dose was adjusted to maintain a leukocyte count of 4,000–6,000/mm^3. Prednisone (0.5 mg/kg/day) was administered postoperatively and tapered to 0.2 mg/kg/day by 1 month. Since 2004, mycophenolate mofetil has been used instead of azathioprine for primary immunosuppression (12, 13). Everolimus has been used for primary immunosuppression since 2010.

Data Collection

Pre-transplant data including recipient's characteristics, complicated bloodstream infection (BSI), and dialysis duration were collected by chart review. The baseline renal function was assessed upon admission. The estimated glomerular filtration rate was calculated using the Cockcroft-Gault formula (14). Perioperative data included the donor's age and sex, and allograft ischemic time. Post-transplant data included mortality, date, and cause of death, date of the first dialysis, early BSI within 30-days after HT, and major postoperative complications.

Statistical Analysis

All statistical analyses were performed using R (version 4.1.0; R Foundation for Statistical Computing, Vienna, Austria). The continuous variables were expressed as the median and interquartile range, and categorical variables were described by frequency values. Comparison of patients with and without RRT before HT was performed using Fisher's exact test to compare categorical variables when observed frequencies were <5 in more than 25% of cells and Mann-Whitney *U* test for continuous variables. Subgroup analyses were performed in patients with transient RRT and patients with persistent RRT until HT. Cox proportional regression was used to identify independent risk factors of 1-year mortality in patients requiring RRT before HT and included all potential predictors with a p-value < 0.1 in the univariate analysis.

The results of multivariable models are reported as the hazard ratio with corresponding 95% confidence intervals. The cumulative incidence of survival curves and ESRD were plotted using the Kaplan-Meier method. The survival rates between groups were compared using the log-rank test. Competing risk analysis was carried out with cumulative incidence of ESRD and death before ESRD by the abovementioned groups. p values < 0.05 were considered statistically significant.

RESULTS

Patient Demographics

This study enrolled 569 patients receiving isolated HT. Sixty-six patients requiring RRT before HT were compared with 503 patients without RRT before HT. Patients with RRT were further divided into two subgroups: 34 transient RRT and 32 persistent RRT.

Table 1 shows the basic patient demographics. The most common etiology of heart failure in patients with RRT was dilated cardiomyopathy (33%) followed by ischemic cardiomyopathy (29%). Patients with RRT were older and had a greater body weight, a higher incidence of diabetes, and a worse baseline renal function. More patients with RRT had United Network for Organ Sharing (UNOS) status 1A (89%), MCS (82%), resternotomy surgery (77%), and previous cardiopulmonary resuscitation (44%). Patients with persistent RRT had a higher rate of diabetes and an even worse baseline renal function than patients with transient RRT.

Short-Term Outcomes

Hospital survival and renal outcome after HT were shown in **Figure 1**. In patients with RRT, 43 (65%) patients survived

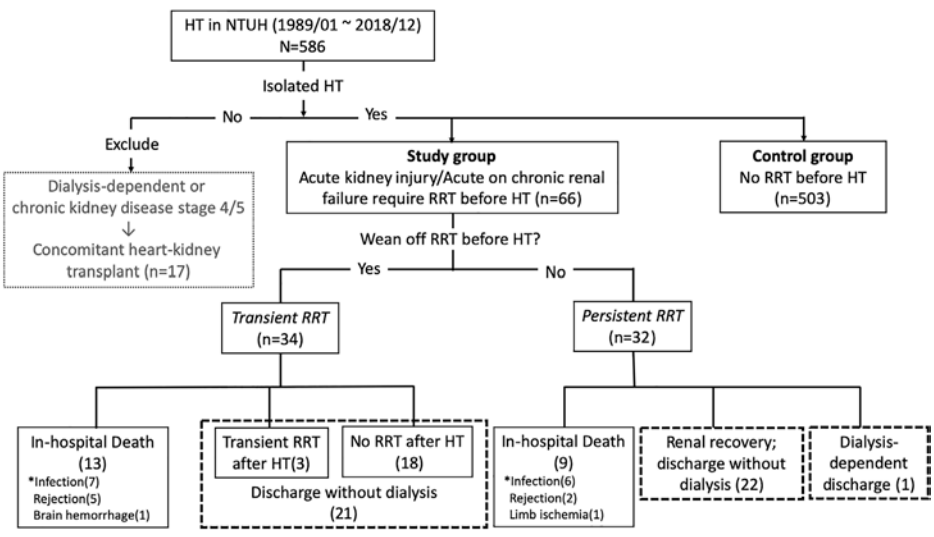


FIGURE 1 | Details regarding the inclusion of study patients. RRT, renal replacement therapy; HT, heart transplantation.

TABLE 1 | Characteristics and clinical outcomes of patients with and without renal replacement therapy (RRT) before heart transplantation (HT).

Variables median (IQR); n (%)	Without RRT *n* = 503	With RRT *n* = 66	*p*-value	Subgroup[a]		
				Transient *n* = 34	Persistent *n* = 32	*p*-value
Age, year	49 (34–58)	51 (40–58)	0.025	52 (39–58)	51 (40–58)	0.948
Woman	84 (17)	10 (15)	0.750	3 (9)	7 (22)	0.180
Body weight, kilograms	62 (54–70)	67 (58–75)	0.002	70 (56–76)	65 (59–75)	0.735
Blood type			0.622			0.238
A	158 (31)	23 (35)		11 (32)	12 (38)	
B	140 (28)	19 (29)		10 (29)	9 (28)	
O	158 (31)	21 (32)		13 (38)	8 (25)	
AB	47 (9)	3 (4)		0	3 (9)	
Comorbidities						
Smoker	112 (22)	16 (24)	0.742	9 (26)	7 (22)	0.777
Hyperlipidemia	131 (26)	20 (30)	0.473	7 (21)	13 (41)	0.109
Diabetes	113 (22)	24 (36)	0.013	8 (24)	16 (50)	0.040
Etiology			<0.001			0.597
Dilated cardiomyopathy	263 (52)	22 (33)		10 (29)	6 (19)	
Ischemic cardiomyopathy	129 (26)	19 (29)		7 (21)	11 (34)	
Acute myocarditis	11 (2)	7 (11)		4 (12)	3 (9)	
Acute myocardial infarction	25 (5)	13 (20)		7 (21)	6 (19)	
Congenital heart disease	18 (4)	2 (3)		1 (3)	1 (3)	
Retransplantation	14 (3)	3 (4)		2 (6)	1 (3)	
Rheumatic heart disease	25 (5)	0		0	0	
Others	18 (4)	0		0	0	
Pre-transplant						
UNOS status 1A	139 (28)	59 (89)	<0.001	31 (91)	28 (88)	0.628
Mechanical ventilation	76 (15)	55 (83)	<0.001	29 (85)	26 (81)	0.748
Intra-aortic balloon pump	69 (14)	34 (52)	<0.001	16 (47)	18 (56)	0.473
Mechanical circulatory support	107 (21)	54 (82)	<0.001	30 (88)	24 (75)	0.210
ECMO	36 (7)	19 (29)	<0.001	9 (26)	10 (31)	0.668
Non-durable VAD ± ECMO	52 (10)	33 (50)	<0.001	19 (56)	14 (44)	0.325
Durable VAD	19 (4)	2 (3)	0.964	2 (6)	0	
Previous cardiopulmonary resuscitation	76 (15)	29 (44)	<0.001	15 (44)	14 (44)	1
Baseline renal function						
Creatinine, mg/dl	1.1 (0.9–1.5)	1.8 (1.1–2.5)	<0.001	1.4 (1.0–2.3)	2.0 (1.5–2.7)	0.015
Blood urea nitrogen, mg/dl	24 (17–34)	34 (24–54)	<0.001	32 (20–43)	41 (27–66)	0.264
eGFR, ml/min/1.73m^2	68 (51–86)	42 (29–67)	<0.001	59 (31–82)	35 (27–47)	0.002
BSI within 2 weeks before HT	6 (1)	8 (12)	<0.001	4 (12)	4 (13)	1
Blood T-bil before HT, mg/dl	1.3 (0.8–2.3)	1.5 (0.9–3.8)	<0.001	1.4 (0.9–3.9)	1.6 (1.0–3.9)	0.646
Length of RRT, days	0	17 (7–35)	<0.001	16 (6–35)	18 (10–35)	0.675
Length of in-hospital waiting, days	2 (0–29)	36 (18–64)	<0.001	51 (23–88)	27 (13–46)	0.020
Donor characteristics						
Age, year	32 (22–44)	39 (28–47)	0.008	37 (28–47)	43 (29–50)	0.495
Body weight, kg	63 (55–70)	65 (58–77)	0.507	69 (60–79)	63 (55–70)	0.083
Woman	141 (28)	20 (31)	0.873	6 (18)	14 (44)	0.033
Intra-operative						
Resternotomy	151 (30)	46 (70)	<0.001	25 (74)	21 (66)	0.485
Allograft ischemic time, min	144 (100–211)	168 (127–228)	0.020	167 (130–212)	177 (116–237)	0.221
Post-transplant						
RRT	71 (14)	43 (65)	<0.001	19 (56)	24 (75)	0.103
Early BSI in 30-day	47 (9)	13 (20)	0.01	7 (21)	6 (19)	0.851
1-year mortality	77 (15)	23 (33)	<0.001	13 (38)	10 (31)	0.552
Follow-up duration, year	7.2 (2.7–12.2)	3.2 (0.1–6.6)	<0.001			

BSI, bloodstream infection; eGFR, estimated glomerular filtration rate; ECMO, extracorporeal membrane oxygenator; T-bil: total bilirubin; UNOS, united network for organ sharing; VAD, ventricular assisted device.

[a]*Patients with RRT, before HT, were divided into two subgroups according to whether they could be weaned from RRT, before HT, or not: transient RRT (weaned from RRT, before HT) and persistent RRT (requiring RRT, until HT).*

discharge without RRT: 21 (62%) and 22 (69%) patients in transient and persistent RRT subgroups, respectively. In the transient RRT subgroup, 3 required a short-term RRT after HT. In the persistent RRT subgroup, 1 was dialysis-dependant after discharge. There was no difference in the rate of survival to discharge without RRT between transient and persistent RRT subgroups.

As shown in **Table 1**, patients with RRT had a higher 1-year mortality rate than patients without RRT (15% versus 33%, $p < 0.001$). The most common cause of post-transplant death in patients without RRT was primary graft failure (10 of 30, 33%) within the first month and infection (20 of 47, 43%) from 1 month to 1 year. However, in patients with RRT, the

TABLE 2 | Cox regression for 1-year mortality in patients requiring renal replacement therapy (RRT) before heart transplantation (HT).

Variable	Univariate			Multivariate		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Recipient						
Age	1.012	0.979–1.046	0.482			
Woman	1.735	0.643–4.68	0.277			
Body weight, kilograms	0.98	0.951–1.01	0.18			
Blood type						
O	1					
A	0.646	0.24–1.737	0.387			
B	0.817	0.304–2.196	0.689			
Smoker	1.438	0.591–3.498	0.424			
Hyperlipidemia	0.761	0.3–1.933	0.566			
Diabetes	0.946	0.401–2.233	0.9			
Previous cardiopulmonary resuscitation	1.806	0.792–4.12	0.16			
Resternotomy	1.787	0.663–4.816	0.251			
UNOS status 1A	0.629	0.187–2.119	0.455			
Mechanical ventilator	0.902	0.307–2.652	0.851			
Intra-aortic balloon pump	0.845	0.373–1.914	0.686			
Mechanical circulatory support						
non-use	1					
ECMO	1.767	0.554–5.638	0.336			
Non-durable VAD ± ECMO	0.618	0.186–2.053	0.432			
Durable VAD	1.872	0.209–16.787	0.575			
Baseline renal function						
Creatinine, mg/dl	1.113	0.803–1.543	0.519			
Blood urea nitrogen, mg/dl	1.008	0.993–1.023	0.278			
eGFR, ml/min/1.73 m^2	0.987	0.97–1.004	0.122			
Diagnosis						
Dilated cardiomyopathy	1					
Acute myocarditis	0.719	0.153–3.387	0.677			
Acute myocardial infarction	0.826	0.249–2.743	0.755			
Ischemic cardiomyopathy	1.084	0.393–2.989	0.877			
Congenital heart disease	2.164	0.27–17.328	0.467			
Retransplantation	1.062	0.133–8.493	0.955			
Pretransplant						
Persistent RRT	1.286	0.564–2.933	0.550			
Length of RRT, days	0.99	0.974–1.005	0.202			
Length of ECMO support, days	0.958	0.892–1.029	0.243			
Length of hospital-stay, days	0.995	0.984–1.005	0.336			
Blood total-bilirubin ≥ 3 mg/dl	3.198	1.405–7.280	0.006	2.534	1.098–5.853	0.029
BSI within 2 weeks	1.774	0.603–5.224	0.298			
Donor						
Age	1.003	0.971–1.036	0.869			
Woman	0.768	0.303–1.948	0.578			
Body weight, kilograms	0.995	0.964–1.027	0.755			
Allograft ischemic time, minutes	1.002	0.996–1.007	0.562			
Posttransplant						
RRT	7.260	1.699–31.015	0.007	5.551	1.280–24.068	0.022
Early BSI in 30-day	4.642	2.001–10.769	<0.001	3.014	1.270–7.152	0.012

BSI, bloodstream infection; CI, confidence interval; eGFR, estimated glomerular filtration rate; ECMO, extracorporeal membrane oxygenator; HR, hazard ratio; UNOS, united network for organ sharing; VAD, ventricular assisted device.

most common cause of death was infection both within the first month (8 of 15, 53%) and from 1 month to 1 year (5 of 8, 62.5%) after HT.

Thirteen patients with RRT (20%) had 16 episodes of early BSI after HT, and the source was the wound for 5 patients (31%), the catheter for 3 (19%), pneumonia for 3 (19%), urosepsis for 1 (6%) and primary BSI for 4 (25%).

Table 2 showed the cox proportional analysis for the risk factors of 1-year mortality. Both univariate and multivariate analysis identified pre-transplant hyperbilirubinemia (serum total-bilirubin > 3 mg/dl) (hazard ratio (HR): 2.534, 95% confidence interval (CI): 1.098–5.853, $p = 0.029$), post-transplant RRT (HR: 5.551, 95% CI: 1.280–24.068, $p = 0.022$) and post-transplant early BSI (HR: 3.014, 95% CI: 1.270–7.152, $p = 0.012$) as significant risk factors of 1-year mortality after HT.

Patients with RRT had a higher incidence of receiving pre-transplant ECMO support than patients without RRT (74%

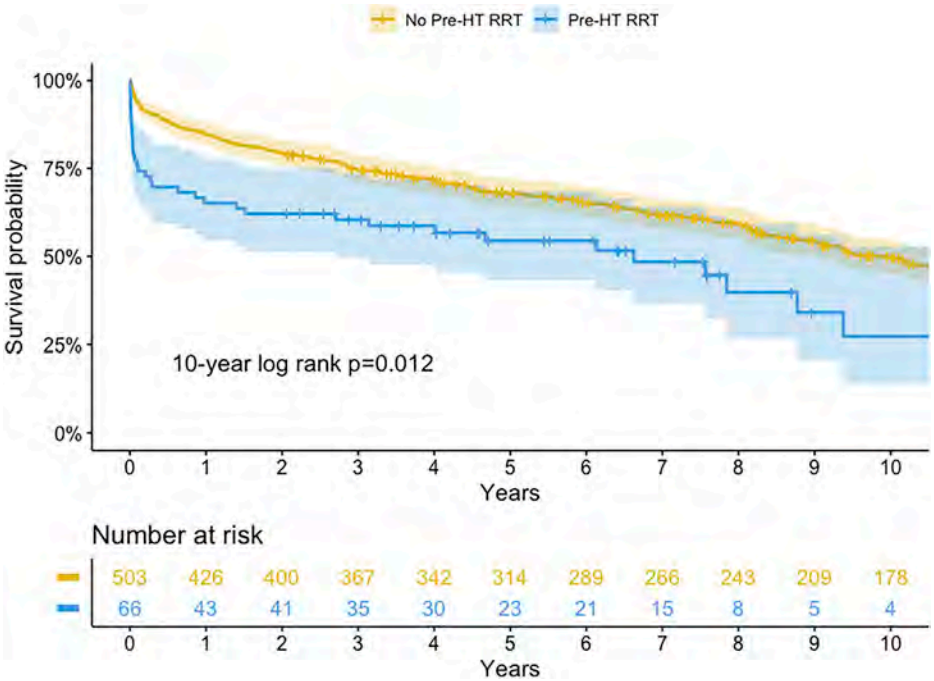


FIGURE 2 | Kaplan-Meier survival curve for patients with and without renal replacement therapy (RRT) before heart transplantation (HT) (log-rank *p*-value = <0.001, 0.011, 0.012 at 1, 5, and 10-year, respectively).

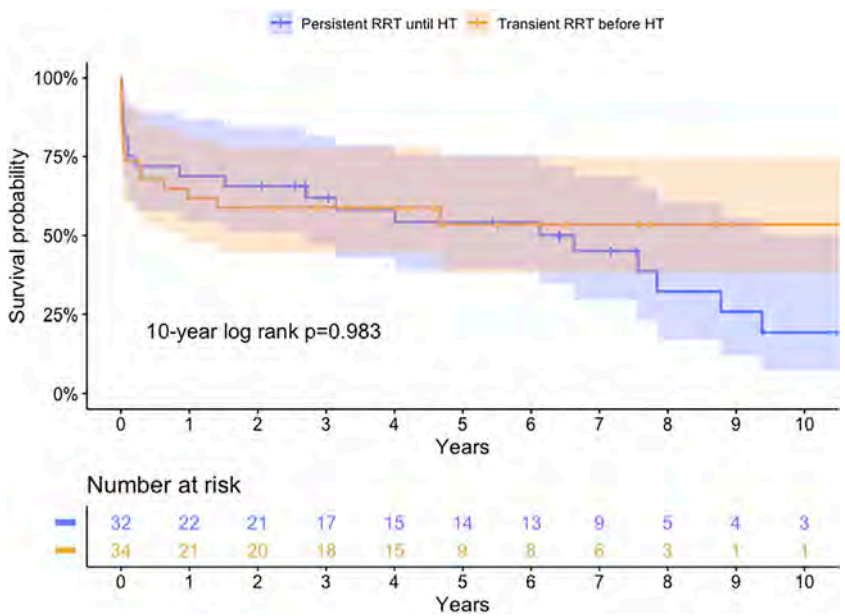


FIGURE 3 | Kaplan-Meier survival curve for transient or persistent renal replacement therapy (RRT) before heart transplantation (HT) (log-rank *p*-value = 0.554, 0.558, 0.983 at 1, 5, and 10-year, respectively).

versus 16%, $p < 0.001$). These incidences were not significantly different between the transient and persistent RRT subgroups (82% versus 66%, $p = 0.120$). Regarding the whole patient population, pre-transplant ECMO support was not a significant risk factor for 1-year mortality after adjusting for pre-transplant RRT (HR 1.355, 95% CI 0.822–2.233, $p = 0.234$). It was also not a significant risk factor for 1-year mortality in patients requiring RRT (**Table 2**).

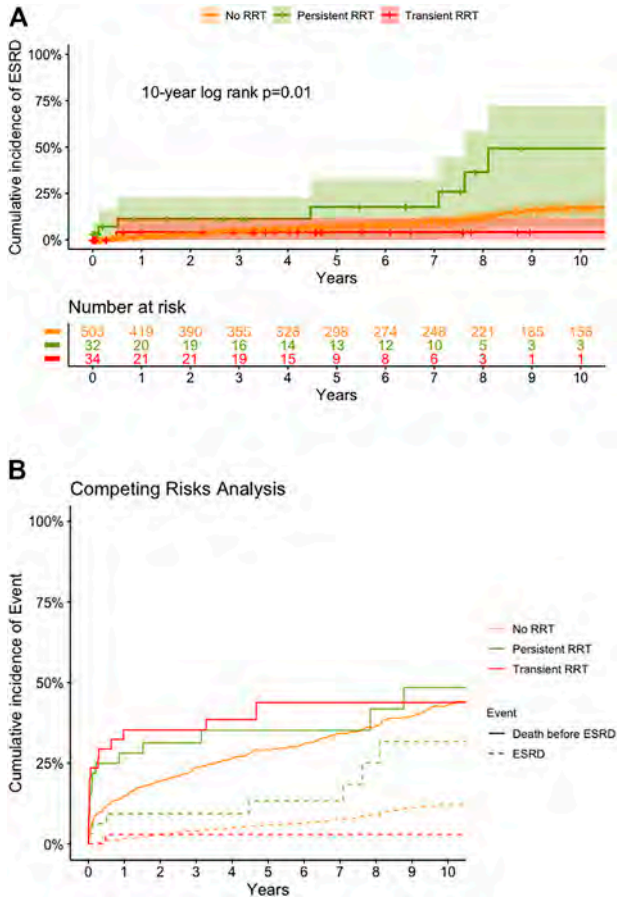


FIGURE 4 | Cumulative incidence of end stage renal disease (ESRD) **(A)** and death before ESRD **(B)**, by competing risk analysis, by various groups. ESRD, end stage renal disease; RRT, renal replacement therapy; HT, heart transplantation.

Long-Term Outcome

The long-term survival is shown in **Figures 2** and **3**. The 1-, 5-, and 10-year survival rates were 65.2, 54.4, and 27.3% in patients with RRT compared to 84.7%, 68.3%, and 51.1% in patients without RRT. Patients with RRT had a significantly lower overall survival rate than those without RRT (Log-rank p-value = 0.012 at 10-year).

We compared the RRT group to heart transplant patients on UNOS 1A status without RRT (n = 144). Survival was worse in patients with RRT than patients on UNOS 1A without RRT (65.2% vs 79.4%; 27.3% vs 44.7% at 1- and 10-year, Log-rank p-value < 0.001 and = 0.092, respectively).

For patients with RRT, the 1-, 5-, and 10-year survival rates were 61.8%, 53.5%, and 53.5% in the transient RRT subgroup compared with 68.8%, 54.2%, and 19.3% in the persistent RRT subgroup. The long-term survival rates were not different between subgroups (Log-rank p-value = 0.983 at 10-year).

The long-term renal outcome in each group is shown in **Figure 4**. The cumulative incidence of late ESRD in patients without RRT was 2.0, 7.4, and 17.3% at 1-, 5-, and 10-year (**Figure 4A**). For patients with RRT, the 10-year cumulative incidence of late ESRD in the transient RRT subgroup was 4.2% with only one patient developing ESRD at 6 months after HT. However, for patients with persistent RRT subgroup, the cumulative incidence of late ESRD was 11.4, 17.7, and 49.2% at 1-, 5-, and 10-year, which was much higher than that in patients without RRT and in the transient RRT subgroup. To correct the competing effect of death and ESRD, we analyzed the cumulative incidence of late ESRD and death before ESRD (**Figure 4B**). Patients with persistent RRT subgroup continued to have a much higher rate of late ESRD (p = 0.010).

Because the immunosuppressive regimen was changed during the observation period, we further stratified the patients into three groups according to different eras: 1989–2003 (n = 187), 2004–2009 (n = 173), and 2010–2018 (n = 209). Among patients from different eras, the long-term survival and the cumulative incidence of ESRD in a 10-year period showed no significant difference (log-rank p value = 0.6 and 0.059, respectively).

DISCUSSION

This is the first study to report on the clinical outcomes of isolated HT in patients who required RRT before HT. Previous studies have shown that pretransplant renal dysfunction was associated with a high incidence of postoperative ESRD and RRT after HT (15). Pretransplant RRT was also associated with a poor outcome after HT (5, 6, 15). However, patients with cardiogenic shock complicating AKI could have renal function recovery following hemodynamic stabilization (16), and early RRT could improve survival and gain a better recovery of renal function after HT (17). The recovery of renal function depends on several factors including patient comorbidity and MCS device-related infection, hemolysis, and thromboembolic events (16, 18). For transplant surgeons, it was very difficult to predict the renal outcome after HT and allocate organ replacement to those transplant candidates with complicating AKI and requiring RRT before HT.

In this study, patients requiring RRT before HT had poor short-term and long-term survival. Several recipient variables have been recognized as risk factors for mortality after HT, including old age, resternotomy, hospitalization, intubation, low estimated glomerular filtration rate, serum total-bilirubin level >2 mg/dl, and use of MCS (5,19). In this study, more than 80% of patients requiring RRT had a high rate of UNOS 1A status, resternotomy surgery, and MCS use. All these factors could contribute to the inferior survival observed in patients requiring RRT.

The clinical outcomes following HT have improved over time (20). The 10-year survival rate among all HT patients in our hospital was >50%. However, in patients with pre-transplant RRT, the 10-year survival rate was comparatively low. As shown in **Figure 2**, most of the mortality in patients requiring RRT occurred within 1 year after HT. After 1 year, the rate of survival decline was not different between patients with and without RRT. Therefore, it was imperative to identify the risk factors associated with 1-year mortality after HT in patients with pre-transplant RRT. Careful patient selection

could achieve better survival after HT in this critical situation. In this study, we identified pre-transplant hyperbilirubinemia, post-transplant RRT, and post-transplant early BSI as the independent risk factors of 1-year mortality. Hyperbilirubinemia was the most significant pretransplant predictor of 1-year mortality after HT.

The occurrence of liver dysfunction was not rare in patients with heart failure and probably even more common in heart transplant candidates. Ischemic liver hypoperfusion and hepatic congestion were the two major pathogenic mechanisms in cardiogenic shock and congestive heart failure (21). Heart failure complicating with liver dysfunction adversely affected prognosis. Furthermore, preoperative liver dysfunction had a significant impact on the survival of patients after HT (22). The presence of pre-transplant hyperbilirubinemia indicated an advanced heart failure and a combination of renal failure and liver dysfunction implied an even worse outcome after HT (23).

According to the ISHLT report, acute graft failure was the most common cause of mortality within the first 30-days after HT (24). In this study, the major cause of 30-day mortality was an infection in patients with pre-transplant RRT. Previous studies have reported that pre-transplant RRT was a major risk factor of post-transplant BSI in HT (2, 25). Both use of RRT and MCS before HT would further increase the risk of BSI before and after HT (12, 26). In our study, 17 (26%) patients with pre-transplant RRT had pre-transplant BSI, and 8 of them (12%) had a positive blood culture within 2-week before HT. Early BSI after HT was one of the major risk factors of 1-year mortality.

CKD and dialysis-dependent ESRD were major long-term complications after HT. The 10-year incidence of developing ESRD was 6% in ISHLT reports (22). We have previously reported that Chinese heart recipients had a higher incidence of developing CKD and dialysis-dependent ESRD than recipients from other countries. The cumulative incidence of late dialysis-dependent ESRD was 16% at 10-year after HT, and the prognosis was poor after RRT (27, 28). In this study, among patients with persistent RRT before HT, 72% of them had renal function recovery after HT and were discharged without RRT. However, the cumulative incidence of developing dialysis-dependent ESRD was 49% at 10-year. Whether a combined HKT could improve survival in these cases was unknown. However, donor shortage made combined organ transplantation difficult. The clinical outcomes of combined HKT were unsatisfactory in heart transplant candidates in UNOS status IA and requiring resternotomy surgery (8, 13). Considering the potential of renal recovery after HT and donor shortage, a staged approach with renal transplant after HT was advisable for those heart transplant candidates requiring RRT after isolated HT (29).

This study has several limitations. First, it was retrospective and the details of residual renal function could not be obtained completely. Second, the small case numbers limited the statistical power to have more independent risk factors of 1-year mortality. Third, the study spanned almost 3 decades, which introduced limitations since there have been significant inevitable practice changes in the management of cardiogenic shock, AKI, and HT over time. Fourth, based on our data, the high incidence of ECMO support among patients requiring RRT may influence renal recovery and outcome. However, the small case number and heterogeneous type of MCS did not allow for further exploration of the impact of ECMO. As there is a possibility of bias, a large registry, propensity score-matched, and multi-center studies are warranted for further exploration of these issues.

This was the first study to focus on the long-term outcomes for patients requiring RRT before HT. Careful patient selection and proper postoperative management in this critical situation are important to achieving better survival rates after HT. Heart transplant candidates with pretransplant RRT and hyperbilirubinemia should be carefully re-evaluated for the eligibility of HT because of an inferior survival rate. Prevention and management of BSI after HT were crucial in patients requiring RRT before HT.

Conclusion

For isolated HT, patients with RRT before HT had a worse short-term and long-term survival. Renal function recovered after HT in the majority of operative survivors. Patients with persistent or transient RRT before HT had similar long-term survival. However, patients with persistent RRT until HT had a higher incidence of late ESRD requiring RRT.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Review Board of the National Taiwan University Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

J-WC and R-BH conceived of the presented idea. J-WC collected the clinical data and performed the analysis. N-KC, C-HW, N-HC, S-CH, H-YY, Y-SC and R-BH contributed data. R-BH supervised the findings of this work. J-WC wrote the paper. All authors discussed the results and contributed to the final manuscript.

FUNDING

This work was supported by the National Science Council of Taiwan (108-2314-B-002-088-MY3).

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

The authors acknowledge statistical assistance provided by the Center of Statistical Consultation and Research in the Department of Medical Research, National Taiwan University Hospital.

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ORIGINAL RESEARCH
published: 28 March 2022
doi: 10.3389/ti.2022.10161

Plasma Biomarkers for Clinical Assessment of Bone Mineral Density in Heart Transplanted Patients—A Single-Center Study at Skåne University Hospital in Lund

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We aimed to identify plasma biomarkers that predict changes in bone mineral density (BMD) and increase the understanding of impaired BMD after heart transplantation (HT). Twenty-eight adult patients were included. Data, including densitometry and 29 plasma proteins, before and 1 year after HT were analyzed. Pre-HT plasma levels of fibroblast growth factor 23 (FGF23) correlated with post-HT T score in lumbar spine, adjusted for age, gender, and BMI (1.72 [95% CI 1.33; 2.22], $p = 0.011$). Change (Δ; post-HT—pre-HT) in plasma levels of melusin correlated to ΔT score from the lumbar spine ($p = 0.028$). Δplasma levels of TR-AP, ITGB2, and Stromelysin-1 correlated to ΔT score from the femoral neck ($p < 0.05$). However, no correlations remained after adjustments for age, gender, and BMI. In conclusion, elevated plasma FGF23 pre-HT predicted an increase in lumbar BMD after HT. However, the results are surprising since FGF23 is known to be inversely correlated with BMD. This may partly be explained by the complex pathophysiology in this particular cohort. Due to the explorative nature of the study and the small sample size, further investigations of biochemical markers on bone metabolism in this patient population are encouraged.

Keywords: heart transplantation, plasma biomarkers, bone mineral density, osteoporosis, bone metabolism

OPEN ACCESS

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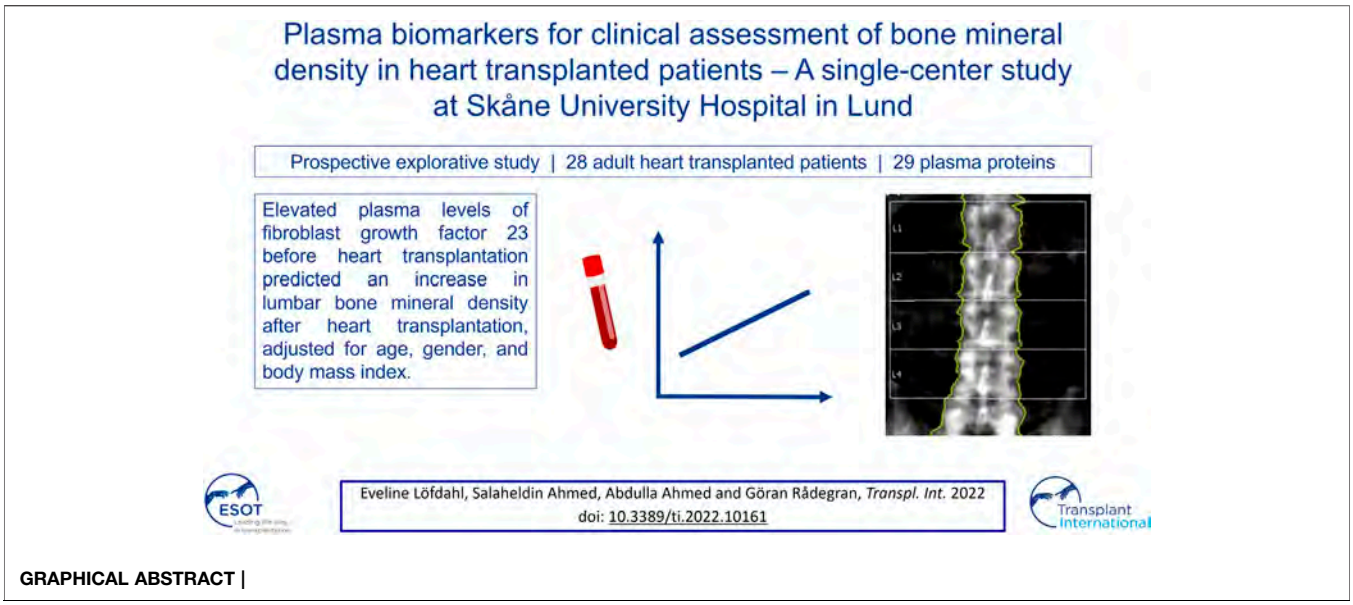
Received: *29 October 2021*
Accepted: *10 February 2022*
Published: *28 March 2022*

Citation:
Löfdahl E, Ahmed S, Ahmed A and Rådegran G (2022) Plasma Biomarkers for Clinical Assessment of Bone Mineral Density in Heart Transplanted Patients—A Single-Center Study at Skåne University Hospital in Lund. Transpl Int 35:10161. doi: 10.3389/ti.2022.10161

Abbreviations: AU, arbitrary units; BMD, bone mineral density; BMI, body mass index; CCN1, CCN family member 1; CI, confidence interval; COL1A1, collagen alpha-1(I) chain; CS, corticosteroids; DXA, Dual-energy X-ray absorptiometry; EDTA, ethylenediaminetetraacetic acid; FGF23, fibroblast growth factor 23; GFR, glomerular filtration rate; GPNMB, transmembrane glycoprotein NMB; hOSCAR, osteoclast-associated immunoglobulin-like receptor; HT, heart transplantation; ITGAV, integrin alpha-V; ITGB2, integrin beta-2; ITGB5, integrin beta-5; IQR, interquartile range; LCPR, Lund Cardio Pulmonary Registry; MEPE, matrix extracellular phosphoglycoprotein; MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; MMP12, macrophage metalloelastase; RANK, receptor activator of nuclear factor κ-B; SD, standard deviation; TIMP4, metalloproteinase inhibitor 4; TR-AP, tartrate-resistant acid phosphatase type 5; WISP-1, WNT1-inducible-signaling pathway protein 1.



GRAPHICAL ABSTRACT |

INTRODUCTION

Osteoporosis is a common condition in patients who have undergone heart transplantation (HT) [1]. It may arise as a side effect of the immunosuppressive therapy given after HT, or as a consequence of various factors related to the heart failure prior to HT, including immobilization, impaired renal function, and heart failure medications [2-7]. Osteoporosis increases the risk of bone fractures which increase morbidity and mortality rates, of which the excess mortality rate within the first year after a hip fracture has been found to range from 8.4% to 36% [8-10]. Also, about 50% of patients who suffer a hip fracture are not able to walk independently afterwards long-term [10]. It has previously been reported that the mortality rate increases 1.5-fold for each standard deviation (SD) decrease in bone mineral density (BMD) in patients with osteoporosis [11]. Hence, impaired bone health constitutes a major limitation for survival and quality of life after HT. Early identification and treatment of osteoporosis are therefore of great clinical interest.

Emerging indicators of bone disease are biochemical markers which reflect the dynamics of bone metabolism, i.e., the process of bone formation and bone resorption [12]. Markers for bone formation reflect the function and recruitment of osteoblasts, including alkaline phosphatase (total and bone-specific), osteocalcin, and procollagen type I N-terminal propeptide, which all can be measured in plasma [13]. Markers for bone resorption, on the other hand, reflect the byproducts of osteoclast activity and include hydroxyproline, pyridinoline, and deoxypyridinoline, which are found in urine, whereas N-terminal and C-terminal crosslinked peptides can be found in both plasma and urine [13].

The current gold standard for assessment of bone strength is BMD which is measured using Dual-energy X-ray absorptiometry (DXA) [8]. Although DXA is widely available and provides a non-invasive method of bone strength assessment, it is also considered a static measurement of bone strength and a relatively expensive investigation, with a reported median cost of $98 per investigation in the Unites States in 2010 [13, 14]. It has been hypothesized that biochemical markers of bone metabolism may prove to be more useful than DXA as they are non-invasive, relatively inexpensive, and due to increasing availability of clinical chemistry analyzers in laboratories [15].

Whether biochemical biomarkers on bone metabolism before HT are useful in assessing bone health after HT is, however, unclear. Therefore, we aimed to identify plasma biomarkers that may predict changes in BMD and increase the understanding of impaired BMD after HT.

PATIENTS AND METHODS

Study Design and Patient Selection

In the present observational cohort study, 29 patients with advanced heart failure were enrolled between October 2011 and July 2015. Patients were evaluated before and 1-year after HT, during the routine clinical evaluations at Skåne University Hospital, Lund, Sweden. Inclusion criteria were adult patients (≥18 years old) available in Lund Cardio Pulmonary Registry (LCPR), a prospective cohort of blood samples and clinical data, and a part of Region Skåne Biobank. Blood samples were collected at the time of inclusion and at the 1-year follow-up. Diagnostic and transplantation procedures were conducted at Skåne University hospital in Lund, Sweden, in accordance with the prevailing guidelines of The International Society for Heart and Lung Transplantation at the time of inclusion [16, 17].

Written informed consents were acquired from all patients upon enrollment. The study was approved by the local ethical board in Lund, Sweden (diary numbers: 2010/114; 2010/442; 2011/368; 2011/777; 2014/92 and 2015/270) and was conducted in agreement with the declarations of Helsinki and Istanbul.

TABLE 1 | Patient characteristics.

Recipient characteristics	Pre-HT			Post-HT		
	N = 28		Missing	*N* = 28		Missing
Age (years)	50	(45; 60)	0	52	(47; 62)	0
Female, *N* (%)	6	(21)	0	6	(21)	0
BMI (kg/m^2)	27	(24; 28)	1	26	(23; 30)	0
Serum creatinine (μmol/L)	106	(88; 121)	0	114	(97; 142)	0
Creatinine based eGFR	65	(57; 82)	0	54	(45; 75)	0
Iohexol-GFR (ml/min/1.73 m^2)	56	(45; 69)	13	53	(46; 78)	2
Daily administration of systemic CS, *N* (%)	1	(4)	0	27	(96)	0
Primary indication for HT			0			
Dilated cardiomyopathy	19	(68)				
Hypertrophic cardiomyopathy	2	(7)				
Ischemic cardiomyopathy	2	(7)				
Other	5	(18)				
BMD (g/m^2)						
Lumbar spine	1.135	(1.028; 1.272)		1.113	(0.944; 1.188)	3
Femoral neck	1.001	(0.946; 1.063)		0.904	(0.818; 0.966)	3
T score (SD)						
Lumbar spine	−0.7	(−1.6; 0.4)		−1.0	(−2.3; −0.2)	2
Femoral neck	−0.7	(−1.0; −0.1)		−1.4	(−1.9; −0.9)	2

Values for continuous variables are expressed as median (IQR), whereas categorical values are expressed as number (%). BMD, bone mineral density; BMI, body mass index; CS, corticosteroids; HT, heart transplantation; (e)GFR, (estimated) glomerular filtration rate; IQR, interquartile range; SD, standard deviation.

Blood Sampling and Protein Analysis

Between October 2011 and February 2017, venous, non-fasting, blood samples were collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes from patients during the routine clinical evaluations before- and at the 1-year follow-up after HT. The blood samples were thereafter centrifuged at 2,000 rpm × 10 min at 20°C and plasma aliquots subsequently stored in LCPR at −80°C.

Twenty-nine proteins related to bone metabolism were analysed in May 2017 using the following multiplex immunoassay panels (Proseek Multiplex cardiovascular II, cardiovascular III and Oncology II panels, Olink Proteomics, Uppsala, Sweden). Proximity extension assay is based on protein specific oligonucleotide-linked antibodies and quantitative microfluidic PCR for protein detection. When a pair of antibodies are in proximity due to binding to the target protein, their respective oligonucleotide strands hybridize, forming a protein-unique DNA reporter sequence, which is subsequently used to quantify the proteins using real-time PCR [18].

The twenty-nine proteins analysed were cadherin-5, CCN family member 1 (CCN1), collagen alpha-1(I) chain (COL1A1), decorin, fibroblast growth factor 23 (FGF23), glypican-1, integrin alpha-V (ITGAV), integrin beta-2 (ITGB2), integrin beta-5 (ITGB5), matrilysin, matrix extracellular phosphoglycoprotein (MEPE), matrix metalloproteinase (MMP)2, MMP9, MMP12, melusin, metalloproteinase inhibitor 4 (TIMP4), osteoclast-associated immunoglobulin-like receptor (hOSCAR), osteonectin, osteopontin, osteoprotegerin, perlecan, prolargin, receptor activator of nuclear factor κ-B (RANK), stromelysin-1, syndecan-1, tartrate-resistant acid phosphatase type 5 (TR-AP), thrombospondin-2, transmembrane glycoprotein NMB (GPNMB), and WNT1-inducible-signaling pathway protein 1 (WISP1).

The proteins' levels were expressed in a log2 normalized protein expression scale (NPX) as arbitrary units, corresponding to the inverted Ct-values, unless otherwise stated, i.e., linear NPX [18]. All panels are validated regarding sensitivity, dynamic range, specificity, precision, and scalability. Information about panel specific validation can be found at www.olink.com/downloads.

Bone Mineral Density and Other Data Collection

Measurements of BMD was collected from clinical records during the transplantation assessment before HT and from the routine check-up 1 year after HT. BMD was expressed in T score (SD) and was obtained using DXA from the lumbar spine and femoral neck.

Other data collected included age (recipient), gender, body mass index (BMI [kg/m^2]), primary indications for HT, and administration of systemic corticosteroids (CS). Glomerular filtration rate (GFR [ml/min/1.73 m^2]) was based on measurement of iohexol clearance or serum levels of creatinine (i.e., estimated [e]GFR). The eGFR was calculated using the CKD-EPI formula, in accordance with the current guidelines from the Kidney Disease: Improving Global Outcomes (KDIGO) working formulation [19].

Study Setup

To explore the predictive value of protein levels and BMD, correlations between pre-HT protein levels and post-HT T score in lumbar spine and femoral neck were performed. Next, to reflect the dynamics of protein levels in relation to the dynamics of BMD, correlations of Δ (delta; post-HT—pre-HT values) protein levels vs. ΔT score in lumbar spine and femoral neck were performed.

TABLE 2 | Regression analyses between pre-HT levels of plasma proteins measured in normalized protein expression scale, expressed in AU, and post-HT T score from the lumbar spine (A) and femoral neck (B). [a]Adjusted with Benjamini & Hochberg (false discovery rate) correction.

(A) Plasma protein	β	(95% CI)	p	Adjusted p[a]
FGF23	1.72	(1.33; 2.22)	<0.001*	0.011*
Osteopontin	2.44	(1.40; 4.27)	0.005*	0.066
Osteoprotegerin	3.21	(1.30; 7.91)	0.018*	0.111
Perlecan	2.24	(1.22; 4.11)	0.016*	0.111
RANK	2.02	(1.17; 3.49)	0.019*	0.111
COL1A1	2.20	(1.14; 4.27)	0.028*	0.135
ITGB2	2.18	(1.03; 4.63)	0.053	0.219
Melusin	1.22	(1.00; 1.50)	0.066	0.240
WISP1	1.78	(0.94; 3.36)	0.087	0.258
ITGB5	2.32	(0.92; 5.88)	0.089	0.258
Stromelysin-1	1.51	(0.93; 2.43)	0.107	0.259
MEPE	1.79	(0.91; 3.54)	0.106	0.259
MMP2	2.59	(0.80; 8.42)	0.126	0.261
MMP9	1.52	(0.92; 2.51)	0.117	0.261
Prolargin	3.43	(0.64; 18.40)	0.164	0.317
Syndecan-1	1.71	(0.79; 3.70)	0.184	0.334
Matrilysin	1.39	(0.78; 2.47)	0.270	0.459
TIMP4	1.48	(0.73; 2.99)	0.285	0.459
Osteonectin	2.10	(0.46; 9.58)	0.349	0.533
Glypican-1	1.60	(0.54; 4.72)	0.403	0.584
ITGAV	1.85	(0.35; 9.80)	0.478	0.660
Cadherin-5	1.38	(0.47; 4.07)	0.566	0.746
Decorin	1.25	(0.50; 3.13)	0.631	0.766
HOSCAR	1.52	(0.28; 8.32)	0.634	0.766
TR-AP	0.83	(0.32; 2.16)	0.703	0.778
Thrombospondin-2	0.79	(0.22; 2.90)	0.724	0.778
MMP12	1.08	(0.76; 1.54)	0.681	0.778
GPNMB	1.11	(0.16; 7.57)	0.917	0.950
CCN1	1.00	(0.41; 2.44)	0.994	0.994
(B) Plasma protein				
Melusin	1.15	(1.02; 1.28)	0.029*	0.758
GPNMB	0.40	(0.14; 1.12)	0.095	0.758
HOSCAR	0.44	(0.18; 1.12)	0.100	0.758
ITGB5	1.54	(0.90; 2.66)	0.130	0.758
Osteopontin	1.33	(0.92; 1.92)	0.138	0.758
COL1A1	1.35	(0.90; 2.03)	0.159	0.758
Thrombospondin-2	0.60	(0.29; 1.24)	0.183	0.758
FGF23	1.10	(0.91; 1.33)	0.313	0.947
Syndecan-1	0.82	(0.52; 1.29)	0.405	0.947
MMP12	0.92	(0.75; 1.13)	0.436	0.947
ITGB2	1.19	(0.75; 1.90)	0.465	0.947
TR-AP	0.82	(0.47; 1.41)	0.478	0.947
Prolargin	0.72	(0.26; 1.96)	0.525	0.947
Osteonectin	1.33	(0.55; 3.23)	0.531	0.947
Stromelysin-1	1.08	(0.81; 1.44)	0.618	0.947
Cadherin-5	0.86	(0.46; 1.61)	0.642	0.947
MMP9	1.07	(0.79; 1.45)	0.682	0.947
MEPE	0.92	(0.61; 1.39)	0.696	0.947
CCN1	1.11	(0.66; 1.85)	0.704	0.947
Perlecan	0.93	(0.63; 1.38)	0.732	0.947
Osteoprotegerin	1.10	(0.61; 1.97)	0.760	0.947
ITGAV	1.16	(0.44; 3.06)	0.767	0.947
Matrilysin	0.96	(0.68; 1.34)	0.799	0.947
Glypican-1	0.93	(0.49; 1.75)	0.816	0.947
RANK	1.04	(0.73; 1.49)	0.819	0.947
Decorin	1.04	(0.62; 1.77)	0.874	0.947
WISP1	1.02	(0.69; 1.51)	0.913	0.947
MMP2	0.97	(0.48; 1.99)	0.941	0.947
TIMP4	0.99	(0.65; 1.49)	0.947	0.947

[a]*Adjusted with Benjamini & Hochberg (false discovery rate) correction.*

*AU, arbitrary units; CCN1, CCN family member 1; CI, confidence interval; COL1A1, collagen alpha-1(I) chain; FGF23, fibroblast growth factor 23; ITGAV, integrin alpha-V; ITGB2, integrin beta-2; ITGB5, integrin beta-5; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; TIMP4, metalloproteinase inhibitor 4; hOSCAR, osteoclast-associated immunoglobulin-like receptor; RANK, receptor activator of nuclear factor κ-B; TR-AP, tartrate-resistant acid phosphatase type 5; GPNMB, transmembrane glycoprotein NMB; WISP1, WNT1-inducible-signaling pathway protein 1. *Indicates statistical significance.*

Statistical Analysis

Linear regression models were employed to describe the relation between each of the plasma protein levels pre-HT and T score from the lumbar spine and femoral neck post-HT, respectively. Similarly, the relation between Δplasma protein levels and ΔT score from the lumbar spine and femoral neck was investigated in linear regression models. We adjusted for multiple testing using the Benjamini and Hochberg (false discovery rate) correction (Q = 5%). We used Pearson correlation coefficients to evaluate the relationship between pre-HT plasma protein levels and post-HT T scores as well as the relationship between Δplasma protein levels and ΔT scores. Simple linear regressions were calculated in order to predict pre-HT plasma levels of FGF23 by GFR based on iohexol clearance and serum levels of creatinine. All analyses were performed in R v.4.1 (R Core Development Team 2021), and a p-value of <0.05 was considered statistically significant. The median and interquartile range (IQR) were calculated for continuous variables.

RESULTS

Study Population

Of the 29 patients, one was retransplanted and was therefore excluded. Of the remaining included 28, pre-HT data was collected at a median of 115 (70; 237) days before HT and post-HT data was collected at a median of 395 (369; 429) days after HT. The most frequent primary indication for HT was dilated cardiomyopathy (68%). Baseline characteristics, as well as follow-up data 1 year after HT, are displayed in **Table 1**.

Maintenance Immunosuppressive Therapy

Immunosuppressive agents were tapered after HT in accordance with local guidelines, previously described elsewhere [20]. A total of 64% received a combination of prednisolone, tacrolimus, and mycophenolate mofetil; 14% received prednisolone, cyclosporine, and mycophenolate mofetil; 14% received prednisolone, tacrolimus, and azathioprine; whereas 7% received other combinations. Only one patient was completely free of systemic corticosteroids at the 1-year post-HT check-up.

Pre-HT FGF23 Correlated Independently With Post-HT T Score in the Lumbar Spine

In linear regression analyses, pre-HT plasma levels of FGF23 correlated with post-HT T score in lumbar spine adjusted for age, gender, and BMI (1.72 [95% CI 1.33; 2.22], $p = 0.011$). All correlations between pre-HT levels of proteins and post-HT T score from the lumbar spine and femoral neck are presented in **Table 2**. Protein levels from both pre-

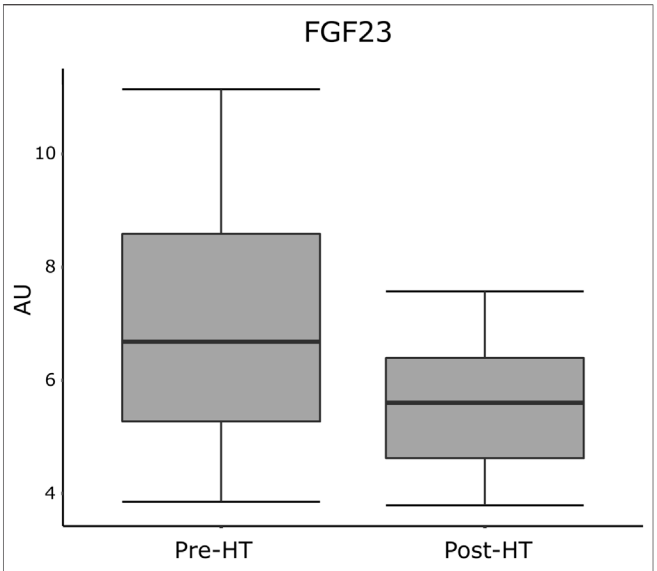


FIGURE 1 | Plasma levels of FGF23 from pre- and post-HT measured in normalized protein expression scale, expressed in AU. AU, arbitrary units; FGF23, fibroblast growth factor 23; HT, heart transplantation.

and post-HT are displayed in boxplots in **Figure 1** (FGF23) and in **Supplementary Figure S1** (remainder). Correlations between pre-HT plasma protein levels and post-HT T score from the lumbar spine and femoral neck are shown in **Supplementary Figure S2**.

In a sub -analysis, pre-HT plasma levels of FGF23 were inversely correlated with pre-HT iohexol based GFR, suggesting a FGF23 factor decrease of 0.99 (95% CI 0.97; 1.00) arbitrary units (AU) ($p = 0.029$). Likewise, pre-HT FGF23 levels decreased with a factor of 0.99 (95% CI 0.97; 1.00) AU by every unit increase in pre-HT creatinine-based GFR, however, this relationship was statistically not significant ($p = 0.072$).

Dynamics of Plasma Protein Levels in Relation to Bone Mineral Density Evolution

Regression analyses between Δplasma protein levels and ΔT score from the lumbar spine and femoral neck are shown in **Table 3**. In the unadjusted analysis, Δplasma levels of melusin correlated to ΔT score from the lumbar spine (1.20 [95% CI 1.03; 1.40], $p = 0.028$). Δplasma levels of TR-AP, ITGB2, and Stromelysin-1 correlated to ΔT score from the femoral neck (1.23 [95% CI 1.07; 1.42], 1.25 [95% CI 1.03; 1.52], and 0.90 [95% CI 0.81; 0.99], respectively, all with $p < 0.05$). However, after adjustments for age, gender, and BMI, no significant correlations remained. Correlations between Δplasma protein levels and ΔT score from the lumbar spine and femoral neck are shown in **Supplementary Figure S3**.

DISCUSSION

Impaired BMD is commonly found in patients who have undergone HT, leading to significant impact on morbidity and mortality [9, 10]. Early risk stratification and prevention of the development of osteoporosis is therefore of great interest. Emerging indicators of bone disease are plasma bone turnover markers which reflect the dynamics of bone metabolism. Such biochemical markers are considered beneficial with regard to availability and cost-effectiveness when compared to DXA which constitutes the current gold standard method for assessment of BMD [15]. Hence, identification of biochemical markers for the prediction of osteoporosis after HT is of particular interest.

The present single-center observational cohort study aimed to identify plasma biomarkers that may predict changes in BMD and increase the understanding of impaired BMD after HT. This may enable better prediction of impaired skeletal health and improve outcome in this patient population. The present study showed that plasma levels of FGF23 before HT correlated with T score in the lumbar spine after HT, independent of age, gender, and BMI. However, no correlations between changes in plasma levels of biochemical markers and T scores were found. The findings suggest that post-HT BMD loss may be predicted by pre-HT measurements of serum FGF23.

A positive correlation was found between pre-HT levels of FGF23 and post-HT T score in lumbar spine. FGF23, mainly secreted by osteocytes and osteoblasts in bone, plays a significant role in bone mineralization by stimulating phosphaturia, as well as suppressing the production of 1,25-dihydroxyvitamin D, resulting in inhibited bone mineralization [21]. Thus, the findings of the present study are contradictory. In a study by Valentin et al. (2013) on mutant mice, it was concluded that FGF23 plasma levels strongly correlates with circulating calcium levels, suggesting that suppressed FGF23 levels protects from hypocalcemia by reduced inhibitory effect on 1,25-dihydroxyvitamin D production [22]. To our knowledge, no previous study on the correlation of FGF23 levels and BMD after HT have been conducted. However, Jovanovich et al. (2013) found in a prospective, longitudinal study of community-dwelling adults aged 65 or older, including >3,000 participants with a median follow-up of 9.6 (IQR = 5.1; 11.0) years, that FGF23 was weakly associated with increased BMD in both lumbar spine and hip, but no associations were detected between FGF23 levels and fracture risk [23]. Similarly, FGF23 correlated positively with BMD in lumbar spine and hip in a study by Marsell et al. (2008), including >3,000 male participants aged 69–80 years [24]. However, the correlations were discovered to be dependent on BMI. Thus, these results partly support those of the present study. In addition, FGF23 levels are also known to increase in relation to progression of kidney dysfunction, which is common in HT candidates [25]. In a previous study at our center, it was concluded that the occurrence of kidney dysfunction, measured by iohexol clearance, increased over time after HT, reaching 25% with CKD stage ≥4 by the fifth post-operative year [26]. It is furthermore known that DXA from the lumbar spine might be overestimated in cases of vascular calcification, which is a common feature in patients with chronic kidney disease [27, 28]. All in all, FGF23 predicted a higher lumbar T score after HT, which may partly be explained by the complex pathophysiological mechanisms in this particular patient cohort.

Plasma levels of FGF23 correlated positively with T score in the lumbar spine, but not with T score in the femoral neck. In a cross-sectional study, Rupp et al. assessed levels of FGF23 and bone microarchitecture in 82 patients with osteoporosis [29].

TABLE 3 | Regression analyses between Δplasma protein levels measured in normalized protein expression scale, expressed in AU, and ΔT score from the lumbar spine (A) and femoral neck (B).

(A) Plasma protein	β	(95% CI)	p	Adjusted p[a]
ΔMelusin	1.20	(1.03; 1.40)	0.028*	0.809
ΔOsteoprotegerin	1.01	(0.48; 2.14)	0.974	0.993
ΔCCN1	0.96	(0.48; 1.94)	0.912	0.993
ΔWISP1	1.02	(0.62; 1.68)	0.935	0.993
ΔCOL1A1	1.34	(0.75; 2.39)	0.337	0.993
ΔITGB2	0.84	(0.42; 1.69)	0.624	0.993
ΔITGAV	0.53	(0.15; 1.88)	0.334	0.993
ΔDecorin	0.58	(0.13; 2.61)	0.485	0.993
ΔMMP2	1.04	(0.54; 2.02)	0.908	0.993
ΔStromelysin-1	0.97	(0.67; 1.40)	0.868	0.993
ΔMatrilysin	0.81	(0.38; 1.72)	0.589	0.993
ΔOsteonectin	3.25	(0.55; 19.20)	0.206	0.993
ΔOsteopontin	1.25	(0.86; 1.81)	0.259	0.993
ΔTR-AP	1.14	(0.67; 1.94)	0.641	0.993
ΔMMP9	1.31	(0.97; 1.77)	0.089	0.993
ΔITGB5	1.55	(0.55; 4.35)	0.417	0.993
ΔSyndecan-1	1.23	(0.84; 1.81)	0.298	0.993
ΔCadherin-5	0.34	(0.09; 1.25)	0.117	0.993
ΔGlypican-1	0.84	(0.31; 2.25)	0.725	0.993
ΔThrombospondin-2	0.77	(0.29; 2.07)	0.615	0.993
ΔMMP12	1.02	(0.65; 1.62)	0.926	0.993
ΔProlargin	0.78	(0.22; 2.79)	0.706	0.993
ΔPerlecan	1.27	(0.49; 3.25)	0.628	0.993
ΔGPNMB	0.49	(0.06; 3.97)	0.512	0.993
ΔhOSCAR	0.88	(0.19; 4.17)	0.877	0.993
ΔTIMP4	1.02	(0.59; 1.77)	0.934	0.993
ΔFGF23	1.04	(0.88; 1.22)	0.669	0.993
ΔMEPE	1.00	(0.42; 2.39)	0.993	0.993
ΔRANK	1.47	(0.86; 2.49)	0.169	0.993
(B) Plasma protein				
ΔTR-AP	1.23	(1.07; 1.42)	0.007*	0.189
ΔITGB2	1.25	(1.03; 1.52)	0.032*	0.435
ΔStromelysin-1	0.90	(0.81; 0.99)	0.045*	0.435
ΔMMP2	0.83	(0.69; 1.00)	0.063	0.457
ΔGPNMB	0.60	(0.33; 1.06)	0.094	0.481
ΔTIMP4	0.88	(0.75; 1.03)	0.116	0.481
ΔFGF23	0.96	(0.92; 1.01)	0.106	0.481
ΔOsteonectin	1.47	(0.88; 2.46)	0.158	0.528
ΔMelusin	1.04	(0.99; 1.09)	0.164	0.528
ΔProlargin	0.77	(0.53; 1.12)	0.184	0.534
ΔCCN1	1.14	(0.93; 1.39)	0.213	0.547
ΔCOL1A1	0.90	(0.76; 1.07)	0.238	0.547
ΔITGB5	1.20	(0.89; 1.62)	0.245	0.547
ΔWISP1	0.92	(0.80; 1.06)	0.280	0.557
ΔDecorin	1.28	(0.82; 2.01)	0.288	0.557
ΔITGAV	0.83	(0.57; 1.21)	0.341	0.618
ΔThrombospondin-2	0.88	(0.65; 1.18)	0.387	0.624
ΔPerlecan	0.88	(0.66; 1.17)	0.381	0.624
ΔOsteopontin	1.04	(0.93; 1.17)	0.471	0.719
ΔMatrilysin	0.94	(0.75; 1.17)	0.569	0.745
ΔGlypican-1	0.92	(0.69; 1.23)	0.575	0.745
ΔMMP12	1.04	(0.91; 1.19)	0.591	0.745
ΔRANK	1.05	(0.89; 1.24)	0.564	0.745
ΔOsteoprotegerin	0.95	(0.75; 1.18)	0.627	0.758
ΔMEPE	0.94	(0.73; 1.22)	0.658	0.763
ΔSyndecan-1	0.98	(0.87; 1.10)	0.714	0.796
ΔCadherin-5	0.95	(0.63; 1.43)	0.801	0.830
ΔhOSCAR	1.07	(0.67; 1.71)	0.791	0.830
ΔMMP9	1.01	(0.91; 1.11)	0.910	0.910

[a]*Adjusted with Benjamini & Hochberg (false discovery rate) correction.*

*Δ, delta (post-HT—pre-HT values); AU, arbitrary units; CCN1, CCN family member 1; CI, confidence interval; COL1A1, collagen alpha-1(I) chain; FGF23, fibroblast growth factor 23; ITGAV, integrin alpha-V; ITGB2, integrin beta-2; ITGB5, integrin beta-5; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; TIMP4, metalloproteinase inhibitor 4; hOSCAR, osteoclast-associated immunoglobulin-like receptor; RANK, receptor activator of nuclear factor κ-B; TR-AP, tartrate-resistant acid phosphatase type 5; GPNMB, transmembrane glycoprotein NMB; WISP1, WNT1-inducible-signaling pathway protein 1. *Indicates statistical significance.*

They concluded that increased levels of FGF23 were associated with impaired trabecular but not cortical bone microarchitecture, after adjusting for age and BMI. This is contradicting to our results, but may be partly explained by overestimations of T score in the lumbar spine, as outlined above, as well as the potential impact of renal dysfunction as pre-HT levels of FGF23 correlated with both measured and estimated GFR pre-HT.

A correlation between the change from pre-HT to post-HT in plasma levels of melusin, a muscle-specific integrin beta1-interacting protein, and the change in lumbar T score was found in the unadjusted analysis. However, no correlation remained after adjustments for age, gender, and BMI. It is well known that beta1 integrins are required for proper bone formation and homeostasis by playing a main role in the recruitment, differentiation, and mineralization of osteoblasts [30-32]. Brunner et al. (2018) reported that, for proper bone formation, beta1 integrins are required at the early stages of osteoblast differentiation *in vivo* [33]. Thus, the findings of the present study may reflect the pathophysiology behind beta1 integrins and their impact on bone formation.

Further, in the unadjusted analysis, the change from pre-HT to post-HT in plasma levels of ITGB2, stromelysin-1, and TR-AP correlated with the change in femoral T score. After adjustments for age, gender, and BMI, however, these correlations were lost. Although TR-AP has been considered a marker for osteoclastic activity, Halling Linder et al. (2017) demonstrated that TR-AP exhibits an inhibitory effect on osteopontin mediated mineralization delay, which is supported by the findings of this study [34, 35]. Also, ITGB2, which is involved in cell adhesion and in promoting intracellular signaling events, has been found to play a key role in the osteogenic processes [36, 37]. Miura et al. (2005) showed that mice lacking CD18, one of the members in beta-2 integrin family, exhibited features of osteoporosis, including decreased BMD, and impaired trabecular microarchitecture. This is consistent with the positive correlation between Δplasma levels of ITGB2 and ΔT score in the femoral neck that was found in the present study [37]. Stromelysin-1 is an activator of procollagenases which promotes cartilage degeneration [38]. In a study by Blom et al. (2007), stromelysin-1-knockout mice demonstrated a significant reduction in cartilage degeneration after induction of osteoarthritis [39]. Whether stromelysin-1 has an impact on the development of osteoporosis in HT patients remains to be established.

The present study provides explorative data on novel biochemical plasma markers for bone metabolism in 28 patients after HT. The major strength of this study was the application of multiplex proximity extension assay, which is known for its high sensitivity and specificity in plasma [18]. Data was independent of fasting and was adjusted for age, gender, and BMI. Moreover, the study was performed at a single-center which facilitated data

collection. Due to the explorative nature of the study, the small size of the patient cohort, as well as absence of a validation cohort, generalizability of the results is limited. Furthermore, the small size of the study restricted statistical adjustments with additional variables, such as comorbidities, medications, CS dose, time on waiting list, vitamin D intake and serum level, as well as calcium and phosphate levels in serum and urine, potentially influencing the BMD and levels of biochemical markers.

In conclusion, the present study showed that elevated plasma levels of FGF23 pre-HT predicted an increase in lumbar BMD after HT, which may be partly explained by the complex pathophysiological mechanisms in relation to the comorbid burden and immunosuppressive therapy in this patient cohort. Further investigations of biochemical markers on bone metabolism, especially FGF23, in larger HT populations are highly encouraged.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethical Board in Lund, Sweden (diary numbers: 2010/114; 2010/442; 2011/368; 2011/777; 2014/92 and 2015/270) and was conducted in agreement with the declarations of Helsinki and Istanbul. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EL: Study design, data collection, data analysis, and writing of the article. SA: Study design, data collection, and writing and reviewing of the article. AA: Study design, data collection, and writing and reviewing of the article. GR: Study design, data acquisition, and writing and reviewing of the article.

FUNDING

This work was funded by unrestricted research grants from the Anna-Lisa & Sven-Eric Lundgren Foundation, as well as from ALF's Foundation, Lund, Sweden. The contributors had no role in the collection, analysis or interpretation of the data, and had no right to restrict the dissemination or publication of the results.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We acknowledge the support of the staff at the Hemodynamic Lab, the Section for Heart Failure and Valvular Disease, VO. Heart and Lung Medicine, Skåne University Hospital, Lund, Sweden, and the Department of Clinical Sciences Lund, Cardiology, at Lund University, Sweden. Exceptional gratitude to Anna Åkesson for her statistical expertise which was invaluable during the analysis and interpretation of the data. We also acknowledge the biobank services and retrieval of blood samples from LCPR at Lab medicine Skåne, University and Regional Laboratories, Region Skåne, Sweden. A special thanks to Anneli Ahlqvist for her efforts in LCPR administration and blood sample management.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10161/full#supplementary-material

Supplementary Figure 1 | Plasma levels of proteins from pre- and post-HT measured in normalized protein expression scale, expressed in AU. AU, arbitrary units; CCN1, CCN family member 1; COL1A1, collagen alpha-1(I) chain; HT, heart transplantation; ITGAV, integrin alpha-V; ITGB2, integrin beta-2; ITGB5, integrin beta-5; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; TIMP4, metalloproteinase inhibitor 4; hOSCAR, osteoclast-associated immunoglobulin-like receptor; RANK, receptor activator of nuclear factor κ-B; TR-AP, tartrate-resistant acid phosphatase type 5; GPNMB, transmembrane glycoprotein NMB; WISP1, WNT1-inducible-signaling pathway protein 1.

Supplementary Figure 2 | (A-AC) Correlations between pre-HT plasma protein levels measured in normalized protein expression scale, expressed in AU, and post-HT lumbar and femoral T scores. AU, arbitrary units; CCN1, CCN family member 1; COL1A1, collagen alpha-1(I) chain; FGF23, fibroblast growth factor-23; ITGAV, integrin alpha-V; ITGB2, integrin beta-2; ITGB5, integrin beta-5; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; TIMP4, metalloproteinase inhibitor 4; hOSCAR, osteoclast-associated immunoglobulin-like receptor; RANK, receptor activator of nuclear factor κ-B; TR-AP, tartrate-resistant acid phosphatase type 5; GPNMB, transmembrane glycoprotein NMB; WISP1, WNT1-inducible-signaling pathway protein 1.

Supplementary Figure 3 | Correlations between Δplasma protein levels measured in normalized protein expression scale, expressed in AU, and ΔT score from the lumbar spine and femoral neck. AU, arbitrary units; CCN1, CCN family member 1; COL1A1, collagen alpha-1(I) chain; FGF23, fibroblast growth factor-23; ITGAV, integrin alpha-V; ITGB2, integrin beta-2; ITGB5, integrin beta-5; MEPE, matrix extracellular phosphoglycoprotein; MMP, matrix metalloproteinase; TIMP4, metalloproteinase inhibitor 4; hOSCAR, osteoclast-associated immunoglobulin-like receptor; RANK, receptor activator of nuclear factor κ-B; TR-AP, tartrate-resistant acid phosphatase type 5; GPNMB, transmembrane glycoprotein NMB; WISP1, WNT1-inducible-signaling pathway protein 1.

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ORIGINAL RESEARCH
published: 31 March 2022
doi: 10.3389/ti.2022.10450

Survival After Lung Transplantation for Chronic Hypersensitivity Pneumonitis: Results From a Large International Cohort Study

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Repeated exposure to antigens *via* inhalation is the primary cause of hypersensitivity pneumonitis, a form of interstitial pneumonia. The chronic form of hypersensitivity pneumonitis leads to progressive loss of respiratory function; lung transplantation is the only therapeutic option for chronically ill patients. The ESTS Lung Transplantation Working Group conducted a retrospective multicentred cohort study to increase the body of knowledge available on this rare indication for lung transplantation. Data were collected for every patient who underwent lung transplant for hypersensitivity pneumonitis in participating centres between December 1996 and October 2019. Primary outcome was overall survival; secondary outcome was freedom from chronic lung allograft dysfunction. A total of 114 patients were enrolled from 9 centres. Almost 90% of patients were diagnosed with hypersensitivity pneumonitis before transplantation, yet the antigen responsible for the infection was identified in only 25% of cases. Eighty per cent of the recipients received induction therapy. Survival at 1, 3, and 5 years was 85%, 75%, and 70%, respectively. 85% of the patients who survived 90 days after transplantation were free from chronic lung allograft dysfunction after 3 years. The given study presents a large cohort of HP patients who underwent lung transplants. Overall survival rate is higher in transplanted hypersensitivity pneumonitis patients than in those suffering from any other interstitial lung diseases. Hypersensitivity pneumonitis patients are good candidates for lung transplantation.

Keywords: lung transplant, hypersensitivity pneumonitis, rare lung disease, respiratory insufficiency, pneumonia, interstitial pneumonia

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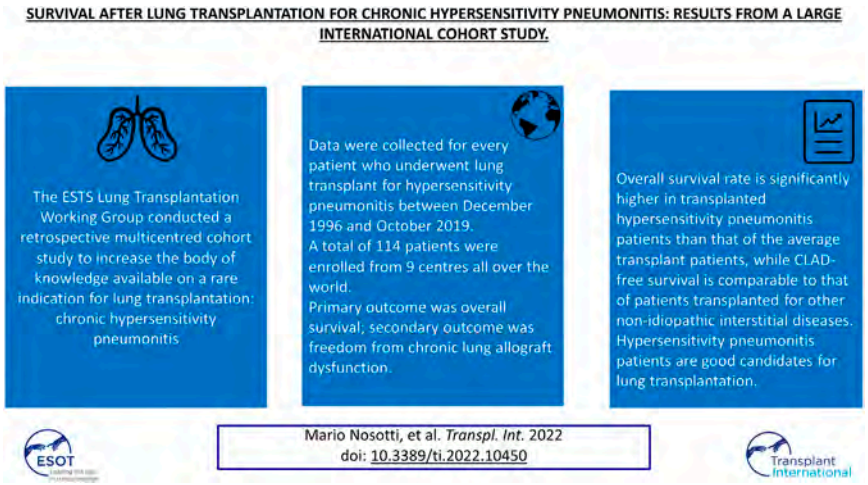
Received: *22 February 2022*
Accepted: *03 March 2022*
Published: *31 March 2022*

Citation:
Nosotti M, Leiva-Juarez M, D'Ovidio F, Van Raemdonck D, Ceulemans L, Keshavjee S, Rackauskas M, Paladini P, Luzzi L, Casado PM, Alvarez A, Inci I, Ehrsam J, Krueger T, Roth A, Rea F, Schiavon M and Rosso L (2022) Survival After Lung Transplantation for Chronic Hypersensitivity Pneumonitis: Results From a Large International Cohort Study. Transpl Int 35:10450. doi: 10.3389/ti.2022.10450

Abbreviations: CI, confidence interval; CLAD, chronic-lung-allograft-dysfunction; ESTS-LTxWG, European society of thoracic surgeons lung transplantation Working Group; HP, hypersensitivity pneumonitis; HR, hazard ratio; ISHLT, International society for heart and lung transplantation.



GRAPHICAL ABSTRACT |

INTRODUCTION

Hypersensitivity pneumonitis (HP) is a rare parenchymal disease prompted by an immunologic reaction to inhaled organic antigens. HP incidence was assessed as 1 per 100,000 inhabitants in Great Britain; its prevalence varies significantly among countries, regions, and according to occupational exposure (1). A study based on insurance claims databases conducted in the United States between 2004 and 2013 estimated the prevalence of HP to be from 1.67 to 2.71 per 100,000 inhabitants (2).

Repeated exposure to one or more stimulating agents triggers the onset of HP in susceptible individuals; these patients develop both a humoral and a cellular reaction, which leads to peribronchiolar chronic inflammatory infiltrates and non-necrotising granulomas. The presence of MUC5B (Mucin 5B) single nucleotide polymorphisms and peripheral blood leukocyte telomere length dysfunction seems to induce pulmonary fibrosis in HP patients (3). The development of fibrosis can follow three different patterns: simple peribronchiolar, subpleural or bridging fibrosis. The latter is quite typical of HP, because of which spreading fibrotic tissue in the interstitium between bronchioles and interlobular septa or areas of subpleural fibrosis accumulates (4).

It is well known that the diagnosis of HP is not straightforward. A 2016 study, conducted on 70 England-based patients affected with interstitial pulmonary disease, assessed the difficulties encountered by international multidisciplinary teams in reaching a consensus on diagnosis and therapeutic approach (5). Very recently, an *ad hoc* board of experts appointed by several scientific societies drew up a practical clinical guideline for diagnosing HP in adults (6). The guideline board developed a systematic approach to diagnostic criteria and established a dedicated algorithm based on computer tomography imaging, exposure evaluation, broncho-alveolar lavage lymphocytosis, and histopathological findings. The board drew a definite distinction between the fibrotic and non-fibrotic forms of HP; the first is clearly associated with a poor prognosis. Indeed, findings of thick fibrosis, fibroblast foci, and microscopic honeycombing in a cohort of 119 patients with HP were predictors of early mortality or lung transplantation (7).

Patients with HP who develop the fibrotic form of HP can generally benefit from lung transplantation; nevertheless, the opportunity to enlist patients who have shown an extreme pathological reaction to a foreign antigen and would be permanently exposed to a graft only partially compatible with their immune system raises some concerns. Being HP a rare clinical occurrence, the scientific literature is lacking dedicated studies on the topic. The European Society of Thoracic Surgeons Lung Transplantation Working Group (ESTS-LTxWG) on lung transplantation deemed necessary to help fill this gap in the literature with a large multicentred retrospective study, given the consistent number of patients with HP enrolled in a previous ESTS-LTxWG study on rare indications for lung transplantation (8).

MATERIALS AND METHODS

This was an international, retrospective cohort study including consecutive patients who received lung transplantation in 9 centres between Europe and North America. Each centre autonomously identified suitable patients and collected the data. Eligible patients were adult individuals with histologically proven HP on native lungs; each centre was responsible for the proper diagnosis of their own patients. Postoperative therapy, as well as periodical clinical assessments, followed the standard of

TABLE 1 | Patients' characteristics.

Variable	Value
Number	114
Male gender	71 (62.3%)
Age, years	57.5 (50–63)
Preoperative diagnosis	102 (89.5%)
Exposure to antigens	
Bird fanciers	21 (18.4%)
Farmers, mushroom growers, gardeners	5 (4.4%)
Pharmaceutical industry workers	3 (2.6%)
Not known	85 (74.6)
Preoperative FEV1%	41.3 (32–54)
Preoperative FVC%	40 (33–51.7)
Preoperative DLCO%[a]	32 (23–38)
Bilateral transplantation	52 (45.6%)
Transplantations by era (2009–2019)	96 (84.2%)
Induction therapy	91 (79.8%)

Data are presented as number and percentage or median and 1st to 3rd quartile; FEV1%: percentage of predicted forced expiratory volume in 1 s; FVC%: percentage of predicted forced vital capacity; DLCO%: percentage of predicted diffusion capacity CO.
[a]*Data from 38 patients only.*

TABLE 2 | Survival outcomes.

Variable	Value
Mortality at 90 days	5 (4.4%)
Overall survival	
Events (mortality)	34 (29.8%)
Median survival (years)	9.2
1-year survival rate	85.2% (from 78.7% to 92.2%)
3-year survival rate	74.4% (from 66.0% to 83.8%)
5-year survival rate	70.4% (from 61.0% to 81.1%)

Data are presented as number and percentage, median or rate and 95% confidence interval.

care in each participating centre. Demographic, surgical, and survival data were collected with standardised database templates to warrant reliable data collection.

The primary objective of the current study was to evaluate the effects of lung transplantation in patients with HP in terms of overall survival, which was calculated from the day of lung transplantation until death or last follow-up. Secondary outcome was chronic lung allograft dysfunction (CLAD). The onset of CLAD in each patient was diagnosed individually according to the consensus report from the Pulmonary Council of the International Society for Heart and Lung Transplantation (ISHLT) (9). The authors also analysed the outcome of single versus double transplantation and induction therapy versus no induction therapy.

Statistical Analysis

Continuous data were presented as mean and standard deviation or median and 1st to 3rd quartile. Categorical variables are shown as absolute and percentage frequencies. Time-to-event data were displayed using non-parametric Kaplan Meier estimators. The hazard ratio (HR) was computed using Cox regression models with Breslow approximation; given the multicentric nature of collected data, a robust sandwich variance estimator was adopted to account for correlated groups of observations. The CLAD variable was treated as a time-varying covariate into Cox models. The proportional hazards assumption was checked using statistical tests and graphical diagnostics based on the scaled

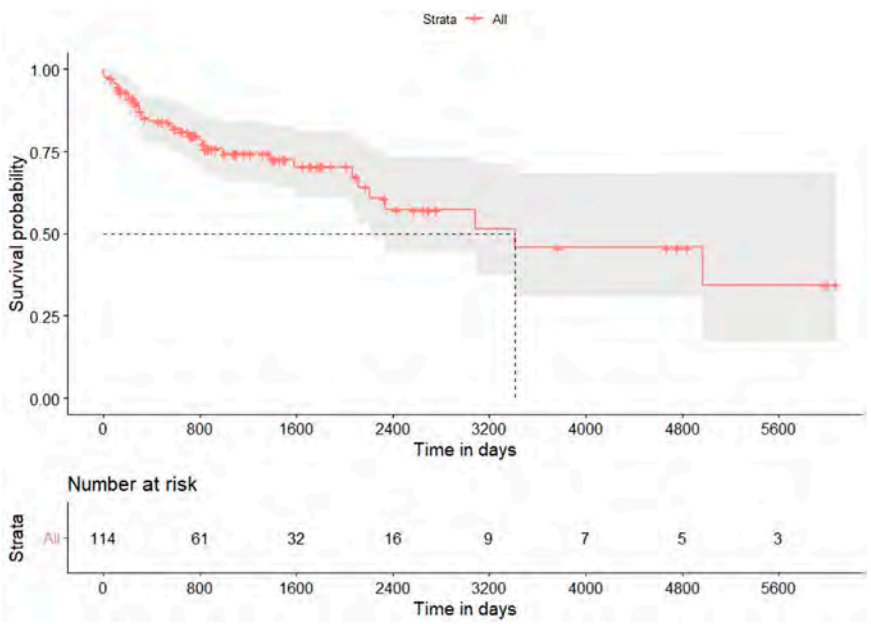


FIGURE 1 | Kaplan-Meier plot for overall survival after lung transplantation for hypersensitivity pneumonitis. Gray area identifies the 95% pointwise confidence intervals.

TABLE 3 | Chronic lung allograft disease in patients who survived 90 days after transplantation.

Variable	Value
Number	108
Male gender	67 (62.0%)
Age	58 (51–63)
Bilateral transplantation	49 (45.4%)
Induction therapy	87 (80.6%)
Median follow-up, days	810 (399–1440)
Patients with diagnosis of CLAD	34 (31.5%)
Median CLAD-free survival, days	1800
CLAD-free survival	
1-year survival (95%CI)	95.0% (from 91.0% to 99.4%)
3-year survival (95%CI)	71.0% (from 61.2% to 82.4%)
5-year survival (95%CI)	49.3% (from 37.9% to 65.8%)

Data are presented as number and percentage or median and 1st to 3rd quartile; CLAD: chronic lung allograft dysfunction.

Schoenfeld residuals. Confidence intervals were computed at 95%, and side p-values were considered significant when < 0.05. All analyses were carried out using R-Cran software, version 3.5.3.

This study followed the principles outlined in the Declaration of Helsinki (2013), was approved by the Institutional Review Board (749_2016bis; Milan 2) and received no financial support.

RESULTS

One-hundred and fourteen patients were eligible and enrolled from seven European and two North American transplantation centres. Participating centres and the respective number of recruited patients are listed in **Supplementary Table S1**. Main clinical characteristics in patient cohort receiving lung transplantation between December 1996 and October 2019 are summarised in **Table 1**. The patient cohort showed a slight prevalence of female patients and a median age of 57 years. The 89.5% of patients were diagnosed with HP prior to listing. However, the antigen responsible for the onset of their lung disease could be identified in only 25.4% of cases. The vast majority of patients in whom the antigen could be identified had been exposed to birds (72.4%). Patients were treated with bilateral transplantation in 45.2% of cases. Induction immunosuppressive therapy rate was 80%. More than 84% of patients were transplanted in the last decade. Median follow-up was 2.25 years.

Five (4.4%) patients died within 90 days of transplantation: three patients experienced cardiovascular events, one had hyperacute rejection, and one suffered from a surgical complication. **Figure 1** shows the Kaplan-Meier plot for overall survival. Survival rate at 1, 3 and 5 years was 85.2%, 74.4% and 70.4%, respectively; median survival was 9.2 years (**Table 2**). Multivariable Cox proportional hazard regression model adjusted for age showed a higher hazard of death over time for patients with CLAD (HR = 9.10; 95% CI from 6.10 to 13.53; $p < 0.001$) and a lower risk of death for patients treated with induction therapy (HR = 0.45; 95% CI from 0.23 to 0.87; p 0.017). Giving that the variable mono/bilateral transplantation violated the Cox proportional hazard assumption, analyses were repeated on each subgroup. Multivariable Cox model adjusted for age showed that patients with single lung transplantation who developed CLAD experienced a higher hazard of mortality compared with patients without CLAD (HR = 8.86; 95% CI from 4.59 to 17.8; $p < 0.001$). Conversely, a refitted Cox model adjusted for age showed that patients with single lung transplantation had a lower risk of mortality if treated with

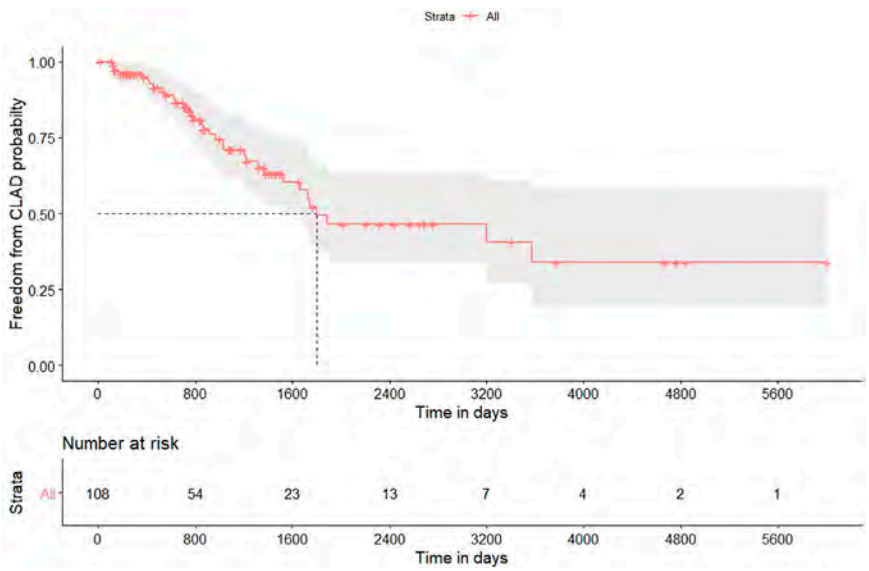


FIGURE 2 | Kaplan-Meier plot for freedom to chronic lung allograft dysfunction in patients who survived 90 days after lung transplantation for hypersensitivity pneumonitis. Gray area identifies the 95% pointwise confidence intervals.

induction therapy (HR = 0.26; 95% CI from 0.11 to 0.61; $p < 0.002$). Cox model adjusted for age showed in the bilateral transplantation group an HR of death for CLAD of 9.74 (95% CI from 7.04 to 13.45; $p < 0.001$); the refitted model adjusted for age indicated a not significant reduction hazard of death related to induction therapy (HR = 0.52; 95% CI from 0.15 to 1.75; $p = 0.289$).

Thirty-four out of 108 patients who survived beyond 90 days after transplantation developed CLAD; **Table 3** reports some characteristics, while **Figure 2** displays the freedom from CLAD Kaplan-Meier graph. CLAD-free survival at 1, 3, and 5 years was 95%, 71% and 49.3%, respectively. Univariable Cox analysis for CLAD identified induction therapy in the single lung transplantation subgroup (HR = 0.48; 95% CI from 0.25 to 0.91; p 0.025) as a protective factor; induction therapy after bilateral transplantation did not reach the statistical significance in the refitted univariable model (HR = 0.62; 95% CI from 0.24 to 1.63; p 0.339).

DISCUSSION

Lung transplantation for HP is infrequent; this condition is usually included in the extensive list of interstitial lung diseases (excluding idiopathic interstitial pneumonia), which only make up 5.7% of all lung transplantations, according to the 2019 Thoracic Organ Transplant Registry report of the International Society for Heart and Lung Transplantation (10). Being HP a rare disease, reports of outcomes after lung transplantation are scarce. To the best of the authors' knowledge, only one paper drafted by Kern and collaborators specifically addressed this issue (11). The researchers compared 31 patients with HP to 91 patients with idiopathic pulmonary fibrosis; patients' survival at 1, 3, and 5 years after transplantation in HP patients was 96%, 89%, and 89%, respectively. Survival rates among HP patients were far higher than those recorded in the idiopathic pulmonary fibrosis cohort. Moreover, a reduced rate of acute cellular rejection was observed in the first year after transplantation in patients with HP. Finally, the authors found two possible HP recurrences after transplantation.

Although the Californian study was excellent in methodology and interpretation, it suffered from the typical lack of external validation of monocentric studies; therefore, some form of multicentric validation would have been advisable. This international collaborative study confirmed the excellent overall survival fixing median survival after lung transplantation for HP at 9.2 years. This result is particularly encouraging in light of the fact that the group of pathologies in which HP is included reaches a median survival of 6.4 years, according to the ISHLT TTX report (10).

Despite survival in this cohort being equivalent either after single or bilateral lung transplantation, the violation of proportional hazard assumption verified through the Schoenfeld residuals test prevented us from performing proper multivariable analyses on the entire patient group. Therefore, by dividing the cohort by transplant type, it has been proved that the CLAD onset had a strong negative impact on survival in both subgroups (HR 8.86 and 9.74 for single and bilateral lung transplantation, respectively). Notwithstanding the excellent survival of patients transplanted for HP, negative effects of chronic rejection were also observed in this cohort (12). Induction therapy impacted positively on survival in the subgroup of patients treated with single lung transplantation. This result is likely linked to the small sample size, given that it has been already shown elsewhere how this variable is protective for both types of lung transplantation (8).

Median CLAD-free survival in our cohort was 4.9 years; this result was satisfactory and congruent with the time span (4.8 years) recorded in the ISHLT TTX report concerning patients transplanted for interstitial lung diseases excluding idiopathic interstitial pneumonia (10). Considering how this patient cohort CLAD included the possible recurrence of HP in addition to bronchiolitis obliterans syndrome and restrictive allograft syndrome, one can speculate that the recurrence of underlying lung disease had a negligible clinical impact. The given study found that induction therapy is likely to have a protective effect against the onset of CLAD only in patients who underwent single lung transplantation.

The current study has some limitations. As a multicentric retrospective study, it is susceptible to selection bias; namely, we have not been able to classify patients according to recent HP guidelines (6) since our data collection ended before their publication. Given the radiological and pathological peculiarities of HP, it is unlikely that incorrect diagnoses were made for enrolled patients, while some cases may have been classified as idiopathic interstitial pneumonia and therefore not included in the current study. Another limitation is the absence of a control group. We took as reference the ISHLT Thoracic Organ Transplant Registry data for patients suffering from idiopathic fibrosis. Anyway, it cannot be entirely excluded that the results obtained by the centres participating in the study were, for some reason, above the international average limiting the difference in survival with HP patients. Moreover, the chance that unknown clinical factors may have affected the observed results cannot be ruled out. In particular, no data on possible recurrence of HP in the graft are available. Among the patients with CLAD the prevalence of the restrictive form was 11.7%; this prevalence is lower than that reported in the literature for the general population of patients transplanted with CLAD. We can speculate that HP recurrence, which has a clinical picture similar to the restrictive allograft syndrome, had a negligible impact on our patient cohort. Another limitation is the high percentage of patients in whom the antigen was not known; theoretically, different antigens could affect the aggressiveness of pulmonary fibrosis and therefore determine different underlying clinical conditions. One can speculate that this possible confounder, which is geographically determined, was mitigated by the international distribution of this patient cohort.

In conclusion, this international multicentric study highlights how patients with HP are good candidates for lung transplantation. Their survival rate is significantly higher than that of the average transplant patients, while CLAD-free survival is comparable to that of patients transplanted for other non-idiopathic interstitial diseases. The problem of possible

recurrence of HP in the graft requires additional studies, although its clinical impact seems very limited.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board (749_2016bis; Milan 2). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MN: conceptualization, supervision, investigation, data curation, formal analysis, writing—original draft, visualization. ML-J and MR: conceptualization, investigation, data curation, writing—review and editing. FD'O, DV, SK, and II: conceptualization, supervision, investigation, data curation, writing—review and editing. LC, LL, AA, JE, TK, AR, and MS: data curation, investigation, writing—review and editing. PP, PC, and FR: data curation, investigation, supervision, writing—review and editing. LR: data curation, investigation, writing—review and editing, visualization.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

Gianluca Bonitta Msc for statistical analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10450/full#supplementary-material

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ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/ti.2022.10182

Differential IgG4-Producing Plasma Cell Infiltration in Non- and Post-Transplant Plasma Cell Hepatitis

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Autoimmune hepatitis (AIH), post-transplant recurrent AIH (rAIH), and plasma cell-rich rejection (PCR) are clinical diagnoses with the shared histopathologic hallmark of plasma cell hepatitis (PCH). As these histologically and serologically indistinguishable diagnoses are differentiated by clinical context, it remains uncertain whether they represent distinct immunologic phenomena. Improved understanding of immunoglobulin subclass 4-producing plasma cells (IgG4-PC) has brought attention to IgG4 as an immunophenotypic biomarker. To date, degree and clinical significance of IgG4-PC infiltration in PCH remain elusive. This retrospective, single-center study assessed IgG4-PC infiltration in AIH, rAIH, and PCR via standardized immunohistochemistry analysis. Identified cases from 2005 to 2020 (n = 47) included AIH (treatment-naïve AIH (tnAIH): n = 15 and AIH-flare on treatment (fAIH); n = 10), rAIH (n = 8), and PCR (n = 14) were analyzed and correlated with clinical characteristics. IgG4-Positivity (# IgG4-PC/# pan-IgG-expressing cells) distribution was heterogenous and overlapping [tnAIH: 0.060 (IQR 0.040–0.079), fAIH: 0.000 (0.000–0.033), rAIH: 0.000 (0.000–0.035), PCR: 0.228 (0.039–0.558)]. IgG4-Positivity was inversely correlated with corticosteroid use (p < 0.001). IgG4-Positivity ≥0.500 was associated with rapid AST improvement (p = 0.03). The variable IgG4-Positivity of AIH, rAIH and PCR suggests diverse and overlapping immunopathologic mechanisms and that current diagnostic schemes inadequately capture PCH immunopathology. We propose incorporation of IgG4-Positivity to refine current PCH classification and treatment strategies.

Keywords: plasma cell hepatitis, alloimmunity, autoimmunity, IgG4, autoimmune hepatitis

OPEN ACCESS

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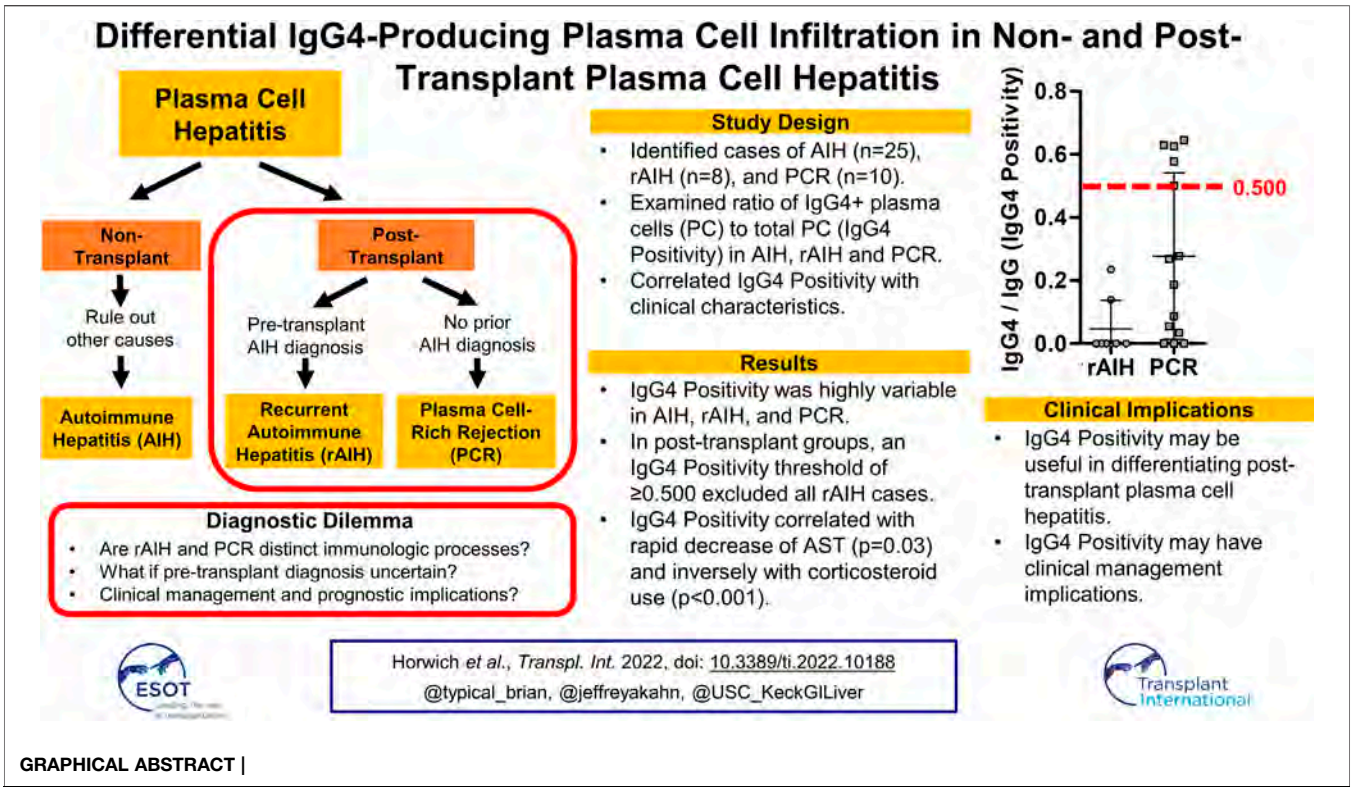
***Received:** 04 November 2021*
***Accepted:** 04 February 2022*
***Published:** 18 March 2022*

Citation:
Horwich BH, Liang TZ, Dodge JL, Chopra S, Kahn JA and Saito T (2022) Differential IgG4-Producing Plasma Cell Infiltration in Non- and Post-Transplant Plasma Cell Hepatitis. Transpl Int 35:10182. doi: 10.3389/ti.2022.10182

INTRODUCTION

Plasma cell hepatitis (PCH) is a pathohistological finding characterized by lymphoplasmacytic portal and lobular inflammation with prominent plasma cells and often with the presence of interface hepatitis, perivenulitis and centrilobular necrosis (1–4). While the term "PCH" was originally used to describe autoimmune hepatitis (AIH), its use has extended to other plasma cell (PC)-rich necroinflammatory disorders including recurrent AIH (rAIH) and PC-rich rejection (PCR) in



GRAPHICAL ABSTRACT |

liver allograft (2, 5–7). Accordingly, the Banff Working Group defines that AIH represents PCH of the native liver, while rAIH and PCR are clinical entities of PCH occurring in the post-LT setting (8–12).

PCH often results in the development of hepatic fibrosis if not promptly and adequately treated with potent immunosuppressants (IS) such as corticosteroids (CS), antimetabolites, and calcineurin inhibitors (13–16). Moreover, IS often fail to sufficiently control hepatic necroinflammation, which eventually leads to liver failure (7, 14, 17, 18). Furthermore, the long-term administration of IS is associated with significant morbidity, including the development of opportunistic infections and neoplasms (19).

Since histopathological and serological assessment do not distinguish between AIH, rAIH, and PCR, their diagnosis is entirely reliant on clinical context, which is coupled with challenges, perhaps ambiguity (8, 12, 20–23). This is especially relevant in differentiating between rAIH and PCR. By convention, PCR refers to PCH in individuals who underwent transplantation for diseases other than AIH (8). Conversely, rAIH refers to PCH occurring in patients transplanted for AIH. Thus, based upon current convention, differentiating between rAIH and PCR may not be plausible in circumstances where pre-LT diagnosis was uncertain (e.g., acute liver failure of unknown etiology or cryptogenic cirrhosis). Furthermore, current PCH classification scheme excludes individuals transplanted for AIH from receiving a diagnosis of PCR. Thus, it remains elusive if rAIH and PCR represent distinctive or overlapping clinical entities. Similarly, there has not been evidence demonstrating that the onset of rAIH is mediated through the recurrence of immunopathology underlying AIH in the native liver, making the nomenclature of rAIH potentially deceiving. Ultimately, the fundamental issue regarding the current classification of PCH is the substantial degree of uncertainty as to whether each disease—as currently classified—represents a unique immunologic phenomenon.

PC, the terminally differentiated B cells, play a major role in the regulation of humoral immunity through the production of immunoglobulin (Ig). PC exhibit highly diverse immunomodulatory effects depending on the classes and subclasses of Ig production such as IgG, A, and M as well as IgG1, 2, 3, and 4, respectively (24). Hence, the Ig classes and subclasses expressed in the infiltrating PC would associate with, at least in part, the immunopathological presentations of PC-mediated disorders (24, 25). In particular, inflammatory disorders with a pronounced infiltration of IgG4-PC have been known to manifest marked tissue fibrosis and favorable response to IS (26). Therefore, chronic inflammatory disorders with IgG4-PC infiltration have emerged as a unique clinical entity, namely IgG4-related diseases (IgG4-RD) (27). The pancreas was the first organ in which IgG4-RD was recognized, namely autoimmune pancreatitis; thereafter this disease entity has been known to affect multiple organs, including the liver parenchyma (27, 28).

Prior studies have demonstrated the infiltration of IgG4-PC in the liver tissue of PCR and native-liver AIH, with a PCR subtype

demonstrating significant enrichment (28, 29). Consequently, the Banff Working Group recommends use of IgG4 immunostaining in the evaluation of post-LT PCH (12). However, this recommendation lacks a specific threshold for IgG4-PC positivity and does not provide guidance with respect to its clinical relevance. One potential reason for this is that there has not been a comprehensive study that cross-sectionally compares the degree of IgG4-PC infiltration between PCH types with a standardized quantification method, significantly limiting its practical use. In particular, the degree of IgG4-PC infiltration in rAIH has not previously been studied. Thus, it remains elusive whether assessment of IgG4 immunostaining may be of diagnostic and therapeutic relevance in the post-LT setting—particularly in differentiating between rAIH and PCR. The primary aim of this study is to characterize PCH diseases by objectively determining the IgG4-PC positivity and evaluate for associations with clinical presentations and outcomes.

MATERIALS AND METHODS

Study Subject Identification

All study procedures were approved by the University of Southern California Institutional Review Board (HS-19-00258). The study subjects were identified with the Department of Pathology Database by querying for reports containing "AIH," "PCR", or "PCH" from 2005 to 2020. The medical record and histopathological finding of all subjects identified through the database were confirmed to meet the diagnostic criteria of AIH (International Autoimmune Hepatitis Group) or PCH (Banff consensus) (12, 30, 31). Individuals with infectious and neoplastic etiologies as the cause of PC infiltration were excluded. All cases enrolled into this study were classified as the following: treatment-naïve AIH (tnAIH), AIH flare while on IS (fAIH), rAIH, and PCR. A diagnosis of tnAIH was defined as meeting probable or definite diagnostic criteria for AIH with no known IS use with activity against AIH prior to biopsy. A diagnosis of fAIH was defined as having a pre-existing diagnosis of AIH on IS with histopathology demonstrating features consistent with recurrence of active AIH disease. A diagnosis of rAIH was defined as allograft histopathology demonstrating features consistent with active AIH disease for which the pre-transplant diagnosis was AIH. A diagnosis of PCR was defined as clinical history and histopathology consistent with criteria outlined by the Banff Working Group (12). No specimen from executed prisoners were used.

Clinical Data Collection

The medical records of identified subjects were reviewed to extract relevant demographic and clinical information.

Specimen Processing and Histopathologic Review

The Formalin-Fixed Paraffin-Embedded (FFPE) liver needle core biopsy specimens of all enrolled cases ($n = 47$) were retrieved. The hematoxylin-eosin (H&E)-stained pathology slides were first reviewed for adequacy of the tissue as determined by 8 or greater portal tracts and at least 1.5 cm in length. All retrieved samples were deemed adequate by these criteria. For individuals with multiple biopsies with the same diagnosis, the initial biopsy specimen available was used. Cases were evaluated for the following histological characteristics: portal inflammation, interface lobular necro-inflammatory activity, perivenular inflammation and the presence of bridging necrosis. Both activity and fibrosis were assessed on the Metavir histological activity and fibrosis score. Any additional pathological findings were also recorded. The tissue specimens were serially sectioned and applied for immunohistochemical stains using anti-human pan-IgG (RWP49 clone: Leica Biosystems) at 1:1,000 dilution and anti-human IgG4 (MRQ-44 from Cell Marque) at 1:500 dilution. All staining was performed using Bond III Leica Autostainer system at the Human Pathology Core. The representative portal tract of each subject identified by an expert pathologist was used to determine the IgG4-Positivity, which was calculated by the number of IgG4 staining positive PC normalized by the number of pan-IgG staining positive PC in the corresponding portal tract of the serially sectioned slides. Counting was done manually on ×400 of both the IgG and IgG4 cells in that portal tract.

Statistical Analysis

Demographic, laboratory, and histopathologic characteristics were summarized as medians with interquartile ranges (IQR) or frequencies with proportions for the overall cohort and stratified by PCH subtype. Characteristics were compared within the respective non-transplant (tnAIH versus fAIH) and transplant (rAIH versus PCR) groups using Wilcoxon-rank sum and Fisher's exact tests, as appropriate.

Measures of IgG4 were characterized as the presence or absence of IgG4-PC and the proportion of IgG4-PC of all IgG-producing cells, which were compared by PCH subtype. The proportion of IgG4-PC was plotted by PCH subtype and separately categorized in tertiles to accommodate the skewed distribution. Laboratory parameters and therapeutic outcomes were evaluated for their association with increasing tertile of IgG4-Positivity using non-parametric trend tests (Stata nptrend) and markedly high IgG4-Positivity in the post-LT groups (≥0.500 vs. <0.500) using Kruskal-Wallis tests (Stata).

Differences were considered statistically significant at $p < 0.05$. Statistical analysis was conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, United States) and Stata 14.0 (StataCorp, College Station, TX, United States).

RESULTS

Demographics and Clinical Data

The demographics and clinical characteristics of the study subjects are summarized (**Table 1**). There was no subject crossover between groups. Median age was lowest for rAIH at 35 years compared to 50 or more years for the other PCH subtypes. All groups demonstrated a female predominance (tnAIH 73%, fAIH 60%, rAIH 87%, PCR 79%). Median BMI ranged from 24.3 to 29.5 (tnAIH 26.8, fAIH 24.3, rAIH 28.8, PCR 29.5), with 38% individuals having BMI>30 at time of biopsy. A family history of autoimmune disorders was more

TABLE 1 | Characteristics of individuals with plasma cell hepatitis.

Parameter	All subjects	Non-transplant			Transplant		
		tnAIH	fAIH	*p*-value	rAIH	PCR	*p*-value
	N = 47	*N* = 15	*N* = 10		*N* = 8	*N* = 14	
Age (years), median	51	52	50	0.70	35	52	0.22
Female, *n* (%)	35 (74)	11 (73)	6 (60)	0.67[a]	7 (87)	11 (79)	1.00[a]
BMI (kg/m^2), median	29.1	26.8	24.3	0.74	28.8	29.5	0.86
Alcohol use, *n* (%)	9 (19)	8 (53)	0 (0)	**0.008**[a]	0 (0)	1 (7)	1.00[a]
Drug use, *n* (%)	3 (6)	1 (7)	0 (0)	1.00[a]	1 (12)	1 (7)	1.00[a]
Family history of autoimmune diseases, *n* (%)	8 (17)	4 (27)	2 (20)	1.00[a]	2 (25)	0 (0)	0.12[a]
Liver disease prior to the liver transplantation, *n* (%)							
Acute liver failure					0 (0)	3 (21)	
Alcohol					0 (0)	0 (0)	
Autoimmune hepatitis					8 (100)	0 (0)	
Cryptogenic					0 (0)	2 (14)	
HBV					0 (0)	2 (14)	
HCV					0 (0)	1 (7)	
NASH/NAFLD					0 (0)	4 (29)	
Other					0 (0)	2 (14)	
Ethnicity, *n* (%)							
White	13 (28)	3 (20)	4 (40)		2 (25)	4 (29)	
Hispanic	3 (6)	2 (13)	0 (0)		0 (0)	1 (7)	
Asian	7 (15)	3 (20)	1 (10)	0.57[a]	0 (0)	3 (21)	0.37[a]
Black	6 (13)	2 (13)	0 (0)		3 (37)	1 (7)	
Other	18 (38)	5 (33)	5 (50)		3 (37)	5 (36)	
Time from Transplant (mo)							
Median		N/A	N/A		9.0	5.6	0.22
Range					2.6–24.3	0.2–25.6	
Immunosuppressants, *n* (%)							
CS	21 (45)	—	7 (70)		7 (87)	7 (50)	0.17[a]
CNI	22 (47)	—	2 (20)		7 (87)	13 (93)	1.00[a]
AZA	3 (7)	—	2 (20)		1 (12)	0 (0)	0.36[a]
MMF	13 (28)	—	0 (0)		3 (37)	10 (71)	0.19[a]
MTOR	1 (2)	—	0 (0)		1 (12)	0 (0)	0.36[a]
Laboratory Data[b]							
Platelet count (K/cumm), median (normal 141–401)	181	202	195	0.95	143	152	0.86
ALP (U/L), median (normal 34–106)	216	155	167	0.68	204	224	0.68
ALT (U/L), median (normal 14–54)	300	349	200	0.29	56	181	0.09
AST (U/L), median (normal 38–126)	306	366	147	0.19	76	155	0.19
TB (mg/dl), median (normal 0.2–1)	5.1	5.3	1.4	**0.03**	1.3	1.1	0.45
Albumin (g/dl), median (normal 3.4–5.3)	3.3	3.1	3.5	0.18	3.6	3.5	0.81
Total protein (mg/dl), median (normal 6.0–8.2)	7.1	7.1	7.3	0.79	7.1	7.0	0.92
IgG, Total (mg/dl), median (normal 600–1,640)	2,149	2,319	2,298	0.68	2,137	1769	0.88
IgA (mg/dl), median (normal 47–310)	377	377	343	0.56	504	298	0.18
IgM (mg/dl), median (normal 50–300)	166	136	273	1.00	231	161	0.08
ANA titer (≥1:80), *n* (%)[c]	26 (62)	13 (87)	5 (56)	0.048	2 (40)	6 (50)	0.81
ASMA (≥20 U), *n* (%)[c]	14 (47)	13 (87)	5 (62)	0.21	[d]	1 (9)	[d]

HBV, Hepatitis B virus; HCV, Hepatitis C virus; NASH/NAFLD, Nonalcoholic steatohepatitis/nonalcoholic fatty liver disease; CS, corticosteroids; CNI, calcineurin inhibitor; AZA, azathioprine; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin inhibitor; TB, total bilirubin; ANA, Anti-nuclear antibody; ASMA, Anti-smooth muscle antibody.

[a]*Fisher's exact test.*

[b]*All subjects were HBsAg negative. One subject in tnAIH was AMA positive. Two subjects (one PCR, one tnAIH) were HCV Ab positive, HCV RNA in these subjects were negative.*

[c]*Percent with data available.*

[d]*No data available.*

Bolded values represent statistically significant difference ($p < 0.05$).

prevalent in the AIH groups (tnAIH 27%, fAIH 20%, rAIH 25%) when compared to PCR (0%). The most common etiologies of pre-transplant liver disease for the PCR group were non-alcoholic steatohepatitis (29%) and acute liver failure of indeterminate etiology (21%).

Calcineurin inhibitor (CNI) and corticosteroids (CS) were the most common IS at biopsy (47% and 45% of all subjects, respectively) (**Table 1**). Apart from one subject on a longstanding stable regimen of etanercept for rheumatoid arthritis, all other subjects in the tnAIH had received no IS prior to biopsy. In the fAIH group, most subjects were on a CS-based regimen (70%) ± azathioprine (29%) or CNI (14%). IS use in the rAIH group were varied, with CNI (87%) or mammalian target of rapamycin (mTOR) inhibitor (12%) base with the addition of CS (87%), mycophenolate mofetil (MMF) (37%), and/or azathioprine (12%). Among PCR, the IS at biopsy were most commonly CNI-based (93%) with MMF (71%) and/or CS (50%). All episodes of PCR were managed with addition or increase of CS ± uptitration of CNI.

TABLE 2 | Histopathologic characteristics of individuals with plasma cell hepatitis.

Parameter	All subjects	Non-transplant			Post-transplant		
		tnAIH	fAIH	*p*-value	rAIH	PCR	*p*-value
	N = 47	*N* = 15	*N* = 10		*N* = 8	*N* = 14	
Lymphocytic Infiltration (cells/tract), median							
Total lymphocytes	137.0	167	105	**0.01**	140	110	0.08
Total plasma cells	37.1	50	20	**0.003**	16	48	<**0.001**
% Plasma cells	30.0	30	20	0.26	10	35	<**0.001**
Fibrosis							
Mild/minimal	24 (51)	6 (40)	3 (30)	1.00[a]	5 (62)	10 (71)	1.00[a]
Moderate/severe	23 (49)	9 (60)	7 (70)		3 (37)	4 (29)	
Portal Inflammation, *n* (%)							
Mild/minimal	9 (19)	1 (7)	1 (10)	1.00[a]	2 (25)	5 (36)	1.00[a]
Moderate/severe	38 (81)	14 (93)	9 (90)		6 (75)	9 (64)	
Lobular inflammation, *n* (%)							
Mild/minimal	30 (64)	4 (27)	7 (70)	**0.046**[a]	8 (100)	11 (78)	0.27[a]
Moderate/severe	17 (36)	11 (73)	3 (30)		0 (0)	3 (21)	
Perivenular inflammation, *n* (%)							
Mild/minimal	30 (64)	7 (47)	8 (80)	0.14[a]	7 (87)	8 (57)	0.19[a]
Moderate/severe	17 (36)	8 (53)	2 (20)		1 (12)	6 (43)	
Bridging necrosis, present, *n* (%)	11 (23)	8 (53)	2 (20)	0.21[a]	1 (12)	0 (0)	0.12[a]
Interface hepatitis, present, *n* (%)	38 (81)	15 (100)	7 (70)	<**0.001**[a]	7 (87)	9 (64)	0.58[a]
Perivenular necrosis, present, *n* (%)	17 (36)	8 (53)	2 (20)	0.18[a]	1 (12)	6 (43)	0.19[a]

[a]*Fisher's exact.*

Bolded values represent statistically significant difference ($p < 0.05$).

TABLE 3 | Prevalence of IgG4-PC in the subtypes of plasma cell hepatitis.

Parameter	All subjects	Non-transplant			Transplant		
		tnAIH	fAIH	*p*-value	rAIH	PCR	*p*-value
	N = 47	*N* = 15	*N* = 10		*N* = 8	*N* = 14	
Prevalence of IgG4-PC, *n* (%)							
IgG4-PC Present	30 (64)	13 (87)	4 (40)	**0.03**[a]	2 (25)	11 (79)	**0.03**[a]
IgG4-PC Absent	17 (36)	2 (13)	6 (60)		6 (75)	3 (21)	
IgG4-Positivity							
IgG4-PC, median	2	2	0	**0.014**	0	10	**0.01**
IgG-PC, median	35	50	20	<**0.001**	16	43	<**0.001**
IgG4-PC/IgG-PC, median	0.040	0.060	0.000	**0.02**	0.000	0.228	**0.02**
IgG4-PC/IgG-PC, IQR	0.000–0.0177	0.040–0.079	0.000–0.033		0.000–0.035	0.039–0.558	
IgG4-Positivity Tertile							
1	17 (36)	2 (13)	6 (60)	0.05[a]	6 (75)	3 (21)	0.06[a]
2	15 (32)	9 (60)	3 (30)		0 (0)	3 (21)	
3	15 (32)	4 (27)	1 (10)		2 (25)	8 (58)	

IgG4-PC: immunoglobulin G subclass 4-positive plasma cells.

IgG-PC: immunoglobulin G-positive plasma cells.

[a]*Fisher's exact test.*

Bolded values represent statistically significant difference ($p < 0.05$).

Histopathological Comparison of Plasma Cell Hepatitis Subtypes

The H&E stained and trichome stained section of liver needle biopsy specimen were reviewed by a liver pathologist for the aforementioned features (**Table 2**). In the non-transplant groups, tnAIH demonstrated greater median total lymphocyte count ($p = 0.01$), median total plasma cell count ($p = 0.003$), severity of lobular inflammation ($p = 0.046$), and presence of interface hepatitis ($p < 0.001$) compared to fAIH. In the post-transplant groups, PCR demonstrated higher median number of plasma cells ($p < 0.001$) and proportion of plasma cells relative to total lymphocyte count ($p < 0.001$) compared to rAIH. However, the overall histologic similarities among PCH subtypes did not allow for definitive differentiation based on the histopathological assessment with H&E staining.

Comparison of IgG4-PC Infiltration

To investigate whether each PCH disease reflects distinctive or overlapping immuno-pathologies, we further examined the specimen by immunophenotyping of infiltrating PC via immunohistochemical analysis of IgG4 expression. IgG4-PC was identified in 30 cases (64%), while the remaining 17 cases did not exhibit the presence of IgG4-PC (**Table 3**). IgG4-PC was not seen in the lobular inflammatory component of any cases.

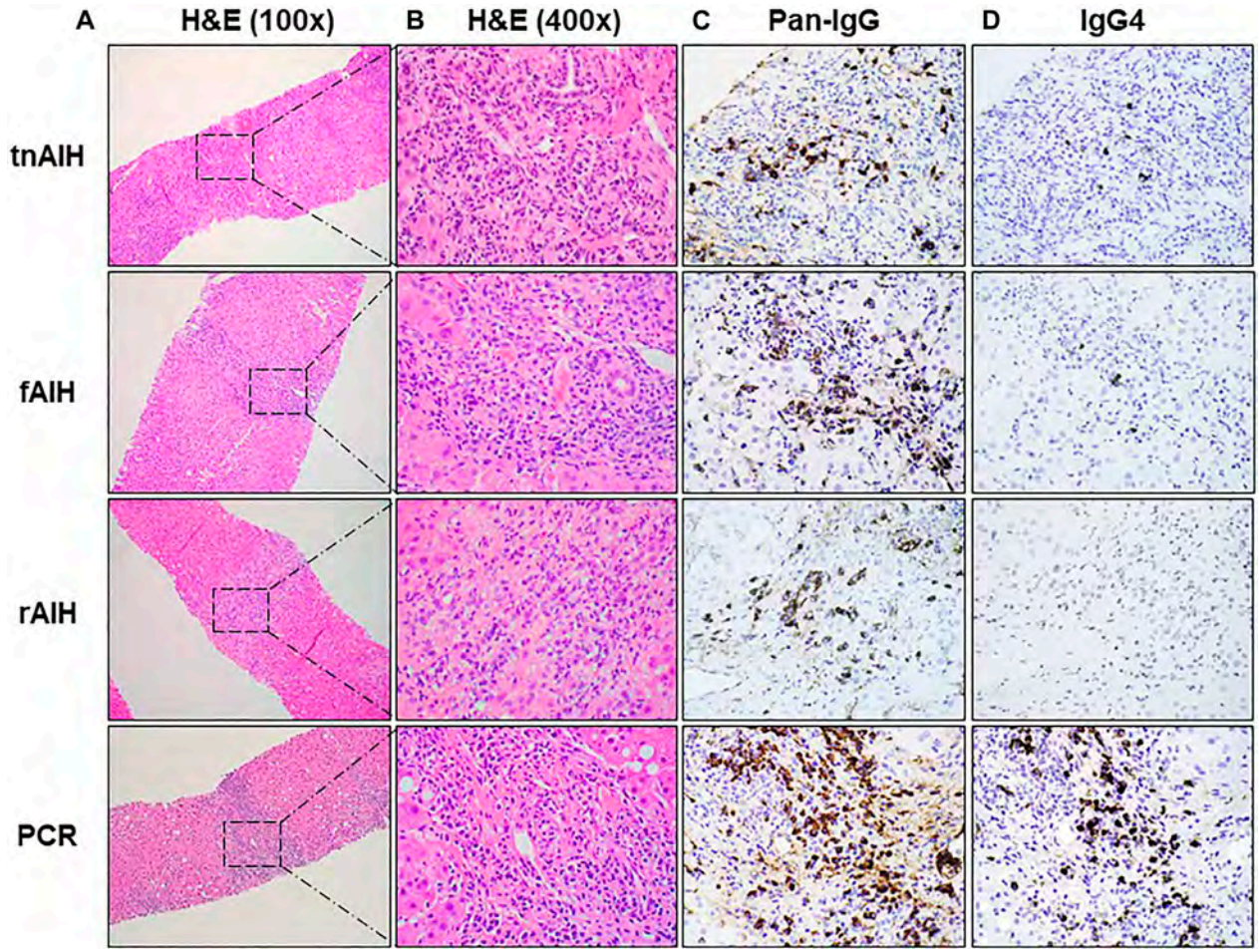


FIGURE 1 | Representative histopathologic and immunohistochemical findings of PCH subtypes: H&E stain of representative portal tract at low magnification (×100) with box with dotted line showing foci of plasma cell infiltration **(A)**, higher magnification (×400) of the selected area of plasma cell aggregates in **(A)** with H&E staining **(B)**, the immunohistochemical (IHC) staining of the representative portal tracts with anti-pan-IgG antibody (×400) **(C)**, and the IHC staining of the corresponding portal tract with anti-IgG4 antibody (×400) **(D)**.

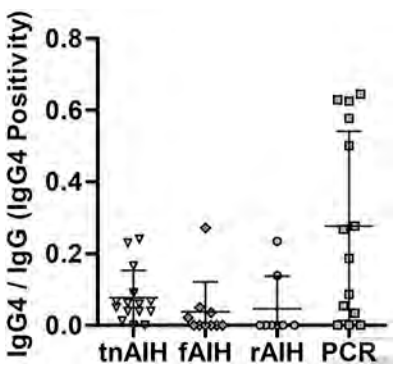


FIGURE 2 | IgG4-Positivity distribution within each PCH subtype. The number IgG4-PC was normalized with the total number of PC in the representative portal tract to determine the IgG4-Positivity. The IgG4-Positivity of each subject are displayed based on their clinical diagnosis: tnAIH (n = 15), fAIH (n = 10), rAIH (n = 8), and PCR (n = 14).

Prevalence of IgG4-PC was highest for tnAIH (87%) followed by PCR (79%), fAIH (40%), and rAIH (25%). The IgG4-Positivity, defined as the number of IgG4-PC over the total number of IgG-expressing PC in the corresponding portal tract, was highly divergent among the PCH types (**Figures 1**, **2**). The diagnosis of PCR demonstrated the highest median IgG4-Positivity (0.228, IQR 0.039–0.558), followed by tnAIH (0.060, IQR 0.040–0.079), rAIH (0.000, IQR 0.000–0.035), and fAIH (0.000, IQR 0.000–0.033).

In the non-transplant groups, tnAIH demonstrated a higher median IgG4-Positivity than fAIH (p = 0.03). In the transplant groups, PCR demonstrated a higher median IgG4-Positivity than rAIH (p = 0.03) (**Table 3**). Five subjects with IgG4-Positivity ≥0.500 were all in the PCR group (**Figure 2**). No rAIH subjects demonstrated this degree of IgG4-Positivity. These observations raise the possibility that PCH is comprised of heterogenous immunophenotypes as the IgG4-Positivty is highly variable among and within PCH subtypes.

TABLE 4 | Clinical and histopathologic characteristics stratified by IgG4-positivity.

Parameters		IgG4-positivity			
	Tertile	**1**	**2**	**3**	***p*-value**
	IgG4-PC/IgG-PC	**0**	**>0–0.087**	**>0.087**	
	n	**17**	**15**	**15**	
Age (years), median		41	50	57	0.11
BMI (kg/m^2), mean		27.41	31.98	27.62	0.64
Female, *n* (%)		13 (76)	12 (80)	10 (67)	0.54
Family history of autoimmune diseases, *n* (%)		2 (12)	2 (13)	4 (27)	0.27
Immunosuppressants (IS), *n* (%)					
CS		14 (82)	4 (27)	3 (20)	<0.001
CNI		9 (53)	4 (27)	9 (60)	0.74
AZA		2 (12)	0 (0)	1 (7)	0.53
MMF		6 (35)	2 (13)	5 (33)	0.86
MTOR		1 (6)	0 (0)	0 (0)	0.24
Other IS		0 (0)	0 (0)	1 (7)	0.21
Laboratory Data					
Pre-Treatment					
WBC (cells/L x109), median (normal 4.1–10.8)		6.2	6.4	4.9	0.71
Neutrophil %, median (normal 40–60%)		60	54	57	0.48
Lymphocyte %, median (normal 20–40%)		25	30	26	1.00
Eosinophil %, median (normal 0–3%)		1.5	1.8	3.7	0.02
Platelet count (K/cumm), median (normal 141–401)		172	204	148	0.22
Na (mg/dl), median (normal 135–145)		137	137	138	0.50
Cr (mg/dl), median (normal 0.40–1.10)		0.81	0.67	0.87	0.83
ALP (U/L), median (normal 34–106)		199	122	213	0.39
AST (U/L), median (normal 38–126)		98	353	107	0.66
ALT (U/L), median (normal 14–54)		120	297	111	0.64
TB (mg/dl), median (normal 0.2–1)		1.3	4.5	1.2	0.70
Albumin (g/dl), median (normal 3.4–5.3)		3.6	3.8	3.3	0.35
Total protein (mg/dl), median (normal 6.0–8.2)		6.8	7.4	7.0	0.41
IgG, Total (mg/dl), median (normal 600–1,640)		1,617	2,495	2,420	0.14
ANA titer (≥1:80), n (%)[a]		9 (60)	11 (79)	7 (54)	0.78[b]
ASMA (≥20 U), n (%)[a]		10 (71)	8 (67)	4 (36)	0.09[b]
On Treatment					
Day 7–10 (% change from pre-treatment, median)					
ALP		−19.4	−15.5	−15.1	0.92
AST		−40.0	−71.1	−65.9	0.16
ALT		−42.8	−44.9	−60.7	0.19
TB		−13.8	−28.6	−27.8	0.45
Day 30–60 (% change from pre-treatment, median)					
ALP		−23.9	−29.3	−25.0	0.74
AST		−42.4	−78.5	−78.9	0.08
ALT		−43.4	−69.9	−74.6	0.13
TB		−15.9	−68.5	−28.2	0.54
Histopathologic data					
Fibrosis, *n* (%)					
Mild/minimal		7 (41)	7 (47)	10 (67)	0.57[b]
Moderate/severe/cirrhosis		10 (59)	8 (53)	5 (33)	
Portal inflammation, *n* (%)					
Mild/minimal		5 (29)	0 (0)	4 (27)	0.78[b]
Moderate/severe		12 (71)	15 (100)	11 (73)	
Perivenular inflammation, *n* (%)					
Mild/minimal		14 (82)	7 (47)	8 (53)	0.04[b]
Moderate/severe		3 (18)	8 (53)	7 (47)	
Lobular inflammation, *n* (%)					
Mild/minimal		17 (100)	5 (33)	8 (53)	0.005[b]
Moderate/severe		0 (0)	10 (67)	7 (47)	
Interface hepatitis, *n* (%)		8 (47)	13 (87)	11 (73)	0.51[b]
Perivenular necrosis, *n* (%)		3 (18)	8 (53)	3 (20)	0.82[b]
Bridging necrosis, *n* (%)		2 (12)	7 (47)	3 (20)	0.54[b]

IS, immunosuppressants; CS, corticosteroids; CNI, calcineurin inhibitor; AZA, azathioprine; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin inhibitor; TB, total bilirubin; ANA, anti-nuclear antibody; ASMA, anti-smooth muscle antibody.

[a]*Percent with data available.*

[b]*Fisher's exact test. Binary comparisons made using "mild/minimal" vs. other where relevant.*

TABLE 5 | Comparison of treatment response of post-LT PCH stratified by diagnosis and IgG4-Positivity.

Laboratory data	Diagnosis			IgG4-positivity		
	rAIH	PCR	*p*-value	<0.500	≥0.500	*p*-value
	N = 8	*N* = 14		*N* = 17	*N* = 5	
Percent change from index biopsy[a] (median)						
At Day 7–10						
ALP	−11.6	−24.9	0.48	−11.9	−25.4	0.37
AST	−32.7	−65.9	0.11	−36.4	−82.9	**0.04**
ALT	−20.2	−61.0	0.34	−20.0	−62.2	0.11
TB	−0.1	−25.8	0.36	−9.1	−40.0	0.16
At Day 30–60						
ALP	−2.9	−34.5	0.10	−23.9	−27.8	0.57
AST	−31.5	−72.7	0.12	−54.5	−91.1	0.27
ALT	−17.8	−68.8	0.12	−36.4	−79.6	0.41
TB	−7.9	−18.3	0.62	−16.7	+46.7	0.36

[a]*Percent change = (Value at Index Biopsy—Value at Day X)/Value at Index Biopsy. Bolded values represent statistically significant difference (p < 0.05).*

Association Between IgG4-Positivity and Clinical Presentation

To better understand the clinical implication of IgG4-PC infiltration in PCH, we evaluated the potential association between IgG4-Positivity, laboratory parameters, histopathology and therapeutic outcomes. Our analysis showed that increasing tertile of IgG4-Positivity was associated with increasing peripheral eosinophil percentage ($p = 0.02$). This is consistent with the findings seen in other IgG4-RD (32). The proportion of subjects with CS use at the time of the biopsy decreased with increasing tertile of IgG4-Positivity ($p < 0.001$). Alternatively, no statistically significant trends were detected between degree of IgG4-Positivity and serum white blood cell count ($p = 0.71$), serum sodium ($p = 0.50$), serum creatinine ($p = 0.83$), ALP ($p = 0.39$), AST ($p = 0.66$), ALT ($p = 0.64$), or total bilirubin ($p = 0.70$) at time of biopsy (**Table 4**). Notably, no significant association was detected between IgG4-Positivity and serum IgG level ($p = 0.14$) or elevated ANA titer (≥1:40) ($p = 0.78$). ASMA positivity decreased by tertile of IgG4-Positivity from 71% to 36% in the lowest to highest tertiles, although this trend did not achieve statistical significance ($p = 0.09$). The relationship between serum IgG4 level and IgG4-Positivity could not be assessed due to limited data, though prior studies have shown that serum IgG4 concentration does not serve as the surrogate for the degree of IgG4-PC infiltration in the liver (28).

Histologically, higher degree of IgG4-Positivity was associated with severe necro-inflammatory change in the histological assessment (**Table 4**). The proportion of subjects with moderate or severe perivenular inflammation ($p = 0.04$) and lobular information ($p = 0.005$) increased with tertile of IgG4-Positivity but was not significantly associated with presence of moderate or severe fibrosis ($p = 0.57$).

To understand the association between IgG4-Positivity and biochemical response to treatment in the post-LT setting, an analysis comparing responsiveness (percent improvement from index biopsy) of liver-related serologic tests (ALP, AST, ALT, TB) of current diagnoses (rAIH and PCR) to IgG4-Positivity strata (<0.500 and ≥0.500) was performed. High IgG4-Positivity (≥0.500), all of which were PCR cases, was associated with greater improvement of AST ($p = 0.04$) at 7–10 days after treatment initiation. However, no significant difference was found when comparing by diagnosis ($p = 0.11$) (**Table 5**). The % improvement in AST at later time point (30–60 days) also increased with increasing tertile of IgG4-Positivity, though did not reach statistical significance ($p = 0.08$) (**Table 4**).

In summary, our analysis suggests that the high degree of IgG4-PC proliferation is associated with the histological features of severe inflammation in non- and post-LT setting, as well as more rapid response to IS in the post-LT setting.

DISCUSSION

This represents the first study to comprehensively evaluate the degree of IgG4-PC infiltration across types of PCH in the non- and post-transplant setting. Notably, our results showed a high degree of IgG4-PC infiltration more frequently associated with the clinical diagnosis of PCR than tnAIH, fAIH, and rAIH. In particular, markedly high IgG4-Positivity (≥0.500) was only found in PCR cases. This implies that IgG4-PC infiltration to this extent is not a general immune response to a liver allograft, but rather appears to be a unique immunopathological fingerprint of PCR (**Figure 2**). We also note that there are cases of PCR with minimal or absent of IgG4-PC infiltration. Accordingly, the overlapping profiles of rAIH and some cases of PCR (IgG4-Positivity <0.500) raises the possibility a shared immunopathology in these two separate clinical entities (**Figure 2**). These findings may support prior hypotheses that a pathophysiologically-distinct *de novo* AIH exists separately from PCR (33). Moreover, there are some cases with relatively high degree of IgG4-PC infiltration (IgG4-positivity >0.20) in AIH cases, especially in tnAIH group. These observations collectively indicate that each PCH disease consists of heterogeneous immunophenotypes. Additionally, we found that differentiation of PCH by IgG4-positivity may be of direct clinical relevance in the post-LT setting. The group with markedly high IgG4-Positivity (≥0.500) appears to demonstrate a more rapid response to directed IS. Consequently, our study suggests that evaluation of IgG4-Positivity may serve as a valuable diagnostic approach in the post-LT setting with corresponding implications in management. Additionally, the lack of rAIH cases with markedly high IgG4-Positivity in our study suggests that IgG4-Positivity may be of diagnostic utility when pre-LT diagnoses is unclear or unknown. Specifically, an IgG4-Positivity threshold of ≥0.500 may effectively rule out a diagnosis of rAIH. This is of potential therapeutic relevance as rAIH has been known to require aggressive immunosuppression to prevent graft loss (14).

To date, there remain substantial gaps in understanding the underlying pathophysiology of PCH, which significantly hinders clinical management of PCH diseases. In particular, there is concern as to whether each PCH disease represents a distinctive condition or a cluster of broader, overlapping conditions than current classification schemes. The latter case might, at least in part, be the basis for the variable degree of response to IS among and within the PCH diseases. Current management strategies of PCH entirely

rely on the empiric use of a variety of IS, which may result in significant toxicity and morbidity such as, but not limited to, the development of infection and malignancy. While the current empiric management strategy is capable of inducing remission in a large proportion of patients, the prognosis of individuals with PCH remains highly variable. In the non-LT setting, transplant-free survival of AIH is 91% and 70% at 10 and 20 years from the initial diagnosis, respectively, with up to 40% progressing to cirrhosis despite treatment (18, 34). Similarly, rAIH is responsible for 1% of all deaths at 5 years post-transplantation due to graft failure and is associated with increased risk of death from infection (14). With regards to PCR, the overall prognosis in adults is not well defined, although studies in pediatric populations demonstrate no significant impact on the prognosis (35, 36). Though PCR typically responds well to increased doses of corticosteroids, it is understood that non- or under-treated patients eventually progress to graft loss (13). As use of IS also carries its own risks (19, 37), an establishment of a specific, molecular-targeted therapeutic strategy that abrogates off-target toxicities provides an opportunity to improve patient outcomes.

Given advancement in understanding of IgG4-RD, there has been increasing interest in the involvement of IgG4-PC in liver diseases. The pioneer work examined the liver tissue of autoimmune pancreatitis patients with liver enzyme abnormalities, in which the dense infiltration of IgG4-PC in the liver parenchyma was observed in nearly all cases (28). Since the histopathological findings also exhibited multiple features commonly seen in AIH, further studies investigating the presence of IgG4-PC in the liver tissue of patients with AIH were performed (38–40). These studies found an infiltration of IgG4-PC in a small proportion of cases based on the criteria of >5 or 10 IgG4-PC per high power field (HPF). In contrast, more pronounced degree of IgG4-PC infiltration, >25 IgG4-PC per HPF was noted in cases of PCR (29). While these findings led to substantial excitement into the field, the definition of the "IgG4-PC infiltration" determined in a binary fashion (e.g. "positive" or "negative") or the number of cells staining positive for IgG4 per HPF might not serve as an objective indicator since it lacks the consideration of the total number of PC aggregated in the corresponding foci. To date, the lack of a standardized evaluation method for IgG4-PC infiltration has severely limited its clinical utility—despite expert acknowledgement of its importance in prior consensus statements (12). To overcome this fundamental issue, we established a rigorous approach to better evaluate the degree of IgG4-PC infiltration. To this end, we determined the frequency of IgG4-PC by normalizing the number of IgG4-PC over the total number of PCs in the corresponding portal tracts using serially sectioned slides (IgG4-Positivity). In addition, we sought to minimize the risk of data interpretation bias resulting from the potential sampling error by enrolling only samples that have at least 8 portal tracts, of which the foci with the representative tracts were used to evaluate the degree of IgG4-PC infiltration. We believe our quantification system allows objective cross-sectional evaluation of IgG4-PC infiltration.

IgG4 is known to have a unique immunomodulatory effect distinct from other types Ig (24). Other than antigen binding, Ig plays an important role in activation of the immune system through two discrete mechanisms: 1)Ig binding to fragment crystallizable (Fc) receptors (FcR) expressed on the cell surface of various immune cells, which augment the cytotoxic and phagocytic function of immune cells, and 2)Ig interaction with complement component 1q (C1q), resulting in activation of classical complement pathway. Of great interest, IgG4 has been considered a noninflammatory IgG compared with other IgG subclasses due to stronger affinity to the inhibitory FcR, Fcγ receptor IIB, and an inferior capacity in complement pathway activation (41, 42). These notions regarding the "non-inflammatory" characteristics of IgG4 appears irrelevant in PCH, at least in our study, as our observations revealed that IgG4-Positivity is associated with higher degree of hepatic necroinflammation. A potential explanation is that IgG4-PCs may serve an anti-inflammatory role when severe inflammation is present. Interestingly, despite the positive association of IgG4-Posivity with inflammation severity, there was no difference with respect to liver biochemistries at biopsy (**Table 4**).

It has been shown that the production of IgG4 involves an activation of unique upstream immune pathway, in which interleukin (IL)-4 and IL-10 secreted from $CD4^+$ (C-X-C chemokine receptor type-5) $CXCR5^+$ T cell, namely Tfh cells, facilitates transdifferentiation of naive B cells to IgG4-PC (43–45). Accordingly, affected organs in IgG4-RD exhibit an abundant infiltration of Tfh cells (45, 46). In addition, the clonal expansion of $CD4^+$ T cells with a cytotoxic function ($CD4^+$ CTLs), which abundantly express SLAM family member 7 (SLAMF7), granzyme A (GZMA), IL-1β, and TGF-β, is observed in the affected organ of IgG4-RD (47, 48). TGF-β is a potent anti-inflammatory cytokine and contributes to the fibrosis development. Thus, development of fibrosis a hallmark feature of IgG4-RD. However, we observed that infiltration of IgG4-PC was not associated with fibrosis severity. This potential discrepancy between IgG4-RD and PCH with IgG4 PC infiltration may be partially due to the close laboratory monitoring of AIH and post-LT patients, resulting in uptitration of IS before the fibrosis development. Alternatively, the immunosuppressive properties of IgG4 and TGF-β might have a stronger impact on disease presentation than the pro-fibrotic effect of TGF-β in PCH.

In general, IgG4-RD is known to be highly responsive to glucocorticoid-based IS. This notion coincides with the immunosuppressive properties of IgG4 as well as the anti-inflammatory effect of TGF-β secreted by the $CD4^+$ CTLs. Accordingly, previous studies by others reported that AIH cases with IgG4-PC infiltration were associated with a comparatively rapid response to steroid therapy to the induction of clinical remission (38, 40). Moreover, the relatively high frequency of IgG4-PC seen in PCR cases in our observation as well as by others (29) may serve as an explanation as to why a less aggressive regimen of IS is generally required for PCR compared to AIH and rAIH (8, 49, 50). This notion is also supported by our finding that markedly high IgG4-Positivity in the post-LT setting (≥0.500) was correlated with rapid improvement of serum AST, for which all cases were in the PCR

group (**Table 5**). Moreover, the strong association of decreasing IgG4-Positivity and CS use is also congruent with existing paradigm of steroid-responsiveness in IgG4-RD (**Table 4**). These observations suggest that evaluating IgG4-Positivity has a direct relevance to clinical management of PCH, particularly in the post-LT setting, as it may be of prognostic value in assessing expected response to corticosteroid therapy.

In summary, this study is the first to cross-sectionally demonstrate the diversity of the immunophenotypic profile of PCH in the non- and post-transplant setting, in the absence and presence of IS, as defined by IgG4-Positivity—with significant differences across and within disease entities. The highly heterogenous IgG4-Positivity across and within PCH entities indicate that current classification of PCH diseases is insufficient in capturing the immuno-pathophysiology. Hence, we propose refining the PCH classification strategy by incorporating the IgG4-Positivity based stratified categorization, particularly in the post-LT setting; which might inform a immunopathology-specific management strategy and prognostication. Toward this goal, further prospective studies with a larger number of subjects treated with a standardized IS regimen, and long-term follow up, are warranted.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BH participated in the conceptual design of the study; BH, TL, SC, JK, and TS participated in the performance of the research. BH and JD participated in the data analysis and manuscript preparation. JK and TS designed the study and oversaw manuscript preparation.

FUNDING

Departmental Research Fund (TS) and USC Research Center for Liver Diseases (P30DK048522) (JD).

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ryuta Kamekura at the Sapporo Medical University School of Medicine for his critical input and discussion.

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GLOSSARY

AIH autoimmune hepatitis
ANA anti-nuclear antibody
ASMA anti-smooth muscle antibody
AZA azathioprine
BMI body mass index
CD4+ cluster of differentiation 4 positive
$CD4^+$ CTLs $CD4^+$ T cells with a cytotoxic function
CNI calcineurin inhibitor
CS corticosteroids
CTL cytotoxic T lymphocyte
CXCR5 C-X-C chemokine receptor type 5
fAIH flare of autoimmune hepatitis
Fc fragment crystallizable
FcR Fc receptor
FFPE formalin-fixed paraffin embedded
GZMA granzyme A
H&E hematoxylin-eosin
Ig immunoglobulin
IgG4 immunoglobulin G subclass 4
IgG4-PC IgG4-producing plasma cells
IgG4-RD IgG4-related disease
IHC immunohistochemical
IL interleukin
IS immunosuppressants
LT liver transplantation
MMF mycophenolate mofetil
mTOR mammalian target of rapamycin
PC plasma cell
PCH plasma cell hepatitis
PCR plasma cell-rich rejection
rAIH recurrent autoimmune hepatitis
SLAMF7 SLAM family member 7
Tfh T follicular helper
TGF-β transcription growth factor-β
tnAIH treatment-naïve autoimmune hepatitis

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ORIGINAL RESEARCH
published: 23 March 2022
doi: 10.3389/ti.2022.10412

Progression of AFP SCORE is a Preoperative Predictive Factor of Microvascular Invasion in Selected Patients Meeting Liver Transplantation Criteria for Hepatocellular Carcinoma

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Microvascular invasion (MVI) is one of the main prognostic factors of hepatocellular carcinoma (HCC) after liver transplantation (LT), but its occurrence is unpredictable before surgery. The alpha fetoprotein (AFP) model (composite score including size, number, AFP), currently used in France, defines the selection criteria for LT. This study's aim was to evaluate the preoperative predictive value of AFP SCORE progression on MVI and overall survival during the waiting period for LT. Data regarding LT recipients for HCC from 2007 to 2015 were retrospectively collected from a single institutional database. Among 159 collected cases, 34 patients progressed according to AFP SCORE from diagnosis until LT. MVI was shown to be an independent histopathological prognostic factor according to Cox regression and competing risk analysis in our cohort. AFP SCORE progression was the only preoperative predictive factor of MVI (OR = 10.79 [2.35–49.4]; p 0.002). The 5-year overall survival in the progression and no progression groups was 63.9% vs. 86.3%, respectively (p = 0.001). Cumulative incidence of HCC recurrence was significantly different between the progression and no progression groups (Sub-HR = 4.89 [CI 2–11.98]). In selected patients, the progression of AFP SCORE during the waiting period can be a useful preoperative tool to predict MVI.

Keywords: hepatocellular carcinoma, microvascular invasion, liver transplantation, recurrence, delta AFP score

OPEN ACCESS

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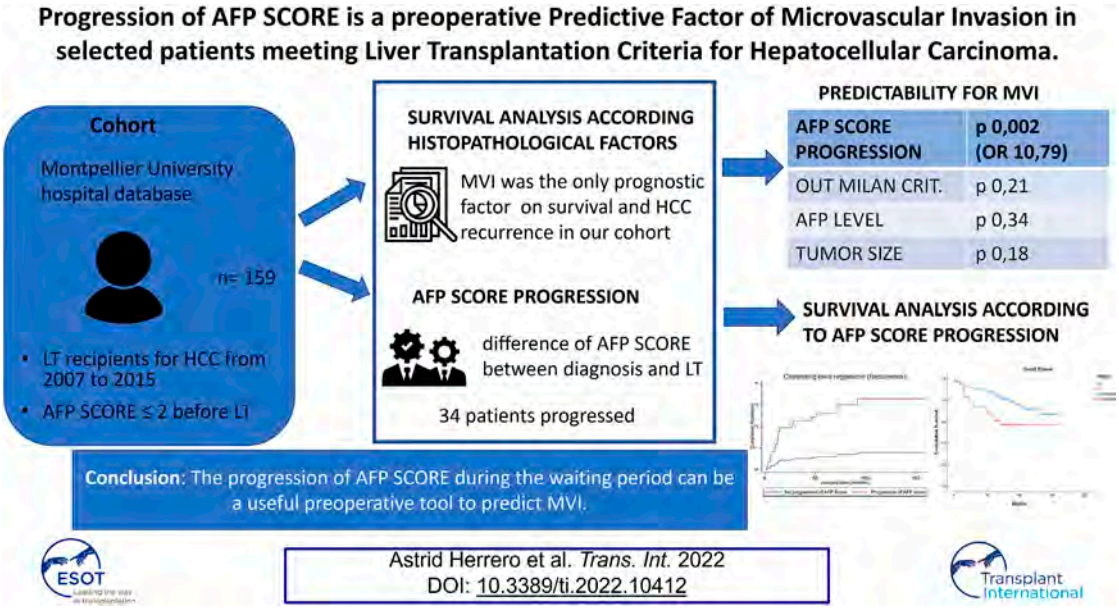
Received: *07 February 2022*
Accepted: *24 February 2022*
Published: *23 March 2022*

Citation:
Herrero A, Boivineau L, Cassese G, Assenat E, Riviere B, Faure S, Bedoya JU, Panaro F, Guiu B, Navarro F and Pageaux G-P (2022) Progression of AFP SCORE is a Preoperative Predictive Factor of Microvascular Invasion in Selected Patients Meeting Liver Transplantation Criteria for Hepatocellular Carcinoma. Transpl Int 35:10412. doi: 10.3389/ti.2022.10412

Abbreviations: AFP, alpha fetoprotein; DFS, disease-free survival; HCC, hepatocellular carcinoma; LT, liver transplantation; MVI, microvascular invasion; OS, overall survival.



GRAPHICAL ABSTRACT |

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide, ranked sixth for cancer incidence and third for cancer-related deaths (1). Liver transplantation (LT) represents the only curative therapy being one of the few tumors treated by organ transplantation when diagnosed at an early stage (2).

Five-year overall survival (OS) ranges from 65 to 80% (3, 4, 5), but it is challenged by two events that cannot be avoided: the waiting period, with the growing risk of dropout (5–10%), and the risk of recurrence (6), highly influenced by the post-transplant immunosuppressive regimen. Due to organ shortage and to the ethical principle of equity, patients with HCC are constantly "competing" with cirrhotic patients, prioritizing patients with more severe disease according to the Model for End-Stage Liver Disease (MELD) score (7, 8, 9).

All these criteria must be integrated before LT for HCC to optimize benefit on survival and limit futile transplants due to tumor recurrence leading to rapid death and graft loss.

Milan criteria, based on tumor size and number of nodules, are considered the benchmark of transplant patients selection, but despite their use, HCC still has a recurrence of 10%–15% (10, 11).

HCC recurrence after LT significantly affects long-term patient survival (12, 13). Microvascular invasion is a well-known risk factor for recurrence, as well as poor differentiation, tumor size, tumor number, and satellite nodules (14, 15, 16). Unfortunately, histological data are only available after LT, and prediction of microvascular invasion before treatment remains one of the main challenges for physicians involved in LT. Alpha fetoprotein (AFP) is the main biomarker that has been shown to predict microvascular invasion, dropout, and recurrence (17, 18, 19). However, only half of patients with HCC have abnormal AFP levels and it cannot be the only variable taken into account (20); previous papers have always focused on cohorts of non-secreting tumors (21).

In France, HCC has become the first indication for liver transplantation, concerning 30% of patients on the waiting list (22). The French Study group for LT reported a new predictive model for HCC recurrence, called AFP SCORE, that was officially endorsed in 2013 by the French organ sharing organization (Agence de la Biomédecine, ABM) (23). The use of an AFP SCORE ≤2 in the last trimester preceding LT has been shown to reduce the risk of HCC recurrence up to 10% (24, 25). Only the last static AFP SCORE value is considered for decision-making in current practice.

While several studies analyzed the interest of dynamic AFP measurements as a possible prediction tool for dropout or post liver transplant recurrence (21), no studies investigating the variation effect on AFP SCORE exist. Yet, being assessed every 3 months during the waiting period by the French organ sharing organization, it could be a relevant and an easy-to-use tool.

The main objective of our study was to evaluate the impact of the AFP SCORE variation during the waiting period to preoperatively predict the microvascular invasion risk in a selected population of LT recipients with a histologically proven AFP SCORE ≤2.

PATIENTS AND METHODS

This is a single institution observational retrospective study, conducted according to the Strengthening and the Reporting of Observational Studies in Epidemiology (STROBE) guidelines of the EQUATOR network (26). All consecutive adult recipients

who underwent LT for HCC from January 2007 to December 2015 were reviewed. All patients gave their informed consent prior to their inclusion in the study. The study was registered in the institutional review board of the Montpellier University Hospital (N° 2018_IRB-MTP_11-23). The inclusion criteria were defined according to the AFP model (23, 24), in accordance with French national guidelines, considering LT for patients with HCC with an AFP SCORE ≤2 at the last trimester preceding LT. HCC was histologically proven on the native liver. Patients with an AFP SCORE >2 at HCC diagnosis but were down-staged by locoregional therapies to fit transplantation criteria and patients who underwent LT for recurrence after a first liver resection or ablation (salvage transplantation) were also included. The exclusion criteria included presence of cholangiocarcinoma, incidental finding of HCC on the explant, and patients transplanted within 3 months after the diagnosis of HCC.

Data regarding LT recipients' age, gender, BMI, primary etiology of cirrhosis, and Child Pugh and MELD scores were collected. Tumor characteristics collected at the diagnosis before any treatment were number of nodules, size of the largest nodule, AFP level, the AFP SCORE, and grading according to the Milan criteria. Histopathology data collected on the explant were size of the largest nodule (mm), number of nodules, tumor differentiation according to the WHO classification, microvascular invasion, macrovascular invasion, and satellite nodules (27).

Management During the Waiting Period and the Follow up

All variables of interest were evaluated every 3 months during the waiting period by CT scan or MRI and blood sample analysis until liver transplantation (based on the national protocol for patients on the waiting list for liver transplantation). Any bridging therapies during the waiting period were decided by the institutional weekly Multi-Disciplinary Team in HCC of the Montpellier University Hospital, in accordance with the European and French guidelines (28, 29). All bridging therapies performed were reported. The delay between HCC diagnosis and the inscription on the waiting list and the delay between the inscription and the liver transplantation was recorded. Over the study follow-up period, the same standard immunosuppressive regimen was followed by LT recipients, consisting of tacrolimus (plus steroids for the first 3–6-month period post-LT) ± mycophenolate mofetil. Follow-up was scheduled every 3 months during the first year after LT, then every 6 months until May 2020. Tumor recurrence was screened performing serum AFP levels and chest and abdominal CT scans or hepatic ultrasounds every 3 months during the 2 first post-operative years, and then twice a year and/or when clinically indicated.

Definition of AFP SCORE Progression

The AFP SCORE (0–9 points) was calculated depending on largest tumor diameter (≤3 cm = 0 points, 3–6 cm = 1 point, >6 cm = 4 points), number of HCC nodules (1–3 nodules = 0 points, ≥4 nodules = 2 points), and pre-LT AFP levels ng/ml (≤100 = 0 points, 101–1,000 = 2 points, and >1,000 = 3 points) (23).

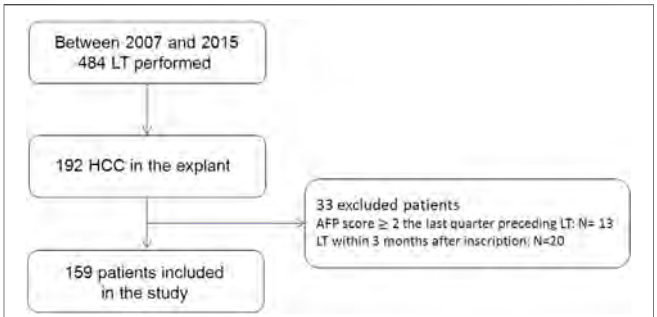


FIGURE 1 | Flow chart of the study population.

The variation of AFP SCORE was calculated from the difference between AFP SCORE at the diagnosis of HCC and AFP SCORE 3 months before LT, regardless of pre-transplant therapy (radiofrequencies, chemo-embolization, or others), as it is part of the natural history of HCC patients on the waiting list. Patients were classified into two groups: progression (ΔAFP ≥ +1) and non-progression (Δ < 1).

Endpoints

The primary outcome was the preoperative prediction of MVI on the explant. MVI was defined as the presence of tumor cells in portal veins, in large capsule vessels, or in a vascular space lined by endothelial cells on microscopy. Pathological specimen were evaluated on 5 mm slices, observed by two expert pathologists, blinded from clinical data (30).

Secondary outcomes were OS and HCC recurrence risk after LT according to AFP SCORE progression.

Statistical Analysis

The categorical data were described by frequencies and percentages, whereas continuous data were described by mean ± standard deviation (SD) or median ± interquartile range (IQR) depending on whether or not they showed a normal distribution. Categorical variables were compared by using the χ^2 test or Fisher exact test, while continuous variables were compared by applying Student's t-test or Mann-Whitney test, when appropriate. Median follow-up (and 95% CI) was computed using the reverse Kaplan-Meier method. Overall and disease-free survival were calculated using the Kaplan-Meier method. First, we aimed to confirm the prognostic impact of MVI on OS through a Cox regression analysis incorporating histopathological data determined from the native liver. Secondly, predictive factors associated with MVI were identified using uni- and multivariate logistic regression models. Owing to the relatively limited number of events, relevant variables with a p value of less than 0.1 were selected for multivariate analysis via a backward procedure, and an internal validation of the model was performed with 150 bootstrap samples to prevent overfitting (cf **Supplementary**

TABLE 1 | Comparative analysis according to the AFP SCORE progression during the waiting period.

Characteristics	Total (*n* = 159)	Progression (*n* = 34)	No progression (*n* = 125)	*p*-value
Age, mean (range)	57.9 (39–72)	59.3 (46–69)	57.5 (39–72)	0.18
Sex, male/Female	136/23	31/7	105/16	0.85
BMI, kg/m^2 median (IQR)	26 (±6)	26 (±6)	26 (±7)	0.58
Etiology of cirrhosis (%)				
Hepatitis C	55 (34)	6 (18)	49 (40)	0.01
Hepatitis B	10 (6)	2 (6)	8 (6.5)	0.91
Alcohol	75 (47)	21 (62)	54 (43)	0.055
NASH	11 (7)	4 (12)	7 (5.6)	0.20
Other	8 (6)	1 (2)	7 (5.6)	0.52
MELD score, Median (IQR)	11 (±9)	10 (±5.25)	12 (±9)	0.13
MELD >30 (%)	2 (1)	0	2 (99)	0.45
MELD <30 (%)	157 (99)	34 (100)	123 (1)	
Child- Pugh score (%)				
A	79 (49)	19 (56)	60 (49)	0.49
B	52 (32)	10 (30)	42 (34)	0.64
C	26 (19)	5 (14)	21 (17)	0.61
Time on waiting list, mean (SD)	7.32 (±5.75)	7.85 (±4.95)	7.18 (±5.9)	0.50
Pre-LT tumor treatment (%)	119 (78)	24 (64)	95 (76)	0.51
Tumor number, (%)				
1	73 (45)	19 (50)	67 (50)	0.81
2	44 (28)	8 (21)	36 (27)	0.55
≥3	42 (27)	11 (29)	31 (23)	0.37
Tumor size, mm, Median (IQR)	18.5 (±17)	24 (±11)	22 (±13)	0.68
Diameter of largest nodule (%)				
≤30 mm	124 (78)	95 (76)	29 (85)	0.28
30–60 mm	32 (20)	27 (22)	5 (15)	0.37
>60 mm	3	3 (2)	0	
AFP at diagnosis, ng/ml, median (range)	6 [1–1,584]	5.95 (2–607)	6 (1–1,584)	0.27
AFP at diagnosis, ng/ml				
≤100	153 (96.5)	120 (96)	33 (97)	0.77
100–1,000	5 (3)	4 (3)	1	0.93
>1,000	1 (0.5)	1	0	
AFP SCORE at diagnosis				
0	111 (70)	28 (82)	83 (66)	0.07
1	27 (17)	5 (15)	22 (18)	0.69
2	13 (8)	1 (3)	12 (10)	0.20
≥3	8 (5)	0	8 (6)	

TABLE 2 | Histopathological features on surgical specimens after liver explant.

MVI (%)	31 (20)
Macrovascular invasion (%)	4 (2.5)
Satellite nodules (%)	19 (11.9%)
Median tumor size (IQR)	30 (20)
Median tumor number (IQR)	2 (2)
Poor differentiation (%)	15 (9.4)

MVI, microvascular invasion; IQR, inter-quartile range; poor differentiation, G3 sec. Edmonson.

TABLE 3 | Cox regression model for overall survival, univariate analysis.

Cox regression model for overall survival according to histopathological features		
	HR (CI 95%)	***p*-value**
MVI	**3.85 (1.98–7.49)**	**0.000**
Poor differentiation	0.89 (0.34–2.29)	0.815
Satellite nodules	0.85 (0.31–2.30)	0.460
Tumor size >30 mm	1.63 (0.85–3.14)	0.139
Tumor nodule >3	1.31 (0.63–2.69)	0.460

Bold values represent statistically significant results
MVI, microvascular invasion; SHR, sub-distribution hazard ratio; CI, confidence interval.

Material) (31). In addition, the area under the ROC (AUROC) curve was computed to capture the predicting ability of the model. Finally, OS was compared in patients with vs. without AFP SCORE progression, using a log-rank test. HCC recurrence after LT was analyzed in a competing risks framework with HCC recurrence and death as competing events. Cumulative incidence curves for HCC recurrence using Fine-Gray proportional sub-distribution hazards models according to the AFP SCORE progression were performed. Log-linearity was checked and continuous variables were transformed whenever necessary (32).

All analyses were performed with the Stata software, version 17 (Stata Corporation, College Station, TX, United States). A p-value < 0.05 was considered significant.

TABLE 4 | competing risk analysis for HCC recurrence according to post-operative histopathological factors.

	Competing risk analysis for HCC recurrence	
	***p*-value multivariate SHR [CI]**	***p*-value univariate**
MVI	**0.000 8.11 [CI 3.13–20.96]**	**0.000**
Poor differentiation	0.403	**0.056**
Satellite nodules		0.72
Tumor size >30 mm		0.47
Tumor nodule >3		0.08

Bold values represent statistically significant results
MVI, microvascular invasion; SHR, sub-distribution hazard ratio; CI, confidence interval.

RESULTS

Patient and Tumor Characteristics

From 2007 to 2015, among 484 liver transplantations performed in our hospital, 192 patients presented with HCC on the native liver and 159 patients met the inclusion criteria (**Figure 1**).

Liver transplantation was performed using full grafts or partial grafts from a split procedure ($n = 2$) from deceased donors. At the diagnosis, the tumor number was 1 in 45% of patients ($n = 73$), 2 in 28% of patients ($n = 44$), and ≥3 in 27% of them ($n = 42$). Median tumor size was 18.5 mm [±17]. The median value of AFP level was 6 ng/ml (range:1–1,584 ng/ml), while AFP SCORE was 0 in 70% of patients ($n = 111$), 1 in 17% ($n = 27$), 2 in 8% ($n = 13$), and ≥3 in 5% of patients ($n = 8$). All patient and tumor characteristics are detailed in **Table 1**. The mean time on the waiting list was 7.32 months (±5.75). Overall, 119 patients (75%) received one or more bridging therapies to control the disease during the waiting period, according to the institutional multidisciplinary team indications (as detailed in the **Supplementary Material**).

Pathological examination of the explanted liver showed that the median tumor size was 30 mm (±20 mm). The HCC nodule was solitary in 55 patients (22%), while 64 LT recipients had three or more lesions (40%), and 89 patients (56%) were within the Milan criteria. Among the 70 LT recipients (44%) beyond the Milan criteria, histological analysis showed a tumor size >50 mm in six patients, four or more nodules in 35 patients; 29 patients had less than three nodules but with a tumor size >30 mm. According to the WHO classification, the tumor was well, moderate, or poorly differentiated in 28, 62, and 10% of patients, respectively. MVI was found in 31 (19.5%) explants. Among them, 14 presented satellite nodules. The histopathological results are shown in **Table 2**.

Survival Analysis According to Post-Operative Histopathologic Factors on the Native Liver

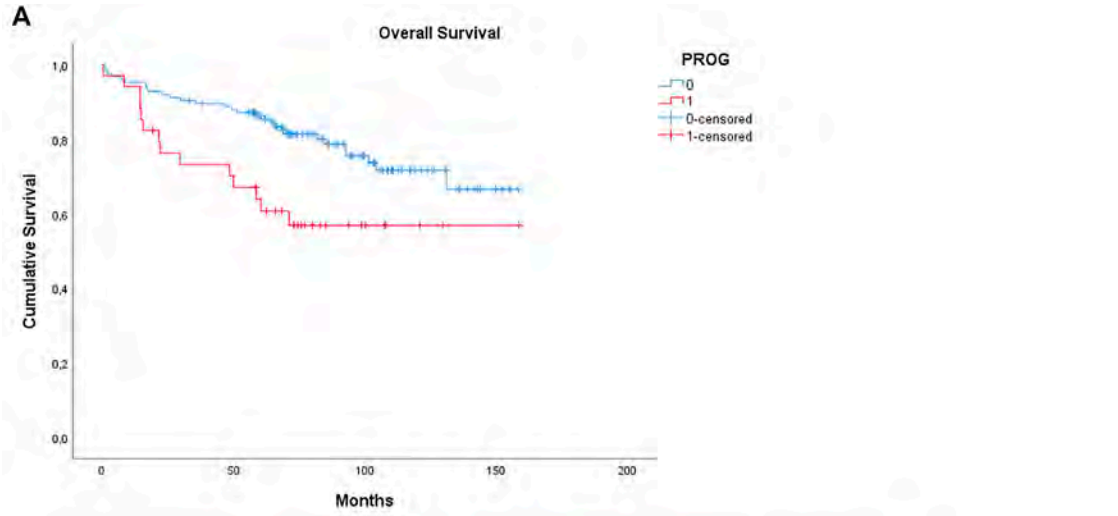
After a median follow-up of 94 months [95% CI: 83–105], a total of 43 patients died (28.1%). Among them, 29 did not recur. HCC recurrence was observed in 19 patients (12%) within a median delay of 13 (range 2–92) months, and 14 of them died after the recurrence (73.6%). The 90-day post-operative mortality was 2.5% (4 patients). Three- and 5-year OS was 86.1% and 81.5%.

Cox regression analysis showed MVI as the only histopathological prognostic factor (among tumor differentiation, tumor size, number of nodules, and the presence of satellite nodules) of overall survival (HR 3.85 [95% CI 1.98–7.49]; $p < 0.0001$) (**Table 3**). Competing risk analysis for

TABLE 5 | Multivariate logistic regression analysis for prediction of MVI in patients undergoing LT for HCC.

	Tot	MVI	No MVI	Univariate analysis		Multivariate analysis	
				Odds ratio (95% CI)	***p*-value**	**Odds ratio (95% CI)**	***p*-value**
Overall *n* (%)	159 (100)	31 (19.5)	128 (80.5)				
Age >60 (%)	81 (50.9)	14 (17.3)	67 (82.7)	0.74 (0.34–1.64)	0.47		
BMI >30 (%)	37 (23.2)	27 (73)	10 (27)	1.78 (0.74–4.22)	0.17		
Cirrhosis etiology (%)							
HCV	55 (34)	6 (11)	49 (89)	0.38 (0.14–1.02)	0.10		
HBV	10 (6)	3 (30)	7 (70)	1.85 (0.45–7.61)	0.28		
Alcoholic	75 (47)	14 (18.6)	61 (81.4)	0.90 (0.41–1.98)	0.74		
NASH	11 (7)	2 (18.2)	9 (81.8)	0.91 (0.18–4.44)	0.95		
Other	8 (6)	3 (37.5)	5 (62.5)	2.63 (0.59–11.6)	0.13		
Waiting time, mean (SD)	6.78 (±5.75)	7.07 (±5.77)	7.38 (±5.79)	1.003 (0.96–1.04)	0.78		
No treatments during waiting time (%)	40 (25.1)	10 (25)	30 (75)	1.55 (0.66–3.66)	0.31		
Tumor size >30 mm (%)	17 (10.7)	6 (35)	11 (65)	**2.55 (0.86–7.55)**	**0.08**	1.49 (0.39–5.7)	0.18
Tumor nodules pre-LT ≥3 (%)	53 (33.3)	10 (19)	43 (81)	0.94 (0.40–2.17)	0.30		
Child- Turgot- Pugh (%)							
A	79 (49.6)	18 (22.7)	61 (77.7)	1.52 (0.68–3.36)	0.29		
B	52 (32.7)	7 (13.4)	45 (86.6)	0.53 (0.21–1.34)	0.18		
C	28 (17.6)	6 (21.5)	22 (78.5)	1.15 (0.42–3.15)	0.77		
MELD, median (IQR)	11 (±9)	11 (±6)	11 (±9)	0.97 (0.90–1.04)	0.42		
AFP pre-LT ng/ml, median (range)	6.65 (1–1,170)	12.7 (1.2–1,170)	5.3 (1–373)	**1.02 (0.99–1.03)**	**0.07**	1.03 (0.98–1.05)	0.34
Within Milan criteria (%)	114 (71.6)	19 (16.6)	98 (83.4)	**0.48 (0.21–1.11)**	**0.08**	0.17 (0.01–1.18)	0.21
Delta AFP SCORE progression	34 (21.3)	18 (53)	16 (47)	**9.69 (3.9–23.4)**	**<0.0001**	**10,79 (CI = 2.35–49.4)**	**0.002**

Bold values represent statistically significant results
BMI, body mass index; HCV, hepatitis C virus; HBV, hepatitis B virus; NASH, non-alcoholic steatohepatitis; LT, liver transplantation; MELD, model for end stage liver Disease; AFP, alpha fetoprotein; MVI, microvascular invasion. OR, odds ratio; CI, confidence interval. Variables with p-value <0.10 underwent multivariate analysis.



Time (months)	Cumulative Survival Rate		Number of remaining patients		p-value
	Progression	Non-Progression	Progression	Non-Progression	
12	64,1	95,2	32	119	**0,01**
36	76,3	92	25	115	**0,01**
60	70,2	86,3	23	94	**0,01**

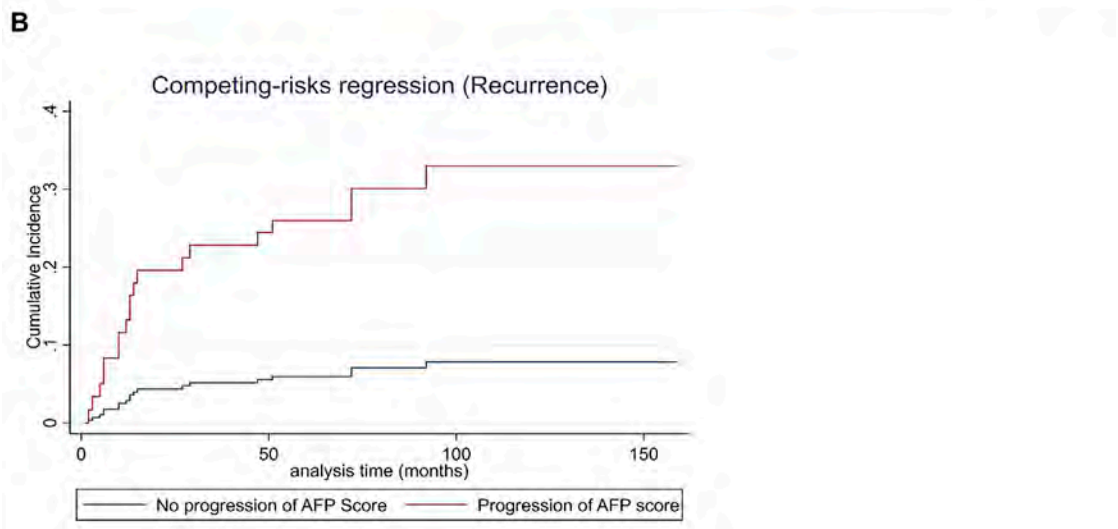


FIGURE 2 | (A) Overall survival and **(B)** competing risk regression for HCC recurrence of patients with and without delta AFP SCORE progression.

HCC recurrence identified MVI as an independent prognostic factor (SHR 8.11 [CI 3.13–20.96]; $p < 0.0001$) (**Table 4**).

AFP SCORE Variation During the Waiting Period

According to the AFP SCORE, 34 LT recipients showed progression during the waiting period.

Progressed and non-progressed patients were statistically comparable with respect to all patient and tumor characteristics, as well as time on the waiting list and bridging therapies. The only statistically significative difference was in cirrhosis etiology where the incidence of progression was lower in the HCV group (**Table 1**).

Primary Outcome: Preoperative Predictive Risk Factors on Microvascular Invasion

Tumor size larger than 30 mm, beyond Milan criteria, AFP value pre-LT, and AFP SCORE progression were associated (i.e., p-value <0.10) with MVI in univariate analysis (**Table 5**). When tested with multivariate analysis, the AFP

SCORE progression was the only independent preoperative risk factor of MVI (OR = 10.79 [95% CI = 2.35–49.4]; p 0.002). A 0.74 AUROC confirmed the good predictive ability of the multivariate model.

Secondary Outcomes: Survival Analysis and Recurrence According to Preoperative AFP SCORE Progression

Three-year and 5-year overall survival was significantly lower in the progression than the non-progression group [(3-year OS 73.2% vs. 89.6%, 5-year OS 63.9% vs. 86.3%; p 0.01] (**Figure 2A**). Cumulative incidence of recurrence significantly differed between the groups of progression and no progression in AFP SCORE (SHR = 4.89 [CI 2–11.98]; p = 0.001 (**Figure 2B**).

DISCUSSION

In the present study, for the first time in literature, we analyzed the predictability of AFP SCORE progression on MVI in a homogeneous population of LT recipients with histologically proven HCC who had AFP SCORE ≤2. Our results showed that AFP SCORE progression, for those on the waiting list for liver transplantation, was the only preoperative factor that enabled prediction of MVI. In contrast, the absolute value of AFP, Milan criteria, number of tumor nodules, and tumor size were not associated with MVI, emphasizing the need to use a composite score as defined by the AFP model.

Furthermore, variation of the AFP SCORE can be easily calculated in clinical practice and could be a relevant preoperative tool for predicting tumor aggressiveness and other related outcomes.

Notably, our patient cohort had already undergone a stringent selection process, thus, a low rate of recurrence was expected. Despite this, 19 recurrences were observed and among them 14 died shortly after. In both overall survival and HCC recurrence, MVI was shown to be a strong prognostic factor in our study population, with HR greater than 3. Despite the strict cohort selection, the augmentation of AFP SCORE by one point, even from 0 to 1—expected to be an irrelevant variation—was shown to enable prediction of MVI, with OR greater than 10.

These results could lead to an optimization of pre- or post-transplantation strategies in terms of prevention and surveillance, enabling adapted treatment strategies, radiological monitoring of patients on waiting lists, and adjustment of immunosuppressive therapy after LT.

The downstaging of the tumor burden in HCC patients awaiting transplantation has been widely shown to be advantageous in terms of survival, both in patients still on waiting lists and in those who dropped out of the criteria (33, 34, 35). Therefore, having a dynamic tool for identifying patients at high risk of MVI while in the waiting period could further identify a subgroup of candidates that could benefit from a different strategy. We suggest that further studies should explore this direction.

Several studies have reported that baseline or follow-up AFP levels are correlated with survival and/or tumor recurrence (36, 37). However, increased AFP levels are inconstant in HCC patients, with only 30%–40% of patients having abnormal values (38). Furthermore, previous studies on the topic have always selected the study population by eliminating non-secreting tumors (21). In contrast, our study included all patients transplanted for HCC who had an AFP SCORE ≤2, regardless of the levels of AFP and other parameters.

In our study, 93% of patients had an AFP level <100 ng/ml, with the median value being 6 ng/ml, which for some reason challenged the tools used to select the correct follow-up strategy. In such circumstances, AFP SCORE progression could identify patients who would benefit from a stricter follow-up. Eventually, contrast-enhanced (18)F-choline or (11)C-choline PET/CT could be useful, alone or combined with (18F)-FDG PET/CT (39, 40).

Actually, in current practice, we do not dispose of any specific tool that can predict MVI preoperatively. The originality of our study is that it demonstrates the utility of a simple preoperative dynamic score evaluation that can strongly predict MVI without performing a biopsy or radiological exam. Previous studies demonstrated the potential utility of MRI or circulating cell free DNA (cfDNA) as dynamic preoperative biomarkers. These biomarkers were found to be independent predictors of MVI (41, 42). However, cfDNA use is not widespread, as it is expensive and difficult to perform regularly in all centers due to the technical procedures necessary for genetic analysis. In contrast, AFP SCORE can be measured easily and without additional cost, as no additional exams are needed. Future studies exploring the association between cfDNA and the dynamic assessment of AFP SCORE may provide physicians with an effective tool and consequently help guide the selection of individualized therapies or treatment monitoring before radiologic and/or biologic progression.

Finally, the parameter of AFP SCORE progression can be integrated into a new predictive score for identifying the risk of tumor recurrence. The interest in creating, validating, and developing such a model is demonstrated by the increasing number of interesting similar papers (43, 44, 45). At the same time, previous efforts have not enabled identification of the best model. We believe that AFP progression could be associated with parameters related to the total tumor burden and tumor aggressiveness (pre-LT AFP levels, total tumor volume). All previous hypotheses require prospective studies on larger populations to be corroborated, but we believe that they must be considered in light of the importance of HCC recurrence in LT recipients and the relevance of MVI to recurrence and survival (46).

Our study's limitations include its retrospective design and relatively small number of events. Therefore, further large-scale, multicenter studies are needed. Another limitation is its use of the AFP SCORE, which at the moment is widely used only in France. Nevertheless, the strengths of our study include its long median follow-up of 94 months, its monocentric character, which

ensured the homogeneous management of all patients using the same surgical and medical teams, and its selection of a patient cohort with no known preoperative MVI or recurrence factors.

To conclude, this study highlights the potentially high relevance of AFP SCORE progression as a simple, dynamic, preoperative predictive factor for MVI in patients undergoing LT for HCC. These findings could lead LT units to adopt new strategies before or after LT to optimize the management of such subgroups of patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional review board of the Montpellier University Hospital (N° 2018_IRB-MTP_11-23). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AH and G-PP were responsible for study conception and design. LB, GC, and BG drafted the manuscript. BR, SF, and JB collected and analyzed data. FN, EA, and FP were responsible for the critical revision 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.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Butterworth for proofreading assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10412/full#supplementary-material

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published: 21 March 2022
doi: 10.3389/ti.2022.10308

Subjective Difficulty Scale in Liver Transplantation: A Prospective Observational Study

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The predictive value of a subjective difficulty scale (DS) after surgical procedures is unknown. The objective of this study was to evaluate the prognostic value of a DS after liver transplantation (LT) and to identify predictors of difficulty. Surgeons prospectively evaluated the difficulty of 441 consecutive liver transplantations from donation after brain death at the end of the surgery by using a DS from 0 to 10 ("the easiest to the hardest you can imagine"). DS was associated with severe morbidity. The risk of graft loss at 1 year remained unchanged from 0 to 6 but increased beyond 6. Graft survival and patient survival of group with DS 7–10 was significantly impaired compared to groups with DS: 0–3 or DS: 4–6 but were significantly impaired for the group with DS: 7–10. Independent predictors of difficult LT (DS ≥ 7) were annular segment 1, transjugular intrahepatic portosystemic shunt, retransplantation beyond 30 days, portal vein thrombosis, and ascites. Of them, ascites was a borderline non-significant covariate (p = .04). Vascular complications occurred more often after difficult LT (20.5% vs. 5.9%), whereas there was no difference in the other types of complications. DS can be used to tailor monitoring and anticipate early complications. External validation is needed.

Keywords: liver transplantation, difficulty, subjective difficulty, technical difficulty, retransplantation

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Received: *14 December 2021*
Accepted: *18 January 2022*
Published: *21 March 2022*

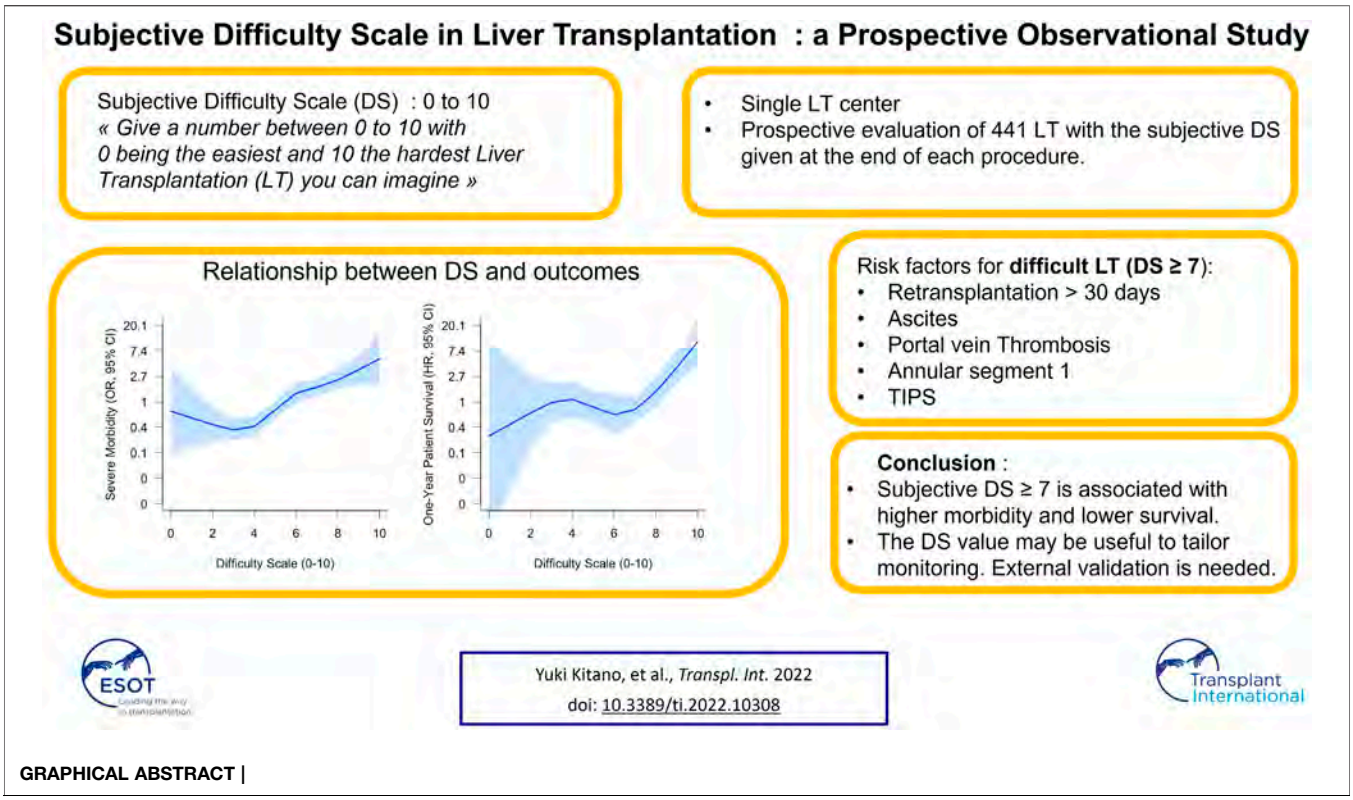
Citation:
Kitano Y, Pietrasz D, Fernandez-Sevilla E, Golse N, Vibert E, Sa Cunha A, Azoulay D, Cherqui D, Baba H, Adam R and Allard M-A (2022) Subjective Difficulty Scale in Liver Transplantation: A Prospective Observational Study. Transpl Int 35:10308. doi: 10.3389/ti.2022.10308

INTRODUCTION

The difficulty in achieving a surgical procedure dramatically varies from one patient to another, independently of its intrinsic complexity (1–5). Several difficulty scoring systems have been published in various surgical fields. These scores are usually built using surrogates of difficulty like blood loss or operation time (3, 6–8), or after selecting risk factors according to expert opinions (4, 5, 9).

This study focused on the technical difficulty of liver transplantation (LT) and proposed a different approach for assessing difficulty. Surgeons prospectively evaluated the difficulty by using a scale ranging from 0 to 10, according to their feeling at the end of the LT.

Abbreviations: BAR, balance-of-risk; DS, difficulty scale; ET-DRI, Eurotransplant Donor Risk Index; ICU, intensive care unit; LT, liver transplantation; MELD, Model for End-stage Liver Disease; PV, Portal vein; ReLT, Retransplantation; RBC, red blood cells; TACE, transarterial chemoembolization; TIPS, transjugular intrahepatic portosystemic shunt; SOFT, survival outcomes following liver transplantation.



GRAPHICAL ABSTRACT |

The prognostic value of such a subjective difficulty scale (DS) is unknown. Balance of Risk (BAR) and Survival Outcomes following Liver Transplantation (SOFT) scores are two validated tools that predict early survival after LT (10, 11). Both include donor and recipient pretransplant variables and cold ischemia time as the unique intraoperative parameter. We hypothesized that the performance of these scores could be improved by adding a subjective DS.

The objectives of this study were to test the impact of DS on outcomes and its added value with regard to validated prognostic models. Lastly, we aimed at identifying preoperative variables that predict difficult LT.

PATIENTS AND METHODS

Study Population and Design

This study included all consecutive patients who underwent LT with a full liver graft from donation after brain death from January 2015 to March 2019 at the Paul Brousse Hospital, Villejuif, France. Every LT involved a fellow, defined here as a "junior" surgeon, and an attending defined as a "senior" surgeon. At the end of each LT, junior surgeons were in charge of entering intraoperative data into a dedicated online questionnaire, including a DS item. Junior surgeons were to give a number ranging from 0 to 10 (0 being the "easiest LT that you can imagine" and 10 being the "most difficult LT you can imagine").

From October 2018 until the end of the study period, both senior and junior surgeons were asked to evaluate the DS, blinded for the evaluation of each other.

LTs without DS were not included. Donor variables were retrieved from the Cristal database of the Agence de la Biomédecine, the French national agency in charge of organ allocation. The design of this study was discussed and approved at our weekly institutional research meeting. This study was achieved in accordance with French legal requirements and the Declaration of Helsinki. Before surgery, patients provided their written consent according to which they permit that data obtained during standard health care can be used for scientific purposes.

CT Scan Review

Pretransplant CT scans were reviewed by YK, blinded for outcomes and DS value. The presence of the following items was assessed:

- annular segment I, defined as a complete inferior vena cava encirclement by hypertrophic caudal lobe.
- significant spontaneous portosystemic shunt (SPSS) ≥ 7 mm in diameter.

Technical Aspect of Liver Transplantation

Total hepatectomy was achieved with caval preservation and transient porto-caval anastomosis in most recipients. The caval anastomosis was done according to the three vein-piggy back

FIGURE 1 | Distribution of DS value across the study population.

technique (12). In the case of huge native liver, or annular segment 1, caval replacement was the preferred option. Portal inflow was obtained with a porto-portal termino-terminal anastomosis. PV thrombectomy was performed when necessary. In the case of a large spleno-renal shunt, left renal ligation or reno-portal anastomosis were decided according to the possibility of using the native portal vein (13). Extra-anatomical PV anastomosis was considered as the last option. For arterial reconstruction, hepatic artery with gastro-duodenal bifurcation was the option of choice.

Postoperative Management

Initial immunosuppression comprised a triple-drug regimen of tacrolimus, mycophenolate mofetil, and corticosteroid. Steroid boluses were used to treat moderate to severe acute rejection episodes after histological documentation. In selected cases, everolimus was introduced to enable early withdrawal of tacrolimus (14). An injected CT scan on day seven was performed routinely to detect vascular abnormalities (15). The post-transplant management and monitoring were done according to our local protocols regardless of the DS.

Statistical Analysis

All statistical analyses were performed using R version 3.5.1.

General overview

Our analysis followed 6 steps:

Step 1: We tested the relationship between DS and severe morbidity and 1-year patient survival.
Step 2: We evaluated the additional predictive value of DS by comparing the performance of BAR and SOFT scores before and after adding the DS.
Step 3: We compared survival according to three levels of difficulty: "easy" (0–3), "intermediate" (4–6), and "difficult" (7–10). Cutoff values to define these categories were arbitrarily chosen.
Step 4: We performed a univariate and multivariate analysis for predicting difficult transplantation.
Step 5: We compared the type of complications according to difficult transplantation.
Step 6: We tested the senior-junior agreement of DS during hepatectomy and implantation.

Methodology

In step 1, the relationship between DS and severe morbidity and 1-year patient survival was explored by using regression and Cox models, respectively.

DS was treated not as an ordinal variable but as a continuous variable for simplicity. Severe morbidity was defined by at least one grade IIIa event according to the Dindo-Clavien classification (16). Since several individuals have evaluated the DS, we sought for the possibility of subject-specific correlation. We tested whether the variable "individuals evaluating the DS" should be considered as a random or fixed variable (*lremTest* package) in the regression model. No significant random effect for this variable was detected, which led us to abandon mixed effect models. We left the variable "individuals assessing DS" in the logistic regression and Cox models as a covariate for more robustness (*rms* packages). Restricted cubic splines were used to relax from the linearity assumption (17). The assumption of proportionality of the Cox model was verified with Schoenfeld residuals.

In step 2, we evaluated the performance of the models without and with DS by using the Area Under Curve (AUC) and Akaike Information Criterion (AIC).

In step 3, graft survival was calculated from the date of LT. Data were censored at the time of last follow-up. The event of interest for graft survival was death or retransplantation, whereas death was the only event of interest used for patient survival calculation. Of note, for 1-year patient survival calculation, patients who died after 1 year from LT were censored. Survival curves were plotted according to Kaplan-Meier method. Survival probabilities were compared by using the log-rank test (*ggplot2* packages).

In univariate analysis (step 4 and 5), continuous variables were expressed as median (range) and compared with the non-parametric Mann-Whitney test. Categorical variables were evaluated using chi-squared or Fisher exact tests, as appropriate. Variables associated with difficult transplant ($p < .10$) were entered into a multivariate regression model. The final choice of the model was guided according to the lowest AIC.

In step 6, we used the Lin concordance correlation coefficient (18) (*DescTools* package) to assess the agreement between junior and senior surgeons.

RESULTS

Of the 631 LT performed during the study period, 525 LT met the inclusion criteria, i.e., a whole liver graft from donation after brain death. After excluding LT without available DS ($n = 84$, 16%), we obtained a study population of 441 LT, including a primary LT in 371 cases and retransplantation in the 70 remaining cases. During the study period, 404 patients underwent a single LT, 17 required two LTs, and one patient was transplanted three times, which represents a total of 422 patients.

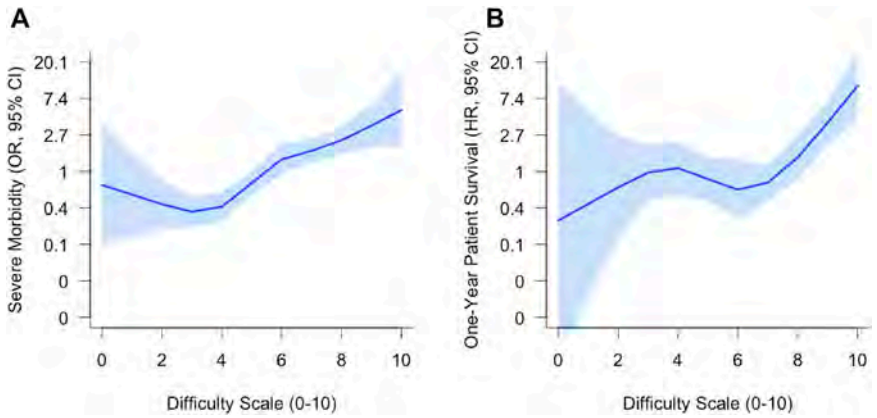


FIGURE 2 | Risk for severe morbidity **(A)** and 1-year patient survival **(B)** according to DS values. Shaded regions indicate 95% confidence bands. HR, Hazard Ratio; OR, Odds Ratio.

TABLE 1 | Performance of SOFT and BAR models with and without DS for severe morbidity, 3-month graft survival, and 1-year patient survival.

Severe morbidity							
Model	**Variables**	**OR**	**95% CI**	***p***	**AUC**	**AIC**	***p*[a]**
One-variable model	SOFT	1.06	1.03–1.09	<.001	.63	545	
Two-variable model	SOFT	1.06	1.03–1.08	<.001	.721	510	
	DS	1.40	1.26–1.57	<.001			<.001
One-variable model	BAR	1.08	1.04–1.12	<.001	.619	549	
Two-variable model	BAR	1.07	1.05–1.13	<.001	.727	510	
	DS	1.48	1.30–1.64	<.001			
3-months graft survival							
Model	**Variables**	**RR**	**95% CI**	***p***	**AUC**	**AIC**	***p*[a]**
One-variable model	SOFT	1.02	1.02–1.38	.227	.632	226	
Two-variable model	SOFT + DS	1.02	1.02–1.38	.441	.715	216	<.001
		1.38	1.14–1.70	.001			
One-variable model	BAR	1.03	.96–1.10	.304	.619	227	
Two-variable model	BAR	1.04	.97–1.11	.25	.720	217	<.001
	DS	1.40	1.16–1.72	<.001			
One-year patient survival							
Model	**Variables**	**HR**	**95% CI**	***p***	**AUC**	**AIC**	***p*[a]**
One-variable model	SOFT	1.07	1.03–1.11	<.001	.664	407	
Two-variable model	SOFT	1.07	1.03–1.11	.001	.709	397	<.001
	DS	1.34	1.12–1.59	.001			
One-variable model	BAR	1.08	1.2–1.14	.007	.626	412	
Two-variable model	BAR	1.08	1.2–1.14	.008	.701	399	<.001
	DS	1.39	1.17–1.66	<.001			

[a]*Comparisons of AUC, with the roc. test function (pROC, package).*

BAR; balance of risk; DS, difficulty scale; SOFT, survival outcomes after liver transplantation; OR, odds ratio.

For our study population, the 3-month and 1-year graft survival were 93% and 87%, respectively. One-year patient survival was 91%. Severe morbidity occurred in 166 (37.6%) LTs. A primary non-function was observed in 16 cases (3.6%).

The DS was evaluated by twelve junior surgeons. The median value of DS was 6, ranging from 1 to 10. DS was comprised between 0–3, 4-6, and 7–10 in 66 (15%), 204 (46.3%), and 171 (38.8%) LTs, respectively. The distribution of DS values is shown in **Figure 1**.

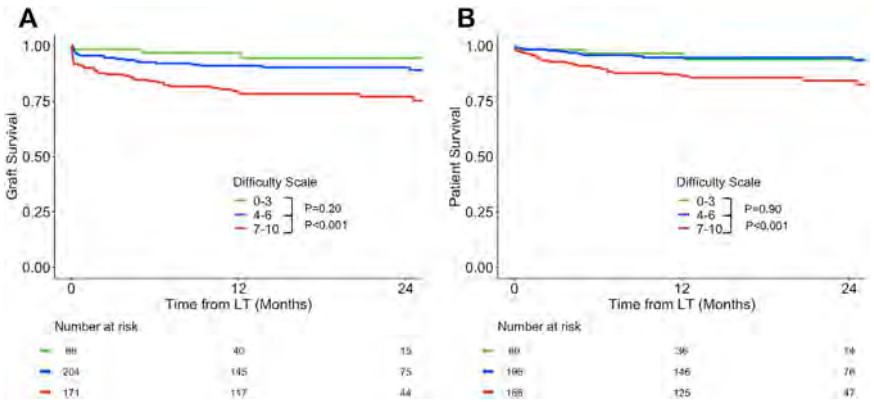


FIGURE 3 | Kaplan-Meier curves for graft survival **(A)** and patient survival **(B)** according to DS 0–3 vs. 4–6 vs. 7–10.

TABLE 2 | Risk factors for difficult LT (DS ≥ 7): Univariable and multivariable logistic regression analysis.

Variables	DS < 7	DS ≥ 7	*p*	Multivariate analysis		
	N = 270 (range or %)	*N* = 171 (range or %)		OR	95% CI	*p*
Recipient						
Male Sex	184 (68.1)	128 (74.9)	.161			
Age, years	55.0 (15.0–71.0)	53.0 (12.0–71.0)	.300			
BMI, kg/m^2	25.2 (15.4–45.7)	25.1 (11.4–46.1)	.741			
MELD score at transplant	19.0 (6.0–40.0)	19.0 (6.0–40.0)	.516			
ICU at the time of transplant	53 (19.6)	29 (17.0)	.564			
Pretransplant dialysis	12 (4.44)	10 (5.85)	.663			
ReLT beyond 30 days	17 (6.30)	32 (18.8)	<.001	4.11	2.18–7.99	<.001
TIPS in place	8 (2.96)	16 (9.41)	.007	2.68	1.06–7.12	.02
Combined Kidney transplant	16 (5.93)	12 (7.02)	.797			
Explant weight, g	1,295 (400–6,290)	1,315 (435–3,665)	.532			
Pretransplant TACE	53 (19.6)	31 (18.2)	.812			
Night time (10 pm–6 am)	43 (15.9)	28 (16.4)	>.99			
Donor						
Male sex	142 (52.6)	100 (58.5)	.266			
Age, years	60.0 (6.00–91.0)	57.0 (14.0–93.0)	.318			
BMI, kg/m^2	24.7 (13.8–51.3)	24.2 (14.6–41.0)	.595			
Weight of the graft, g	1,332 (700–2,425)	1,400 (685–2,795)	.168			
GW/recipient BW ratio	1.8 (.7–4.3)	1.8 (.8–5.9)	.601			
Explant weight/recipient BW ratio	1.7 (.7–10.5)	1.7 (.6–6.9)	.965			
Pretransplant CT scan						
Ascites[a]	103 (39.0)	95 (56.2)	.001	1.64	1.07–2.51	.04
Annular segment 1	6 (2.27)	25 (14.9)	<.001	6.58	2.71–18.49	<.001
Annular segment 1 and Piggy Back caval anastomosis	3 (1.1)	17 (10.1)	<.001			
Portosystemic shunt	120 (45.5)	116 (69)	<.001			
Portal vein thrombosis	25 (9.5)	38 (22.6)	<.001	2.17	1.20–3.95	.01
PVT Yerdel 1–2[b]	25 (9.5)	30 (17.5)	<.001			
PVT Yerdel 3	0 (0)	8 (4.8)				
Scoring systems						
BAR	8 (1–22)	8 (1–22)	.571			
D-MELD	1,050 (162–5,312)	1,064 (153–3,400)	.387			
SOFT	9 (3–36)	12 (0–45)	.004			
ET-DRI	1.47 (.95–2.86)	1.44 (.97–2.71)	.938			

BAR; balance of risk; BMI, body mass index; BW, body weight; D-MELD, Donor age X MELD, score; ET-DRI, European Transplant—Donor Risk Index; GW, graft weight; ICU, intensve care unit; MELD, Model for end-stage liver Disease; PVT, portal vein thrombosis; RBC, red blood cell; SOFT, survival outcomes following liver transplantation; TACE, transarterial chemoembolization; TIPS, transjugular intrahepatic portosystemic shunt.

[a]*Ascites was defined regardless of its volume, according to intraoperative finding at laparotomy.*

[b]*Yerdel classification (30).*

() indicates range for continuous variables and % for categorical variables.

TABLE 3 | Oberved probabilities for difficult LT (DS ≥ 7) according to the number of risk factors (Annular segment 1, ReLT after 30 days, Ascites, Portal vein thrombosis, TIPS).

Observed probability of DS ≥ 7	
No. Factor	**No. DS ≥ 7/overall number**
0	46/177 (26%)
1	59/169 (35%)
2	45/65 (69%)
3+	18/21 (86%)

TABLE 4 | Complications according to DS.

Type of complications	DS < 7	DS ≥ 7	*p*
	N = 270	*N* = 171	
Early allograft dysfunction[a]	57 (21.1%)	49 (28.7%)	.091
Vascular complications[b]	16 (5.9%)	35 (20.5%)	<.001
Biliary complications[c]	9 (3.3%)	5 (2.9%)	>.99
Hemorrhage[d]	31 (11.5%)	24 (14.0%)	.520
Infection	71 (26.3%)	56 (32.7%)	.177
Renal failure[e]	18 (6.7%)	21 (12.3%)	.064

[a]*According to Olthoff et al.*
[b]*Thrombosis or stenosis of the hepatic artery, the portal vein or caval anastomosis diagnosed on imaging regardless of the management.*
[c]*Stenosis or biliary fistula.*
[d]*Hemorrhage requiring laparotomy or hematoma on imaging requiring transfusion.*
[e]*Stage III acute kidney injury (KDIGO Classification).*

Association Between Difficulty Scale Value and Severe Morbidity and One-Year Survival

As shown in **Figure 2**, a continuous increase in the risk of severe morbidity as the DS increases was observed. In contrast, the hazard risk of death within the first year remained stable from 0 to 5 and started to increase from 6 to beyond.

Additional Predictive Value of Difficulty Scale

The predictive value of BAR and SOFT models are given in **Table 1**. An increase of AUC and a decrease of AIC for all models were observed when adding the DS. The AUC of the models (with and without DS) were compared, and tests were significant for each model, indicating that DS improves the predictive value of each model for severe morbidity, 3-month graft survival, and 1-year graft survival.

Survival According to DS 0–3 vs. 4–6 vs. 7–10

Graft survival and patient survival are reported in **Figure 3**. Graft survival of the group with DS ≥ 7 was significantly lower than graft survival with DS: 4-6 or DS: 0–3. Graft survival rates were 79% (95% CI: 73–85%), 91% (95% CI: 87%–95%), and 96% (95% CI: 93%–100%) at 1 year for the group DS: 7–10, DS: 4–6, and DS: 0–3, respectively. There was no difference between the two other groups DS 0–3 and DS: 4–6. Similar findings were observed for patient survival. One-year patient survival rates were 85% (95% CI: 82%–92%) in group DS 7–10 vs. 95% (95% CI: 92%–98%) and 97% (95% CI: 92%–100%) in the group with DS: 4–6 and DS: 0–3, respectively.

Predictive Factors of Difficult LT (DS ≥ 7)

Univariate analysis is shown in **Table 2**. Transplant recipients with DS ≥ 7 had ascites, annular segment 1, PV thrombosis, or portal cavernoma more often. A previous transjugular intrahepatic portosystemic shunt (TIPS) was more present in this group. This group was also more likely to include ReLT > 30 days. The final multivariate model included five independent predictors of transplant with DS ≥ 7: previous TIPS (OR: 2.67 [1.06–7.11]), ascites (OR1.64 [1.07–2.51]), Portal Vein thrombosis (OR 2.17 [1.20–3.95]), annular segment 1 (OR 6.57 [2.71–18.48]), ReLT > 30 days (OR 4.11 [2.18–7.98] **Table 3**). Of note, ascites was a borderline non-significant variable in this multivariable model.

Observed proportions of difficult transplant according to the number of factors are given in **Table 3**. It ranges from 26% to 86% in transplant without risk factors and at least three risk factors.

Complications Associated With Difficult Liver Transplantation

The type of surgical complications, according to LT difficulty DS < 7 vs. DS ≥ 7, is shown in **Table 4**. A higher proportion of vascular complications was observed after difficult LT (20.5% vs. 5.9%; $p < .001$). In contrast, there was no difference in the other types of surgical complications between the two groups. However, the proportion of renal failure tends to be higher in the difficult LT group (borderlin significance).

Agreement Between Junior and Senior Surgeons

The DS values given by the junior and senior are given in **Figure 4**. Diameters of points vary according to the number of evaluations. Points distributed on the diagonal line corresponds to perfect agreement. Points above the diagonal lines indicate that LT was considered more difficult by the senior surgeon, whereas points below refer to harder transplant from the junior point of view. Overall, the agreement was satisfactory. The concordance coefficient correlations (95% CI) were .65 (.51–.76) and .78 (.69–.86) for hepatectomy and implantation, respectively.

DISCUSSION

The technical difficulty is inherently subjective. In previous studies, the technical difficulty in surgery was assessed by using various surrogates. The originality of our study was to prospectively evaluate the difficulty according to the surgeon's subjective feeling at the end of the transplantation.

We observed that DS correlates with morbidity and even 1-year survival. The importance of intraoperative factors to

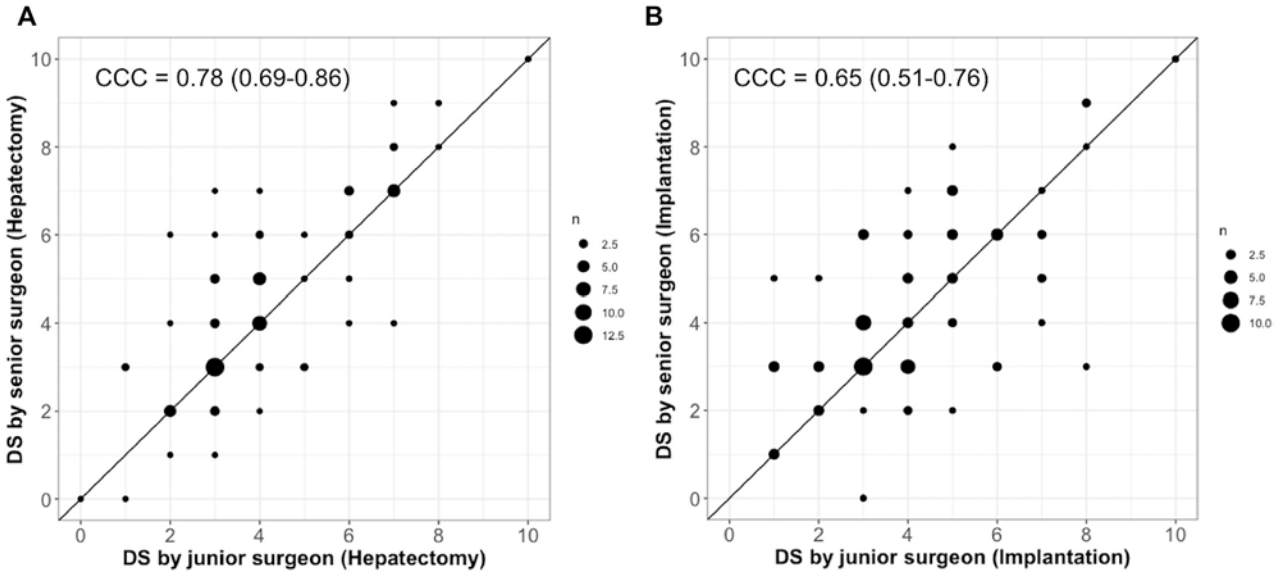


FIGURE 4 | Agreement of DS between junior and senior surgeons for hepatectomy **(A)** and implantation **(B)**.

improve the predictive ability of pretransplant models has been recognized (19, 20). Adding the sole DS was sufficient to improve two validated pretransplant models, namely the BAR and SOFT scores, which means that DS should be not be used in lieu of these models but in conjunction.

As expected, the DS was associated with some objective variables like duration of surgery or transfusion volume, already known to impact outcomes (21, 22). The main strength of the DS is to reflect some subjective predictors of outcomes such as the surgical field exposure, the quality of tissues, and the easiness to achieve vascular or biliary anastomosis, which cannot be captured by usual metric tools. The DS can be seen as a summary of the numerous factors of difficulty, all contributing directly or indirectly to outcomes. This latter point may explain the predictive value of the DS.

The risk of death within the first year started to sharply increase beyond 6, suggesting that this cutoff value of seven carries a relevant clinical meaning. Five independent factors of "difficult" transplant were identified. Of them, late retransplantation is not a surprising finding. Adhesions, sometimes filled by portal hypertension, and modified anatomical landmark makes ReLT more challenging than primary transplantation (23, 24). A complete encirclement of the retrohepatic inferior vena cava is known to increase the difficulty and the risk of total hepatectomy with caval preservation (25). Preexisting TIPS is also associated with an increased risk of bleeding during total hepatectomy, especially in cases of misplacement (26). PV thrombosis may compromise the portal inflow, essential for graft function recovery. In most cases, eversion thrombectomy is sufficient to restore a sufficient portal flow. In the presence of a complete thrombosis of the PV and superior mesenteric vein, other more technically demanding strategies are needed to obtain adequate portal perfusion. The impossibility of restoring sufficient portal flow may force to consider technically demanding strategies, which consist of anastomosing the graft PV to the recipient superior mesenteric vein, gastric, choledochal varices, or left renal vein (27, 28).

Identifying "difficult" transplants with pretransplant variables yields some logistics advantages. Recipient laparotomy should begin as early as possible to limit cold ischemia time. DS highlights some technical difficulties such as annular segment 1 or portal vein thrombosis and may serve to better define the surgical strategy before LT. Complex transplantation may also require a team of two experienced surgeons. It may also guide the graft choice and avoid the combination of a marginal graft and complex transplantation associated with poor results (29).

The DS may also be of interest in the early post-transplant period. Some patients after "technically easy" LT are likely good candidates for enhanced recovery protocol, whereas recipients with high DS may potentially benefit from tailored monitoring, including daily Doppler and systematic CT scan. However, the possibility to tailor monitoring according to DS remains a hypothesis, and a more refined difficulty scale (evaluating each step, for example) might be a more efficient approach to anticipate outcomes.

The DS proposed here is prone to biases. An important variation in the evaluation according to experience, surgical skills, and timing of surgery could be expected. A surgeon's "feeling at the end of LT" can be affected by innumerable variables, including the type of procedure, time of day, surgeon or assistant exhaustion or mood, issues with anesthesia, instruments, staff personnel, and many other factors, some even unrelated to surgical or medical aspects. As a result, the same case, potentially with the same outcome, could be

subjectively evaluated by the surgeon differently in contrasting circumstances. In addition, the agreement across centers may not be warranted, depending on recruitment, number of cases, and type of disease treated. We also observed acceptable agreement between the senior and junior surgeon evaluations, suggesting that DS keeps a reasonable degree of reproducibility, despite its subjectivity. Discordant values in the DS were mainly observed in the intermediate range of difficulty, whereas "difficult" and "easy" were less subject to disagreement. The present study carries some limitations, in addition to its monocentric nature. The DS has not been evaluated in 16% of LT. We decided not to use multiple imputations because DS is the primary variable of interest. The comparisons of the study population with the group of LTs without DS showed significant differences for junior surgeons but neither for recipient characteristics nor intraoperative data.

The DS did not evaluate specifically for total hepatectomy and graft implantation in the whole cohort. A pretransplant DS would also have been helpful to test predictive variables and study the discrepancy between pre- and post-transplant DS. Validation of the DS prognostic value and the risk factors for complex transplant on an independent cohort is necessary to test the reproducibility and the relevancy of the DS in routine.

In conclusion, end-transplant DS predicts morbidity and 1-year survival after liver transplantation. Its value may be helpful to adapt monitoring and facilitate the early diagnosis of complications.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The design of this study was discussed and approved at our weekly institutional research meeting. This study was achieved in accordance with French legal requirements and the Declaration of Helsinki. Before surgery, patients have given a written consent according to which they permit that data obtained during standard health care can be used for scientific purposes.

AUTHOR CONTRIBUTIONS

YK and M-AA designed the study. YK, DP, EF-S, and M-AA performed the research. YK and M-AA wrote the manuscript. DC, DA, EV, RA, AC, HB, and NG contributed important reagents.

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
published: 22 March 2022
doi: 10.3389/ti.2022.10437

Living Donor Liver Transplantation vs. Split Liver Transplantation Using Left Lateral Segment Grafts in Pediatric Recipients: An Analysis of the UNOS Database

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Split and LDLT in pediatric patients have the potential to decrease wait times and waitlist mortality. Using UNOS-STAR data, we compared outcomes of pediatric patients undergoing LDLT and SLT using LLS grafts. The baseline characteristics and post-operative outcomes were compared between groups. Actuarial graft and patient survival were analyzed with Kaplan-Meier curves. Between 2010 and 2019, 911 pediatric LT were included in the analysis (LD graft group, n = 508, split graft group, n = 403). LD graft recipients spent more time on the waitlist vs. the split graft group (60 (22–138) days vs. 46 (16–108) days; p = 0.007). LD recipients had a lower rate of graft failure, found in 9.8% of patients compared with 14.6% in the split graft group (p = 0.02). HAT was the most common graft failure cause, with similar rates. Graft and patient survival at 1-, 3-, and 5-years was comparable between LDLT and SLT. In subgroup analyses, patients with biliary atresia, those ≤10 kg or ≤10 years old receiving an LD graft showed improved graft survival. In conclusion, LDLT is associated with a lower rate of graft failure in pediatric patients. The use of LLS regardless of the type of donor is a safe way to facilitate access to transplantation to pediatric patients with acceptable short and long-term outcomes.

Keywords: pediatric liver transplantation, outcomes, graft, waitlist, survival

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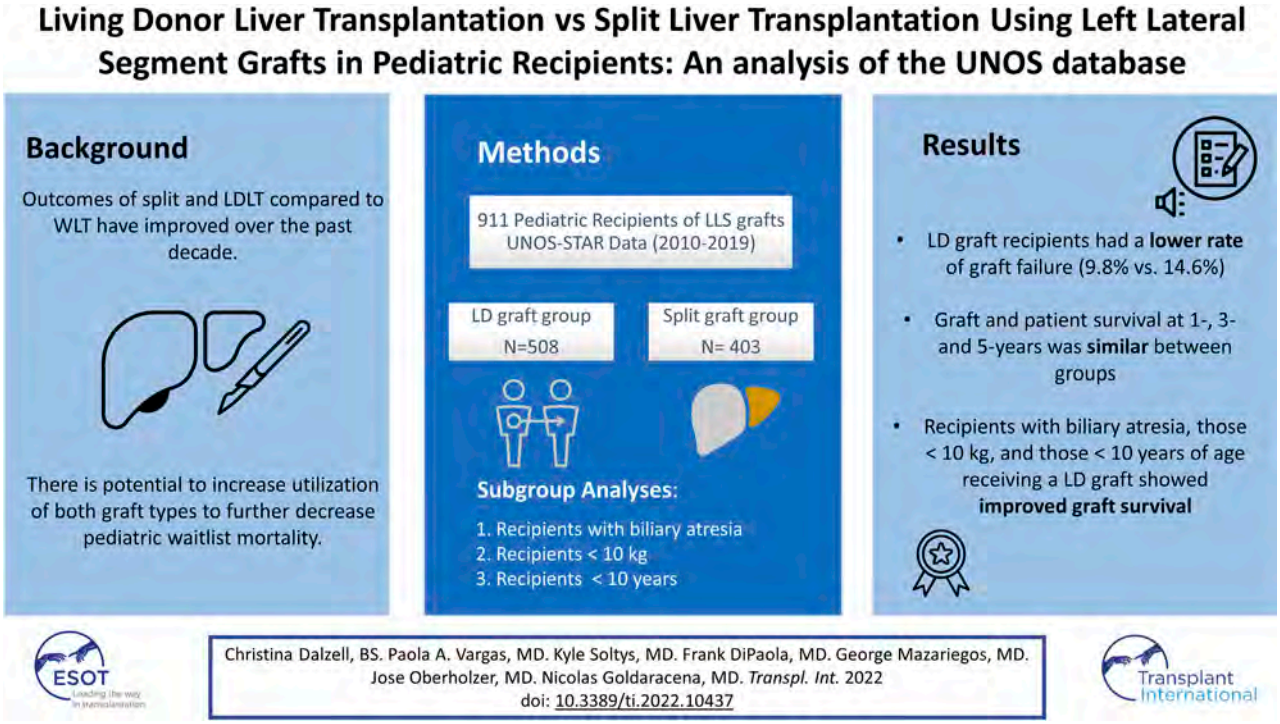
Received: *15 February 2022*
Accepted: *03 March 2022*
Published: *22 March 2022*

Citation:
Dalzell C, Vargas PA, Soltys K, Dipaola F, Mazariegos G, Oberholzer J and Goldaracena N (2022) Living Donor Liver Transplantation vs. Split Liver Transplantation Using Left Lateral Segment Grafts in Pediatric Recipients: An Analysis of the UNOS Database. Transpl Int 36:10437. doi: 10.3389/ti.2022.10437

Abbreviations: DDLT, deceased donor liver transplantation; HAT, hepatic artery thrombosis; LDLT, living donor liver transplantation; LLS, left lateral segment; PVT, portal vein thrombosis; SLT, split liver transplantation; WLT, whole liver transplantation.



GRAPHICAL ABSTRACT |

INTRODUCTION

The use of split liver transplantation (SLT) and living donor liver transplantation (LDLT) have revolutionized the field of pediatric liver transplantation (LT)—with the potential to increase the availability of organs and decrease waitlist mortality (1). Pediatric LT creates a unique need for alternative graft types due to limited access to whole organs from pediatric deceased donors. Both techniques have created interest in better understanding the complications that arise and the factors that contribute to acceptable outcomes.

The number of pediatric LT performed in the US has remained stable over the past decade (2). Out of the 551 pediatric LT performed in 2019, LDLT and SLT accounted for 14.3% and 20.3% respectively, both increased from a decade prior (2). In 2019, 55% of pediatric candidates waited less than 1 year compared to 45.1% a decade prior (2). In this regard, over 90% of pediatric patients on the waitlist undergo LT, with a 5-years patient survival rate ranging from 81 to 93%, depending on the primary diagnosis (2, 3).

Deceased donor splitting into an LLS and an extended right graft, not only provides access to LT for pediatric recipients but also the possibility to utilize the remnant liver graft in a larger size recipient (pediatric or adult) (3, 4). The transplant community has recently supported the evaluation of a regional variance to support split liver transplantation within affiliated centers (5). The potential of a single organ to benefit two patients in need is countered by logistical challenges—including increased cold ischemia time, geographic barriers, surgical complexity, and manpower logistics (1). On the other hand, for LDLT, LLS is removed from a healthy donor (3, 4). Theoretical benefits of LDLT include faster access to transplantation, earlier timing of surgery before clinical decompensation, less cold ischemia time, expansion to patients who would otherwise not qualify for a deceased donor liver, and potential immunologic advantages in related donors (6–8).

Over the past decade, the outcomes of split and living donor vs. the whole LT in the pediatric population have improved (9). Some studies have shown a potential increase in graft failure and complications in SLT when compared to whole LT or LDLT, whereas other studies have shown no impact of graft type on graft or patient outcome (10, 11). Few studies have directly compared these techniques in a pediatric population, and studies comparing outcomes based on the type of grafts among these patients are even more scarce. This study aims to compare patient and graft outcomes in the pediatric population following LDLT and SLT using LLS grafts.

MATERIALS AND METHODS

Study Design

Data for this retrospective study were obtained from the United Network for Organ Sharing (UNOS) Standard Transplant Analysis and Research file (STAR). The UNOS-STAR database does not include any patient or transplant center identifiers. The study

TABLE 1 | Donor characteristics according to donor type.

Donor variables	LD graft group (*n* = 508)	Split graft group (*n* = 403)	*p*-value
Donor age (years)	32 (26–37)	12 (7–17)	<0.001
Donor Male Gender (%)	213 (41.9)	263 (65.3)	<0.001
Donor weight (kg)	70.6 (61.3–83.0)	48.8 (25–64)	<0.001
Donor height (cm)	167.6 (162.5–175.2)	154.9 (124–170)	<0.001
Donor BMI	24.9 (22.5–28.0)	20 (16.8–22.6)	<0.001
Cold Ischemia Time (hours)	1.51 (1.0–2.33)	7.5 (6.2–9.0)	<0.001

Median (IQR). Abbreviations: BMI, body mass index; LD, living donor.

population included all pediatric recipients (<18 years of age) who received a LLS graft from a deceased donor (SLT) or a living donor (LD) in the United States from January 1, 2010, to March 2019. Recipients ≥18 years, patients receiving a right lobe, right trisegment graft, full left lobes, whole liver grafts, donation after circulatory death, multi-organ transplants, re-transplantations, and those transplanted prior to 2010 were excluded from the analysis. The records of the remaining pediatric liver transplant recipients were then analyzed.

Three additional subgroup analyses were performed to assess outcomes with the two techniques in challenging pediatric populations. The first one compared the outcome of both techniques in pediatric patients with biliary atresia. While a second analysis evaluated recipients ≤10 kg, the third analysis assessed both techniques in those patients who were ≤10 years of age at the time of transplant. Demographic variables for donors, recipients, and postoperative outcomes were compared in each set of patients.

Donor and Graft Data

The following donor characteristics were analyzed and compared between groups: age, gender, weight (kg), height (cm), body mass index (BMI), and cold ischemia time (CIT) (hours).

Since November 2007, the OPTN has identified organs with the potential to be split as those that met the following criteria: donor less than 40 years old on less than 1 vasopressor, transaminases no greater than 3 times the upper limit of normal, and BMI of 28 or less. However, although these are meant as guidelines, the final decision to split or not an organ is based on each transplant center's criteria and expertise.

In the deceased donor group, some of the left lateral segment grafts resulted from a reduction of a full graft rather than a true split into two grafts. For descriptive purposes, we described these grafts also as splits although the extended right graft was not used for another recipient.

Recipient Data

The following recipient data were analyzed and compared between groups: age, gender, weight (kg), height (cm), BMI, laboratory values at transplant such as international normalized ratio (INR), albumin level, serum creatinine, and total bilirubin level. Preoperative data such as ascites grade, history of portal vein thrombosis (PVT), previous upper abdominal surgery, indication for transplant, Pediatric End-Stage Liver Disease (PELD) score at transplant, patients transplanted under status 1, and the total number of days on the waitlist were also analyzed.

Status 1 variable was comprised of patients with Status 1A and 1B. Status 1A and 1B are the only medical priority exceptions to PELD scores in pediatric patients and account for less than 1% of liver transplant candidates at any given time. Status 1A patients are those with a diagnosis of acute liver failure with a life expectancy of less than 7-days. Status 1B includes patients with hepatoblastoma, certain metabolic disorders, and chronic liver disease with a MELD or PELD greater than 25.

Postoperative Outcomes

Recipient surgical outcomes were analyzed by assessing the length of hospital stay (LOS), and the reported incidence of graft failure causes such as hepatic artery thrombosis (HAT), other vascular thrombosis, primary non-function (PNF), infection, PVT, biliary related graft failure, diffuse cholangiopathy, hepatitis *de novo*, recurrent disease and hepatic outflow obstruction. Re-transplantation rates and mortality were also analyzed by actuarial graft and patient survival.

Statistical Analysis

Baseline donor and recipient demographics, as well as clinical characteristics, were presented as median (interquartile range) for continuous variables, and counts (percent) for categorical variables, unless stated otherwise according to the distribution of the data. For categorical variables, the *Chi-square* test and *Fisher's Exact Test* were used for comparison between groups accordingly. *Independent t-test* and *Mann-Whitney U test* were used for comparisons of continuous variables as appropriate. Kaplan-Meier method was used to analyze survival between study groups and were compared using the log-rank test. The outcome for graft and patient survival were calculated by using the variables "pstatus" and "gstatus"-Boolean most recent patient status (based on composite death date)- respectively. These variables reflect the death date reported for the patient as deceased, as verified by external sources. For all analyses, two-tailed *p*-values ≤0.05 were considered statistically significant. All analyses were performed using IBM SPSS Statistics Version 26.

RESULTS

During the study period, a total of 911 pediatric patients who received an LT with an LLS graft were identified in the UNOS-

TABLE 2 | Recipient characteristics according to donor type.

Recipient variables	LD graft group (*n* = 508)	Split graft group (*n* = 403)	*p*-value
Age at transplant (years)	1 (0–3.7)	1 (0–3)	0.44
Male Gender (%)	246 (48.4)	205 (50.9)	0.46
Anthropometrics at transplant			
Weight (kg)	9.1 (7.0–17.17)	9.6 (7.0–15.7)	0.08
Height (cm)	73 (65.0–98.8)	74 (64–97)	0.35
BMI at transplant	16.7 (15.5–18.2)	16.8 (15.4–18.3)	0.59
Lab values at transplant			
INR	1.4 (1.1–2.0)	1.4 (1.1–2.2)	0.14
Albumin	3.1 (2.6–3.7)	3.1 (2.6–3.7)	0.79
Creatinine	0.20 (0.20–0.31)	0.27 (0.20–0.38)	0.002
Total bilirubin	9.3 (2.1–17.9)	6.6 (1–16.8)	0.005
Ascites			
Absent (%)	143 (28.1)	120 (29.8)	0.14
Slight (%)	99 (19.5)	59 (14.6)	
Moderate (%)	52 (10.2)	55 (13.6)	
N/A (%)	213 (41.9)	169 (41.9)	
PVT history (%)	15 (3.0)	15 (3.7)	0.33
Previous upper abdominal surgery (%)	306 (60.2)	193 (47.9)	0.001
Diagnosis			
Biliary atresia (%)	291 (57.2)	157 (38.9)	<0.001
Metabolic diseases (%)	44 (8.6)	76 (18.8)	
Tumor related (%)	24 (4.7)	41 (10.1)	
Acute liver failure (%)	38 (7.4)	39 (9.6)	
Cholestatic disorders (%)	19 (3.7)	25 (6.2)	
Other cirrhotic (%)	78 (15.3)	62 (15.3)	
PSC (%)	14 (2.7)	3 (0.7)	
PELD at listing	11 (2–20)	12 (0–21)	0.77
Calculated PELD at transplant	16 (5–25)	16 (4–24)	0.28
Status 1 (%)	66 (13)	96 (23.8)	<0.001
Time on wait list (days)	60 (22–138)	46 (16–108)	0.007

Median (IQR). Abbreviations: BMI, body mass index; INR, international normalized ratio; LD, living donor; N/A, not applicable; PELD, pediatric end-stage liver disease score; PVT, portal vein thrombosis; PSC, primary sclerosing cholangitis.

STAR database. Of those, 508 (55.8%) underwent LT with a graft from a living donor (LD graft group) and 403 (44.2%) received a split liver graft from a deceased donor (Split graft group).

Donor and Graft Data

Donors in the LD graft group were older when compared with donors from the split graft group (32 (IQR: 26–37) years vs. 12 (IQR: 7–17) years, respectively; $p < 0.001$). However, it is important to notice that the maximum donor age in the LD graft group was 59 years, while the older donor in the split graft group was 52 years. There was a significantly lower percentage of male donors in the LD graft group when compared to the split graft group (41.9% vs. 65.3%; $p < 0.001$). Anthropometric measurements, including weight (kg), height (cm), and BMI were significantly higher in the LD group ($p < 0.001$ for all). In congruence with the nature of the groups, CIT was significantly shorter in the LD graft group when compared with the split graft group (1.5 (1.0–2.3) hours vs. 7.5 (6.2–9.0) hours; $p < 0.001$) (**Table 1**).

Recipient Preoperative Data

Age at transplant and anthropometric measurements were similar between groups. Baseline characteristics such as the presence of ascites, and history of PVT were also similar. More patients in the LD group had a history of previous abdominal surgery (306 (60.2%)) vs. the split graft group (193 (47.9%)) ($p = 0.001$). PELD scores at the time of listing and LT were similar between groups. However, fewer recipients were listed under status 1 in the LD graft group compared with the split graft group (66 (13%) vs. 96 (23.8%); $p < 0.001$). The most common indication for pediatric LT was biliary atresia, occurring in 57.2% of recipients in the LD group, and in 38.9% of recipients in the split graft group. The LD group had significantly longer time spent on the waitlist (LD group = 60 (22–138) days vs. split graft group = 46 (16–108) days) (**Table 2**). The longer waitlist time was still observed after excluding recipients with status 1 (LD group $n = 442$, 69 (30–154) days, vs. Split graft group $n = 307$, 56 (21–132) days, $p = 0.027$).

Postoperative Outcomes

Length of hospital stay was significantly shorter in the LD graft group (16 (11–26) days vs. 20 (13–33) days, respectively; $p < 0.001$). The overall graft failure rate was significantly lower in the LD graft group (LD group = 9.8% vs. Split graft group = 14.6%; $p = 0.027$). The most common cause of graft failure in both groups was HAT, which occurred at similar rates between groups (**Table 3**). Other vascular thrombosis as the cause of graft failure were more common in the LD group (LD group = 13 (2.6%) vs. Split graft group = 7 (1.7%);

TABLE 3 | Post-operative outcomes according to donor type.

Postoperative outcomes	LD graft group (*n* = 508)	Split graft group (*n* = 403)	*p*-value
LOS post LT (days)	16 (11–25)	20 (13–33)	<0.001
Graft failure (%)	50 (9.8)	59 (14.6)	0.027
<30-days	23 (46)	26 (44.1)	0.49
>30–90 days	3 (6)	4 (6.8)	0.59
>90-days	24 (48)	29 (49.2)	0.52
Graft failure causes			
Hepatic artery thrombosis (%)	13 (2.6)	11 (2.7)	0.19
<30-days	8 (61.5)	7 (63.6)	0.62
>30–90 days	0	0	
>90-days	5 (38.5)	4 (36.4)	0.62
Other vascular thrombosis (%)	13 (2.6)	7 (1.7)	0.046
<30-days	11 (84.6)	7 (100)	0.52
>30–90 days	0	0	
>90-days	2 (15.4)	0	0.41
Portal vein thrombosis (%)	6 (1.2)	5 (1.2)	0.74
<30-days	4 (66.7)	4 (80)	0.57
>30–90 days	0	0	
>90-days	2 (33.3)	1 (20)	0.62
Infection (%)	3 (0.6)	5 (1.2)	0.39
<30-days	1 (33.3)	1 (20)	0.64
>30–90 days	0	0	
>90-days	2 (66.7)	4 (80.0)	0.67
Biliary related (%)	1 (0.2)	2 (0.5)	0.54
<30-days	0	0	
>30–90 days	0	0	
>90-days	1	2	0.54
Diffuse cholangiopathy (%)	0	1 (0.2)	0.036
Hepatitis *de novo* (%)	0	1 (0.2)	0.31
Recurrent disease (%)	2 (0.4)	2 (0.5)	0.44
Hepatic outflow obstruction (%)	1 (0.2)	0	0.63
Primary Non-Function (%)	4 (0.8)	6 (1.5)	0.017
Re-transplant (%)	28 (5.5)	31 (7.7)	0.18
Mortality (%)	21 (4.1)	27 (6.7)	0.08
1-/3-/5-y graft survival (%)	93/89/85	90/86/79	0.058
1-/3-/5-y patient survival (%)	97/95/95	95/93/91	0.11

Median (IQR). Abbreviations: LT, liver transplant; LD, living donor; LOS, length of hospital stay.

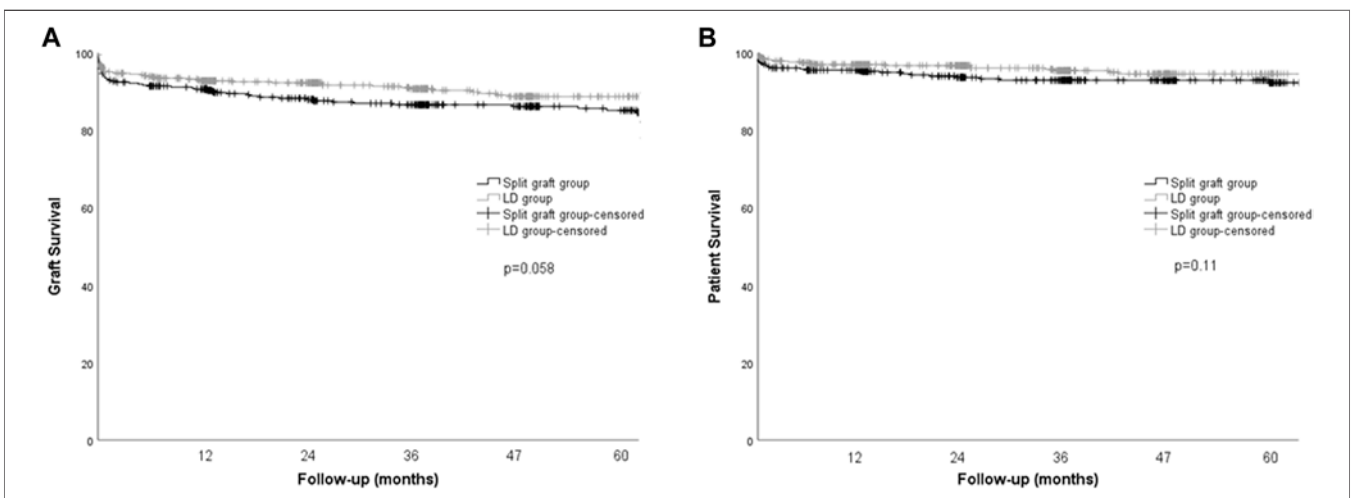


FIGURE 1 | Kaplan-Meier curves for graft and patient survival in pediatric recipients according to donor type. **(A)** Graft survival, $p = 0.058$, log rank (Mantel-Cox). **(B)** Patient survival, $p = 0.11$, log rank (Mantel-Cox).

TABLE 4 | Subgroup analysis on pediatric recipients with biliary atresia diagnosis according to donor type.

	LD group (*n* = 291)	Split graft group (*n* = 157)	*p*-value
Donor variables			
Donor age	32 (26–37)	11 (6.5–15)	<0.001
Donor male gender (%)	115 (39.5)	109 (69.4)	<0.001
Donor weight (kg)	69.8 (61.2–83)	39.9 (23.5–60)	<0.001
Donor height (cm)	167.6 (162.5–175.2)	149 (122.5–165)	<0.001
Donor BMI	25 (22.6–27.8)	19 (15.9–21.2)	<0.001
Cold ischemia time (hours)	1.5 (1–2.4)	7.5 (6.2–9.3)	<0.001
Recipient variables			
Age at transplant	0 (0–1)	0 (0–1)	0.12
Male gender (%)	127 (43.6)	69 (43.9)	0.95
Anthropometrics at transplant			
Weight (kg)	7.7 (6.4–10.4)	7.7 (6.3–9.9)	0.68
Height (cm)	67.4 (63.4–78.5)	67.5 (62.7–75)	0.38
BMI at transplant	16.6 (15.4–18.2)	16.8 (15.4–18.2)	0.55
Lab values at transplant			
INR	1.4 (1.1–1.9)	1.4 (1.2–1.9)	0.19
Albumin	3 (2.5–3.3)	2.8 (2.4–3.3)	0.35
Creatinine	0.2 (0.1–0.3)	0.2 (0.1–0.3)	0.32
Total bilirubin	11.2 (5–18.3)	11.9 (4–18)	0.88
Ascites			0.14
Absent	57 (19.6)	31 (19.7)	
Slight	65 (22.3)	30 (19.1)	
Moderate	33 (11.3)	30 (19.1)	
N/A	136 (46.7)	66 (42.0)	
PVT history	8 (2.7)	5 (3.2)	0.75
Previous upper abdominal surgery	249 (85.6)	134 (85.4)	0.9
PELD at listing	12.5 (5.7–19)	14 (9–19)	0.62
Calculated PELD at transplant	17 (9–25)	18 (10–23)	
Status 1	8 (2.7)	9 (5.7)	0.11
Days on waitlist	69 (34–152)	67 (30–124)	0.34
Postoperative outcomes			
LOS (post-tx)	16 (11–24)	19 (14–33)	<0.001
Hepatic artery thrombosis	3 (1)	5 (3.2)	0.02
Other vascular thrombosis	3 (1.9)	4 (1.4)	0.03
Primary non-function (%)	1 (0.3)	1 (0.6)	0.004
Re-transplant	6 (2.1)	10 (6.4)	0.01
Mortality	7 (2.4)	10 (6.4)	0.03
1-/3-/5-y graft survival (%)	96/94/94	90/89/81	0.004
1-/3-/5-y patient survival (%)	98/97/97	94/93/92	0.055

Median (IQR). Abbreviations: LT, liver transplant; LD, living donor; LOS, length of hospital stay.

TABLE 5 | Subgroup analyses on pediatric recipients according to donor type for smaller recipients.

Recipient variables	Recipients ≤10 kg			Recipients ≤10 years		
	LD graft group (*n* = 275)	Split graft group (*n* = 212)	*p*-value	LD graft group (*n* = 451)	Split graft group (*n* = 375)	*p*-value
Wait list time (days)	52 (23–109)	43 (14–94.7)	0.02	57 (22–131)	47 (16–107)	0.03
1-/3-/5-y graft survival (%)	94/90/88	89/85/76	0.011	94/90/88	91/86/78	0.015
1-/3-/5-y patient survival (%)	97/95/95	94/91/89	0.06	97/95/95	96/93/91	0.16

Median (IQR). Abbreviations. LD, living donor.

p = 0.046). PNF rate was significantly lower in the LD group (LD group = 4 (0.8%) patients vs. Split graft group = 6 (1.5%) patients; p = 0.01). Diffuse cholangiopathy was the cause of graft failure in 1 patient who received a split graft and did not occur in any LD recipients (p = 0.036). Other reported causes of graft failure can be found in **Table 3**. Analysis of graft failure and important causes within 30-days, 90-days and over, yielded no significant difference in rates among the LD donor group vs. the split graft group (**Table 3**).

Re-transplantation rates and mortality were not significantly different between groups. Graft and patient survival at 1-, 3-, and 5-years were also similar between the LD and split graft groups (**Figure 1**).

Subgroup Analysis: Recipients With Biliary Atresia

To identify significant factors influencing outcomes amongst groups, using a more homogeneous sample we performed a subgroup analysis of recipients with biliary atresia receiving a graft from a LD ($n = 291$) vs. those receiving a split liver graft ($n = 157$). Baseline recipient characteristics were similar between groups. Importantly, in this subanalysis time spent on the waitlist was similar in both groups (69 (34–152) days in the LD group vs. 67 (31–125) days in the split graft group; $p = 0.34$). The proportion of male donors was lower in the LD group vs. the split graft group (40% vs. 69%, respectively; $p = <0.001$). Additional donor characteristics appear in **Table 4**. As seen in the main analysis, LOS was significantly shorter in the LD group (LD group = 16 (11–24) days vs. split graft group = 20 (13–33) days, $p = <0.001$). Although improved 1-, 3- and 5-years graft survival was found in the LD group (96/94/94%, vs. 90/89/81; $p = 0.004$), patient survival at 1-, 3-, and 5-years remained comparable between groups (98/97/97% vs. 94/93/92%; $p = 0.055$).

Subgroup Analysis: Recipients ≤10 kg

To assess the outcomes following LT in small pediatric recipients, a subgroup analysis including patients with a body weight ≤10 kg was performed. During the study period, a total of 487 LT were performed in pediatric patients with a body weight ≤10 kg. From this, while 275 (56.5%) were performed with an LD graft, 212 (43.5%) were performed using a split LLS graft from a deceased donor. Patients in the LD group spent more time on the waitlist (LD group = 52 (23–109) days vs. Split graft group = 43 (14–95) days; $p = 0.02$). As in the main analysis, fewer patients in the LD graft group were listed as status 1 when compared to recipients in the split graft group (24 (8.7%) patients vs. 43 (20.3%) patients, respectively; $p = <0.001$).

Graft survival at 1-, 3-, and 5-years was significantly higher in the LD group (94%, 90%, 88%) vs. the split graft group (89%, 85%, 76%; $p = 0.011$). Patient survival at 1-, 3-, and 5-years was similar between groups (**Table 5**).

Subgroup Analysis: Recipients ≤10 Years Old

Recipients ≤10 years old (LD group = 451 vs. Split graft group = 375) were also analyzed. Again, the LD graft group had significantly longer wait times (LD group = 57 (22–131) days vs. Split graft group = 47 (16–107) days; $p = 0.03$). Fewer patients in the LD graft group were status 1 at the time of transplant when compared with patients in the split graft group (59 (13.1%) vs. 86 (22.9%), respectively; $p < 0.001$). Graft survival at 1-, 3-, and 5-years was significantly higher in the LD group (94%, 90%, 88%) than the split graft group (91%, 86%, 78%) ($p = 0.015$). Patient survival at 1-, 3-, and 5-years was similar between groups (**Table 5**).

DISCUSSION

This study compares UNOS data on LDLT and SLT using the left lateral segment in the pediatric population within the last decade. Our analysis revealed improved post-operative outcomes including shorter hospital stays and lower rates of graft failure in living donor recipients. Our study also revealed that patients with a diagnosis of biliary atresia, those who weighed <10 kg or were <10 years old at the time of transplant showed an improved graft survival at 1-, 3-, and 5-years when they received an LLS graft from a living donor.

Previous studies including both graft types have shown acceptable graft and patient outcomes with living donor and split grafts compared to whole liver grafts (6,9, 12-14). A study by Mogul et al. comparing 15-years trends in pediatric LT using the SRTR database showed improvement in LDLT and SLT in both, graft and patient survivals from 2002–2009 to 2010–2015. Unfortunately, SLT and LDLT outcomes were not directly compared. In the later study period of 2010–2015, 1-year survival after LDLT was higher than of whole liver transplantation (WLT), and there was no difference between SLT and WLT (9). This improvement in graft and patient survivals using these techniques over the past decade is consistent with other studies (15). In a study by Kehar et al. from the largest pediatric LT program in Canada, 1-, 5-, and 10-years graft and patient survival rates after LDLT were significantly higher than after deceased donor LT (DDLT), with no difference in surgical or medical complications (6). The graft failure rate was also higher in DDLT recipients, in accordance with our study (6). As expected, CIT in our study was significantly longer in the split graft group. This has been shown to be a predictor of prolonged stay and is associated with reduced graft function and survival (16, 17). Analysing the impact that the splitting technique used (*in situ* vs. *ex vivo*) has on CIT and therefore on graft injury and postoperative outcomes would have been interesting. However, due to the variability of the data set, this was not able to be evaluated in our study and requires further assessment in future studies. Importantly, CIT is a variable that can be modified with improved logistics between centers performing split liver transplantation and calls for a system that would allow for protection of these otherwise ideal allografts by minimizing the obstacles that extend cold ischemic times by a seven-fold difference. Indeed, the deceased donors in this study were from younger donors with significantly lower BMIs leaving extended CIT as the primary difference to explain increased graft loss, ischemic cholangiopathy, and primary non-function rates.

The appropriate lower limit of donor age for SLT has not been defined in the literature. There have been a few studies focused on evaluating the use of split livers from pediatric donors. In a study by Cescon et al., 43 livers were split from pediatric donors less than age 15. Forty left lateral segment grafts were transplanted into pediatric recipients while 39 extended right grafts were transplanted into 11 children and 28 adults (18). Two-year

patient and graft-survival were similar in recipients of grafts from donors <40 kg or >40 kg, between pediatric and adult recipients, and between recipients of ERG or LLS (18). Complications rates were also similar in recipients of donors <40 kg or >40 kg (18). In a more recent study by Gao et al., the outcomes of 16 pediatric recipients of pediatric split liver grafts were analyzed. The split liver grafts came from 8 pediatric donors less than 7 years of age (19). At a 3-months follow-up, both graft and patient survival were 100%. The only surgical complication was portal vein stenosis, reported in 1 patient (19). This study also defined criteria for optimal split liver grafts in pediatric donors which includes a graft to recipient weight ratio of 2–4% (19). Thus, although pediatric split liver donors are not commonly performed, there are institutions with experience in this technique and the selection of appropriately sized recipients for the LLS and extended right grafts. Hence, despite the low average age of split liver donors in the present study, this has not been found to be associated with higher rates of complications in the literature (18, 19).

There is an association between decreased wait time and waitlist mortality in adult recipients after LDLT (20, 21). In the pediatric community, LDLT has been supported because of its potential to decrease wait time and waitlist mortality in this vulnerable patient population as well as the ability to perform the transplant earlier in the disease course (1). In 2019, Kehar et al. showed decreased wait times in LD recipients when the primary etiology was cholestatic liver disease, including biliary atresia. However, wait time was similar between DDLT and LDLT when all diagnoses were analyzed together (6). Opposite to what was expected or reported before, an interesting finding of the present analysis is the fact that patients receiving a left lateral segment graft from an LD spent more time on the waitlist (6, 20, 21). Wait times were significantly longer also in the additional analyses of patients <10 kg or <10 years old but not in patients with biliary atresia. This can potentially be explained by several contributing factors. First, significantly more patients receiving a split graft from a deceased donor were status 1 at the time of transplant. This implies that more patients in that study group had a higher priority on the waiting list, favoring their faster access to deceased donor grafts optimal for splitting. Also, given the higher priority of status 1 patients, an offer of a potential optimal split liver graft from a deceased donor can come up before an adequate living donor work-up is completed in a timely manner. Second, some groups might opt to work-up living donors but only proceed with living donation if no deceased donor is available in a timely manner based on recipient condition or wait to proceed with LT once recipients have grown and safely achieved an adequate size. Moreover, some groups might not consider living liver donation for critically ill patients that have faster access to deceased donor organs. Over the past decade, as the number of LDLT and SLT has increased in the pediatric population, so has the number of candidates listed as status 1A or 1B, which may have affected the difference in wait time in our analysis. We chose not to exclude status 1A patients in our study. While this may have impacted our ability to detect differences in LD and split graft groups, the large number of patients keeps our study representative of the actual transplant population (22). Lastly, the number of patients with biliary atresia was significantly higher in the LD group. Therefore, we decided to perform a subgroup analysis to evaluate the outcomes between patients with biliary atresia, finding similar results as in the main analysis, except, for a similar time on the LT waitlist between both groups.

Biliary and vascular complications in pediatric recipients have been a reason of concern when using living donors and split liver grafts. Historically, partial grafts have been associated with a higher risk of vascular complications (11, 23). Ebel et al. performed a study using multicenter data from the Society of Pediatric Liver Transplantation (SPLIT) database to evaluate the predictors of HAT. In contrast to previous publications, the authors found a decreased risk of HAT in recipients of split, reduced, or living donor grafts compared to whole grafts (OR 0.59, $p < 0.001$) (24). Furthermore, a study by Alexopoulos et al. in pediatric patients ≤7 kg undergoing liver transplant for biliary atresia showed a lower incidence of vascular thrombosis in the technical variant patients than in whole liver recipients (LD (6%) and deceased donor partial liver grafts (5%) compared with whole grafts (13%); $p < 0.002$) (25). In 2020, Boillot et al. performed a retrospective study to identify prognostic factors for 1-year graft and patient survival. Vascular complications including hepatic artery and portal vein thrombosis and or stenosis had no impact on 1-year graft or patient survival (11). Our study revealed no significant difference in the incidence of HAT or PVT but did show an increased incidence of "other vascular thrombosis" as the cause of graft failure in living donor recipients. After the subanalyses, HAT was more common in recipients with biliary atresia that received a split graft, as well as recipients who were <10 kg or <10 years old when compared with LD recipients. However, the low incidence of this cause of graft failure overall makes it difficult to draw conclusions on its implication for these particular groups of patients.

LDLT has been associated with higher rates of biliary complications in the pediatric and adult populations when compared to whole grafts (7, 26). A retrospective analysis by Laurence et al., showed no difference in the rates of biliary complications in pediatric recipients of living donor (14.6%) and deceased donor (18.4%) transplantation. In terms of surgical techniques, Roux-en-Y reconstruction was associated with lower complication rates when compared with duct-to-duct reconstruction (27). In our study using the UNOS database, there was a single reported case of diffuse cholangiopathy in a patient in the split graft group and none in the living donor group. Unfortunately, detailed data about biliary complications or more specific causes of graft failure related to the biliary system were not documented in the UNOS database, which makes it difficult to draw conclusions over this complication in this manuscript.

Subgroup analyses were performed to better understand the challenges associated with low body weight (<10 kg) of pediatric recipients and younger age at the time of transplant (<10 years old). Historically, weight above 10 kg has been predictive of graft survival and associated with improved outcomes (10, 28). Smaller recipient size increases the technical complexity of the surgery

and has been associated with higher rates of vascular and biliary complications (29). However, recent studies have shown no difference in clinical outcomes, allowing for earlier transplantation in these patients (8, 30). In the previously mentioned study by Alexopoulos et al., LD and DDLT recipients ≤7 kg had superior 1-, 5-, and 10-years graft survival compared with WLT (25). In our study, graft survival at 1-, 3-, and 5-years was higher in the LD group, suggesting that LDLT may provide additional benefit to pediatric patients with low body weight.

Age at the time of transplant is also an important consideration in pediatric liver transplant outcomes. Historically, infants <12 months have the highest pretransplant mortality rate (2). In 2019, the subgroups of children less than 1 year of age (29.9%) and 1–5 years old (29.9%) were the largest age groups on the waiting list (2). A study by Byun et al. in 2014 showed no difference in survival outcomes in LDLT recipients <12 months when compared with older children (31). Our analysis showed postoperative outcomes consistent with recipients <10 kg, including improved 1-,3-, and 5-years graft survival and decreased re-transplantation in LD recipients. The decreased incidence of re-transplantation in LD recipients either <10 years old or <10 kg is an interesting finding in our study. Prognostic factors and indications for pediatric re-transplantation have been elucidated, but the association with the type of graft has not been studied to our knowledge (32, 33).

Limitations of this study include the inherent challenges of registry data. Primary diagnoses for transplant in the pediatric UNOS database were felt to be under documented and some categories redundant. The individual causes of graft failure account for a small percentage of the number of deceased donors and living donor cases included. Specifically, the "other vascular thrombosis" as a category of graft failure may have overestimated the incidence of the complication. This is further compounded by the low numbers of liver transplants occurring in pediatric patients in general over the time period. As with all registry data, the onus falls on the transplant center performing the surgery to document the correct complications and reason for graft failure. In addition, lack of detailed information limited analysis of important variables that were not recorded/available in the dataset (i.e., presence of vascular anomalies, graft/recipient weight ratios, technical complications) as well as the possibility to control for additional confounders among the study groups. However, detailed analysis and subgroup analysis, as well as the scarcity of reports in literature comparing outcomes following these techniques in pediatric recipients are amongst the strengths of the present manuscript.

In conclusion, this study demonstrates that LDLT is associated with a lower rate of graft failure in pediatric patients. Patient survival at 1-, 3-, and 5-years is comparable between LDLT and SLT. In patients with a diagnosis of biliary atresia, those with a body weight <10 kg or those <10 years old, LDLT is associated with improved graft survival and decreased need for re-transplantation. The use of LLS regardless of the type of donor could represent a safe way to facilitate access to transplantation to pediatric patients with acceptable outcomes.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The data that support the findings of this study are available from the United Network for Organ Sharing (UNOS) Standard Transplant Analysis and Research file (STAR). The UNOS-STAR database does not include any patient or transplant center identifiers. Restrictions apply to the availability of these data, which were used under license for this study.

ETHICS STATEMENT

Due to the retrospective nature of the study and abstraction of de-identified data from the national registry, it was deemed exempt by our Institutional Review Board.

AUTHOR CONTRIBUTIONS

CD and PV participated in writing of the paper, analysis, interpretation of data, and drafting of the manuscript. KS, FD, GM, and JO participated in critical revision of the manuscript for important, intellectual content. NG participated in conception and design of the manuscript, writing of the paper, analysis and interpretation of data, and drafting of the manuscript. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
published: 28 March 2022
doi: 10.3389/ti.2022.10419

Current Trends in Organ Preservation Solutions for Pancreas Transplantation: A Single-Center Retrospective Study

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Due to the high vulnerability of the pancreas to ischemia-reperfusion injury, choices regarding preservation solution markedly affect pancreas transplant success. A retrospective single-center analysis of 380 pancreas transplants (2000–2019) was performed to correlate current preservation solutions with transplant outcomes. Early graft failure requiring transplantectomy within 30 days post-transplant occurred in 7.5% for University of Wisconsin (UW) group (n = 267), 10.8% of Celsior (CS) group (n = 83), 28.5% of Histidine-Tryptophan-Ketoglutarate (HTK) group (n = 7), and none for Institut Georges Lopez-1 (IGL-1) group (n = 23). The most common causes of technical failures in this cohort included abdominal hemorrhage (8.4%); graft pancreatitis (3.7%); fluid collections (2.6%); intestinal complications (6.6%); and vascular thrombosis (20.5%). Although IGL-1 solution provided lower surgical complication rates, no significant differences were found between studied groups. Nevertheless, HTK solution was associated with elevated pancreatitis rates. The best graft survival was achieved at 1 year using UW and IGL-1, and at 3 and 5 years using IGL-1 (p = 0.017). There were no significant differences in patient survival after a median follow-up of 118.4 months. In this setting therefore, IGL-1

Abbreviations: BMI, Body Mass Index; CIT, Cold Ischemia Time; CS Celsior; DGF, Delayed Graft Function; DM, Diabetes Mellitus; HTK, Histidine-Tryptophan-Ketoglutarate; HR, Hazard Ratio; IGL-1, Institut Georges Lopez-1; IQR, Interquartile Range; ISGPS, International Study Group for Pancreatic Surgery; LR, Log-rank Test; PAK, Pancreas After Kidney; PPASS, Pre-Procurement Pancreas Suitability Score; PTA, Pancreas Transplant Alone; PTx, Pancreas transplantation; SPK, Simultaneous Pancreas-Kidney; UW, University of Wisconsin.

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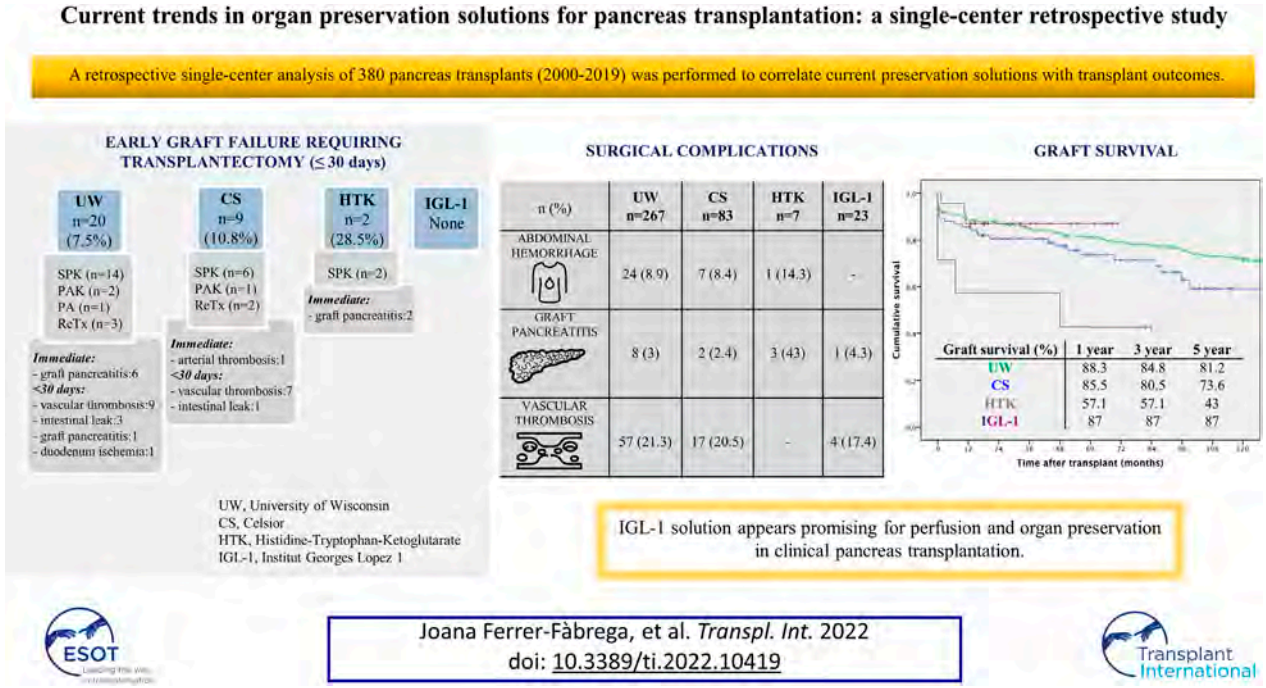
Received: 09 February 2022
Accepted: 25 February 2022
Published: 28 March 2022

Citation:
Ferrer-Fàbrega J, Folch-Puy E, Lozano JJ, Ventura-Aguiar P, Cárdenas G, Paredes D, García-Criado Á, Bombí JA, García-Pérez R, López-Boado MÁ, Rull R, Esmatjes E, Ricart MJ, Diekmann F, Fondevila C, Fernández-Cruz L, Fuster J and García-Valdecasas JC (2022) Current Trends in Organ Preservation Solutions for Pancreas Transplantation: A Single-Center Retrospective Study. Transpl Int 35:10419. doi: 10.3389/ti.2022.10419

solution appears promising for perfusion and organ preservation in clinical pancreas transplantation, compared to other commonly used solutions.

Keywords: pancreas transplantation, graft survival, preservation solution, ischemia-reperfusion, pancreatitis, postoperative outcomes



GRAPHICAL ABSTRACT |

INTRODUCTION

For patients with diabetes mellitus (DM) type 1, pancreas transplantation (PTx) is the only therapeutic option capable of normalizing blood glucose and minimizing secondary complications of diabetes, resulting in an increase in the survival and an improved quality of life (1). According to data from the International Pancreas Transplant Registry, more than 56,000 PTx's were carried out worldwide between the first operation in the 1960s and 2017 (2). In Spain, with 12 accredited centers, 2,006 PTx's have been performed since the program started in 1983 (3-5).

The maintenance of organ viability from donation to transplantation is a decisive factor for the adequate function and survival of the graft, especially in organs such as the pancreas, which is highly susceptible to ischemic damage. Preservation has become a key challenge due to the increasing use of marginal donors, in whom the functionality of the organ is most affected (6,7).

In this scenario, four preservation solutions are currently in use for pancreas transplantation. University of Wisconsin (UW) solution has been considered for organ perfusion in abdominal organ transplantation since the late 80s (8). It features a potassium concentration that mimics the intracellular medium and uses hydroxyethyl starch (HES) as the oncotic agent. In contrast, Histidine-Tryptophan-Ketoglutarate (HTK) and Celsior (CS) solutions, which were originally designed for cardiac graft protection, have the advantage of a much lower viscosity, providing more rapid cooling and better washout during organ procurement. Meanwhile, Institut Georges Lopez 1 (IGL-1) preservation solution was introduced in the early 2000s for the maintenance of abdominal organs and, although clinical experience in PTx with this solution is limited, initial results have been promising (9). Its composition resembles that of UW, with inversed potassium/sodium contents and replaced HES [with a tendency to induce red blood cell aggregation (10)] with 35-kDa molecular-weight polyethylene glycol (PEG35), a neutral, water-soluble, non-toxic polymer that acts like a colloid (11).

At present there is no universal consensus regarding the optimal preservation solution in the setting of PTx albeit UW solution continues to be recognized as the "gold standard" (12). Considering that early technical failure remains the Achilles' heel of pancreas transplantation, there is a growing need within the scientific community for new solutions with superior preservation properties and reduced side effects.

In recent years, the Pancreatic Transplant Unit at Hospital Clínic of Barcelona has routinely used IGL-1 as a preservation solution for PTx from its own donors. The aim of this study was

to compare the effectiveness of the four currently in-use preservation solutions on the outcome of PTx regarding early pancreatic graft function as well as long-term patient and graft survival. Secondly, postoperative surgical complications were also evaluated, as well as their relation with ischemia-reperfusion injury.

MATERIAL AND METHODS

Study Design

Five hundred ninety-one consecutive pancreas transplants were performed at the Hospital Clínic of Barcelona from 1983 through to the end of 2019. A prospectively assembled database of all pancreas transplants from January 2000 to March 2019 was reviewed, i.e. since surgical technique and immunosuppression strategies were standardized. The patient cohort included 380 patients who underwent PTx: 312 (82.1%) simultaneous pancreas-kidney (SPK); 27 (7.1%) pancreas after kidney (PAK) and 3 (0.8%) pancreas transplant alone (PTA). In addition, 38 (10%) patients received a pancreas retransplantation. Data from this cohort were stratified into four groups according to the organ preservation solution employed (UW, CS, HTK and IGL-1). UW and CS were used throughout the whole period of analysis, HTK from January to December 2013 while IGL-1 has been in use from 2014 to the present.

This study protocol was approved by our institutional review board (HCB/2020/0499) and complied with the ethical standards of the Helsinki Declaration of 1975.

Donor Characteristics

Graft pancreas acceptance criteria was performed based on the consensus document of the National Transplant Organization described in 2005 and updated in 2018 (13). Donor analyzed characteristics included: age; gender; cause of death; body mass index (BMI); cold ischemia time (CIT); pre-procurement pancreas suitability score (PPASS); perfusion volume, and amylase/lipase levels.

During organ procurement, both abdominal aorta and portal vein cannulation (dual perfusion) were used to perfuse the organs (perfusion time 8–10 min). The perfusion volume differed depending on the surgeon criteria to obtain a clear effluent via vena cava. The standard, whole-pancreas graft included the entire pancreas and a duodenal segment.

Recipient Characteristics

The indications for PTx were patients with DM who met the inclusion criteria according to the protocol established in our institution (14). Venous systemic drainage was performed between graft portal vein and recipient vena cava or right iliac vein. Arterial supply for the pancreatic graft was done through the anastomosis of the recipient right iliac primitive artery to the graft superior mesenteric artery or the common iliac graft artery, depending on the backtable reconstruction (15). For exocrine secretion, enteric drainage was created "side-to-side", either by duodeno-jejunostomy (from January 2000 to April 2016) or duodeno-duodenostomy anastomosis (from May 2016 to March 2019).

TABLE 1 | Components and function of the various preservation solutions compared in the study.

	UW	CS	HTK	IGL-1	Function
mOsm/L	320	320	310	290	—
Na^+	30	100	15	120	Maintenance of osmotic balance
K^+	125	15	10	25	Maintenance of osmotic balance
Cl^-	—	—	50	—	Maintenance of osmotic balance
Mg^{2+}	5	13	4	—	Maintenance of osmotic balance
Ca^{2+}	—	0.25	0.015	0.5	Maintenance of osmotic balance
HCO^{3-}	5	—	—	—	Buffer
SO^{4-}	5	—	—	5	Buffer
PO^{4-}	25	—	—	25	Buffer
HES (g/L)	50	—	—	—	Oncotic agent, impermeant
PEG35 (g/L)	—	—	—	1	Oncotic agent, impermeant
Mannitol	—	60	30	—	Impermeant, membrane stabilizer
Lactobionate	100	80	—	100	Impermeant, membrane stabilizer
Raffinose	30	—	—	30	Impermeant
Allopurinol	1	—	—	1	Antioxidant
Histidine	—	30	180	—	Antioxidant, buffer
Tryptophan	—	—	2	—	Antioxidant, membrane stabilizer
Glutathione	3	3	—	3	Antioxidant
Ketoglutarate	—	—	1	—	Energy metabolism substrate
Adenosine	5	—	—	5	Energy metabolism substrate
Glutamate	—	20	—	—	Energy metabolism substrate

Concentrations are expressed in mmol/L, unless otherwise specified.
HES, indicates hydroxyethyl starch; PEG35, polyethylene glycol 35 kDa; UW, University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1.

The demographic recipient factors included age; gender; BMI; DM type-1; time of DM evolution (DM *vintage*), and type and duration of dialysis (Dialysis *vintage*). In addition, surgical complications were defined according to the modified Clavien Dindo classification (16) as any postoperative event related to the procedure within the 90 days following the transplant. Postoperative hemorrhage was classified according to the definition of the International Study Group for Pancreatic Surgery (ISGPS) (17). As there was a lack of consensus regarding a clear definition of graft pancreatitis, it was considered the case when it was readily apparent that it had arisen intraoperatively from ischemia-reperfusion injury and its related-complications such as pancreatic abscesses, and peripancreatic fluid collections. Other entities were also considered such as sterile or infected abdominal fluid collections either diagnosed by ultrasound/abdominal computed tomography or evidenced by clinical symptoms. Intestinal complications included duodenum-related leaks and small-bowel obstruction.

Early pancreatic graft function was evaluated both by biochemical parameters (peak serum amylase and lipase levels in the first 48 h together with insulin requirements) and by clinical outcomes, including the need of transplantectomy within 30 days of transplantation.

Immunosuppression

Routine immunosuppression in SPK and PAK consisted of different regimens administered following the institutional protocol, which varied according to the date of transplant

TABLE 2 | Relationship between preservation solutions and clinicopathological features of donors.

	Total *n* = 380	UW *n* = 267	CS *n* = 83	HTK *n* = 7	IGL-1 *n* = 23	*P*
Age (years)	32 (21–40)	30 (20–39)	37 (29–45)	43 (33–47)	30 (19–39)	0.803[a] 0.042[b] <0.001[c]
Gender M/F	224 (58.9)/156 (41.1)	164 (61.4)/103 (38.6)	45 (54.2)/38 (45.8)	4 (57.1)/3 (42.9)	11 (47.8)/12 (52.2)	0.266[a] 0.642[b] 0.251[c]
Cause of death						
-Trauma	197 (51.8)	153 (57.3)	31 (37.3)	2 (28.6)	11 (47.8)	0.561[a]
-Anoxic damage	21 (5.5)	14 (5.2)	5 (6)	-	2 (8.7)	0.100[b]
-CVA	146 (38.4)	90 (33.7)	44 (53)	4 (57.1)	8 (34.8)	0.012[c]
-Others	16 (4.2)	10 (3.7)	3 (3.6)	1 (14.3)	2 (8.7)	
BMI (kg/m^2)	23.4 (21.5–25.3)	23.2 (21.3–25.2)	23.4 (22.3 25.5)	24.2 (23.1–27.3)	23.6 (20.8–25.6)	0.839[a] 0.418[b] 0.065[c]
Pancreas CIT (hours)	10.1 (8–12)	10 (8–12)	11 (9–12.1)	8.3 (6–10.3)	8.2 (7.1–10.1)	0.001[a] <0.001[b] 0.115[c]
Kidney CIT (hours)	12.3 (10–14.3)	12.3 (10–14.3)	12.8 (10.2–14.7)	10.8 (9.4–14.1)	11.2 (9.9–12.8)	0.262[a] 0.188[b] 0.600[c]
PPASS	16 (14–18)	16 (14–18)	17 (14–18)	17 (15–20)	17 (14–18)	0.637[a] 0.683[b] 0.043[c]
Perfusion Volume (L)	6.8 (6.0–7.4)	6.5 (6.0–7.0)	6 (5–6.1)	7 (6–7.5)	7.5 (7–8)	0.014[a] 0.002[b] 0.099[c]
Amylase (IU/L)	84 (47–164.2)	86 (48–172)	73 (39–146)	51 (39–63)	94 (57–294)	0.629[a] 0.202[b] 0.112[c]
Lipase (IU/L)	45 (17–109)	50 (20–126)	22 (11–85.5)	29 (8.2–55.7)	33 (6–79)	0.088[a] 0.820[b] 0.091[c]

Continuous variables are expressed as median (interquartile ranges) and categorical variables as frequencies (percentages).
Comparison of the analyzed variables have been made between UW, CS, and IGL-1, groups. For HTK, group only a descriptive analysis is displayed.
aIGL-1, vs. UW; bIGL-1, vs. CS; cUW, vs. CS.
M, indicates male; F, female; CVA, cerebrovascular accident; BMI, body mass index; CIT, cold ischemia time; PPASS, pre-procurement pancreas suitability score; UW, University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1.

including monoclonal antibody (OKT3), anti-interleukin-2 monoclonal antibody (basiliximab), rabbit anti-human lymphocytes polyclonal antibodies (thymoglobulin) among others, as standard induction therapy. Maintenance immunosuppression was based on triple therapy with calcineurin inhibitor (cyclosporine A until 2005 vs. tacrolimus introduced in the late 90s), mycophenolate and steroids.

Anticoagulant Therapy and Antibiotic Prophylaxis

Our standard anticoagulation protocol included enoxaparin 20 mg every 12 h, starting 8-h post-surgery and maintained until patient discharge (in the absence of thrombotic/hemorrhagic complications), and aspirin 50 mg/d starting at 12-h post-surgery until discharge (100 mg/d).

Vancomycin plus third-generation cephalosporin (from 2000 to 2014) or ertapenem (from 2015 to 2019) were used as antibiotic prophylaxis in the perioperative period. Fungal prophylaxis with fluconazole was universally used in all recipients. Cytomegalovirus prophylaxis was provided by ganciclovir or valganciclovir, depending on glomerular filtration rates.

Statistical Analysis

Categorical variables are expressed as frequencies (%), percentages and continuous variables such as median and interquartile range (IQR). Categorical variables were analyzed by use of Fisher's exact or χ^2 test. Mann-Whitney U test or the Kruskal Wallis in the case of nonparametric distribution were used for the analysis of continuous variables. Due to the limited number of cases for HTK group, and the resulting bias that may arise in subgroup analysis, we have deemed it appropriate to

TABLE 3 | Relationship between preservation solutions and clinicopathological features of recipients.

	Total *n* = 380	UW *n* = 267	CS *n* = 83	HTK *n* = 7	IGL-1 *n* = 23	*P*
Age (years)	40 (35–45)	39 (34–44)	42 (37–47)	45 (33–49)	47 (37–53)	0.003[a] 0.218[b] 0.001[c]
Gender M/F	240 (63.2)/140 (36.8)	170 (63.7)/97 (36.3)	55 (66.3)/28 (33.7)	3 (42.9)/4 (57.1)	12 (52.2)/11 (47.8)	0.369[a] 0.231[b] 0.696[c]
BMI (kg/m^2)	22.7 (20.9–25.6)	22.4 (20.6–25.5)	23 (20.7–25.7)	22.5 (21.2–26.1)	23.1 (21.8–25.5)	0.198[a] 0.581[b] 0.402[c]
DM type						<0.001[a]
-DM I	374 (98.4)	266 (99.6)	80 (96.4)	7 (100)	21 (91.3)	0.017[b]
-Others	6 (1.6)	1 (0.4)	3 (3.6)		2 (8.7)	0.015[c]
DM *vintage* (years)	26 (21–31)	25 (21–30)	28 (22–33.2)	32 (25–34)	27 (21–37)	0.182[a] 0.904[b] 0.020[c]
Dialysis *vintage* (months)	26.5 (17.4–36.7)	26 (18–36.7)	26.8 (19–36.1)	47.3 (26.4–52.3)	24 (11.5–35.3)	0.361[a] 0.322[b] 0.917[c]
Type of dialysis						
-Hemodialysis	213 (56)	151 (56.6)	36 (43.4)	5 (71.4)	12 (52.2)	0.846[a]
-Peritoneal dialysis	85 (22.4)	63 (23.6)	22 (26.5)	2 (28.6)	5 (21.7)	0.992[b]
-Pre-emptive	30 (7.9)	20 (7.5)	9 (10.8)		2 (8.7)	0.469[c]
-No dialysis	52 (13.7)	33 (12.3)	9 (10.8)		4 (17.4)	
Transplant type						
-SPK	312 (82.1)	224 (83.9)	62 (74.7)	7 (100)	19 (82.6)	0.933[a]
-PAK	27 (7.1)	16 (6)	9 (10.8)	—	2 (8.7)	0.847[b]
-PA	3 (0.8)	2 (0.7)	1 (1.2)	—	—	0.281[c]
-Retransplant	38 (10)	25 (9.4)	11 (13.3)	—	2 (8.7)	
Induction therapy						
-Basiliximab	151 (39.7)	116 (43.5)	32 (38.5)	3 (42.8)	-	<0.001[a,b]
-Thymoglobulin	192 (50.5)	114 (42.7)	51 (61.5)	4 (47.2)	23 (100)	0.001[c]
-Others	37 (9.8)	37 (13.8)	—	—	—	
Graft reconstruction						
-SA-SMA	350 (92.1)	249 (93.3)	72 (86.7)	7 (100)	22 (95.7)	0.823[a]
-"Y" iliac graft	27 (7.1)	15 (5.6)	11 (13.3)	—	1 (4.3)	0.191[b]
-Others	3 (0.8)	3 (1.1)	—	—	—	0.012[c]
Intestinal anastomosis	—	—	—	—	—	
-Duodeno-jejunostomy	337 (88.7)	256 (95.9)	67 (80.7)	7 (100)	7 (30.4)	<0.001[a,b,c]
-Duodeno-duodenostomy	43 (11.3)	11 (4.1)	16 (19.3)	—	16 (69.6)	
Transplant Era						
-2000–2009	226 (59.5)	220 (82.4)	6 (7.2)	—	—	<0.001[a,c]
-2010–2019	154 (40.5)	47 (117.6)	77 (92.7)	7 (100)	23 (100)	0.336[b]

Continuous variables are expressed as median (interquartile ranges) and categorical variables as frequencies (percentages).
Comparison of the analysed variables have been made between UW, CS, and IGL-1, groups. For HTK, group only a descriptive analysis is displayed.
aIGL-1, vs. UW; bIGL-1, vs. CS; cUW, vs. CS.
M, indicates male; F, female; BMI, body mass index; DM, Diabetes Mellitus; SPK, Simultaneous Pancreas-Kidney; PAK, Pancreas After Kidney; PA, Pancreas Transplant Alone; SA-SMA, Splenic Artery - Superior Mesenteric Artery; UW, University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1.

provide a detailed description of the immediate post-transplant complications instead of including it for comparison with other groups.

The following variables have been included in the univariate and multivariate analysis as potential risk factors for early graft survival: donor demographics (age, gender, cause of death, body mass index, amylase and lipase values, and P-PASS); donor procurement factors (preservation solution, total perfusion volume, cold ischemia time); era of transplant (before and after 2010); recipient demographics (age, gender, body mass index, DM type, DM vintage, dialysis vintage, type of dialysis, transplant type, and induction therapy). Other factors related to

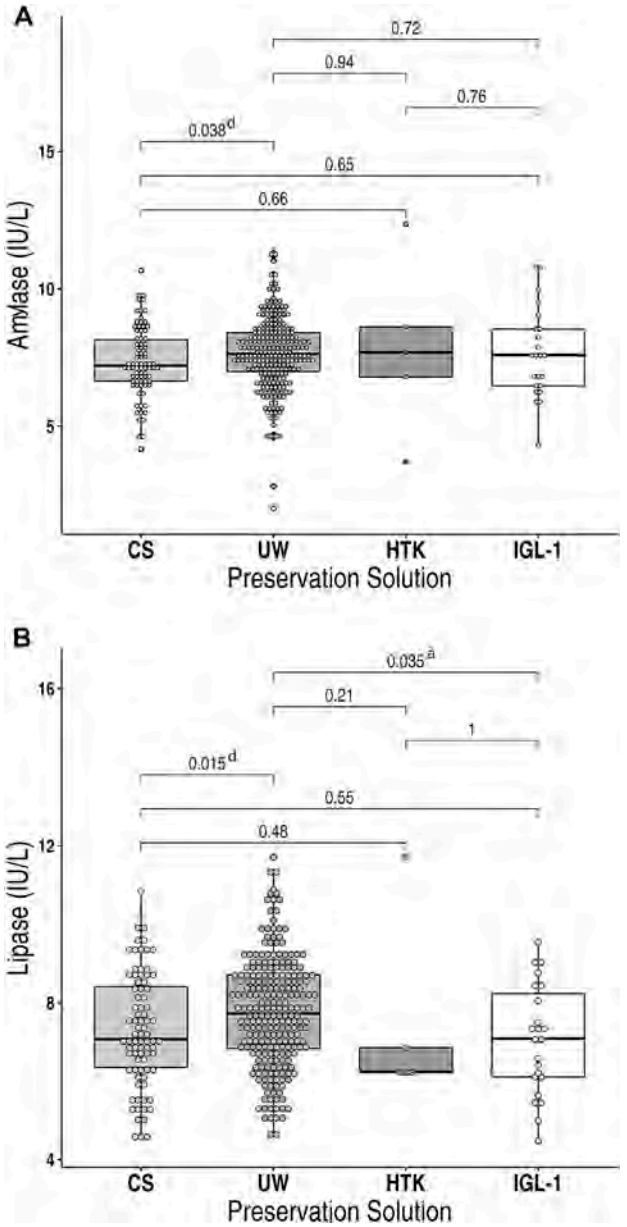


FIGURE 1 | Box Plot showing **(A)** post-transplant serum amylase levels and **(B)** post-transplant serum lipase levels in recipients of pancreas allografts preserved in either CS, UW, HTK or IGL-1 preservation solution. Measured values were converted into logarithmic values. Boxes represent the interquartile ranges and whiskers extend to the minimum and maximum values. The median values are shown within the boxes. UW, University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1. [a]IGL-1 vs. UW; [d]CS vs. UW.

surgical management and technique included were the type of arterial reconstruction in the backtable, the type of vascular (arterial and venous) anastomosis and the intestinal anastomosis technique used in the recipient.

Patient and graft survival were assessed using Kaplan–Meier curves and compared with the log-rank test (LR) and Breslow. Numeric covariates were dichotomized by their median. Patient survival was calculated from the time of transplant to death or the end of follow-up. Pancreas graft survival was calculated from the time of transplant until any of the following: the need for graft removal; the return to permanent insulin therapy dependency; retransplant or death/end of follow-up with a functioning graft. p values of less than 0.05 were considered statistically significant. Significant covariates were subjected to multivariate cox regression analysis.

Statistical calculations were made using SPSS for Windows software (IBM SPSS Statistics version 20.0, 1989–1995; Chicago, IL) and R statistical software (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

RESULTS

Demographic Profile

A total 380 PTx's were performed in our center with the use of four different preservation solutions, which differed in terms of their chemical composition (**Table 1**). Some 267 (70.3%) patients were perfused with UW, 83 (21.8%) with CS, 7 (1.8%) with HTK, and 23 (6.1%) with IGL-1. HTK was introduced in 2013 but was associated with a high and unexpected incidence of graft pancreatitis, prompting us to cease using it and convert to IGL-1.

The four groups had similar characteristics regarding donors as shown in **Table 2**. HTK and CS groups presented older donor age as compared to IGL-1 and UW ($p < 0.05$). IGL-1 and HTK exhibited shorter CIT ($p < 0.05$), with significantly larger volumes of perfusion solution as compared to CS and UW ($p < 0.05$). The preservation solutions did not differ regarding gender, cause of death, BMI, PPASS and the levels of lipase. Nevertheless, in relation to donor amylase levels, HTK group presented lower values compared to others.

Recipient demographics showed no significant differences with respect to gender, BMI, dialysis *vintage*, type of dialysis and type of transplant (**Table 3**). By contrast, recipients in the IGL-1 group were older compared with UW group ($p = 0.003$) and had the lower proportion of patients with DM I compared to others. Thymoglobulin was the most frequently used drug as induction therapy for CS, HTK and IGL-1 groups.

Surgical Technique

There were, by far, more SPK compared to PAK and PTA in the UW, CS and IGL-1 group (**Table 3**). Patients transplanted with HTK solution corresponded solely to SPK technique.

For the vascular reconstruction of the pancreatic graft during backtable, arterial anastomosis between the splenic artery and the superior mesenteric artery was performed in the majority of cases for all analyzed groups. Regarding enteric exocrine drainage procedures, most UW, CS and HTK-preserved grafts were transplanted intraperitoneally (duodeno-jejunostomy), except for IGL-1, for which duodeno-duodenostomy technique was used in 69.6% of cases.

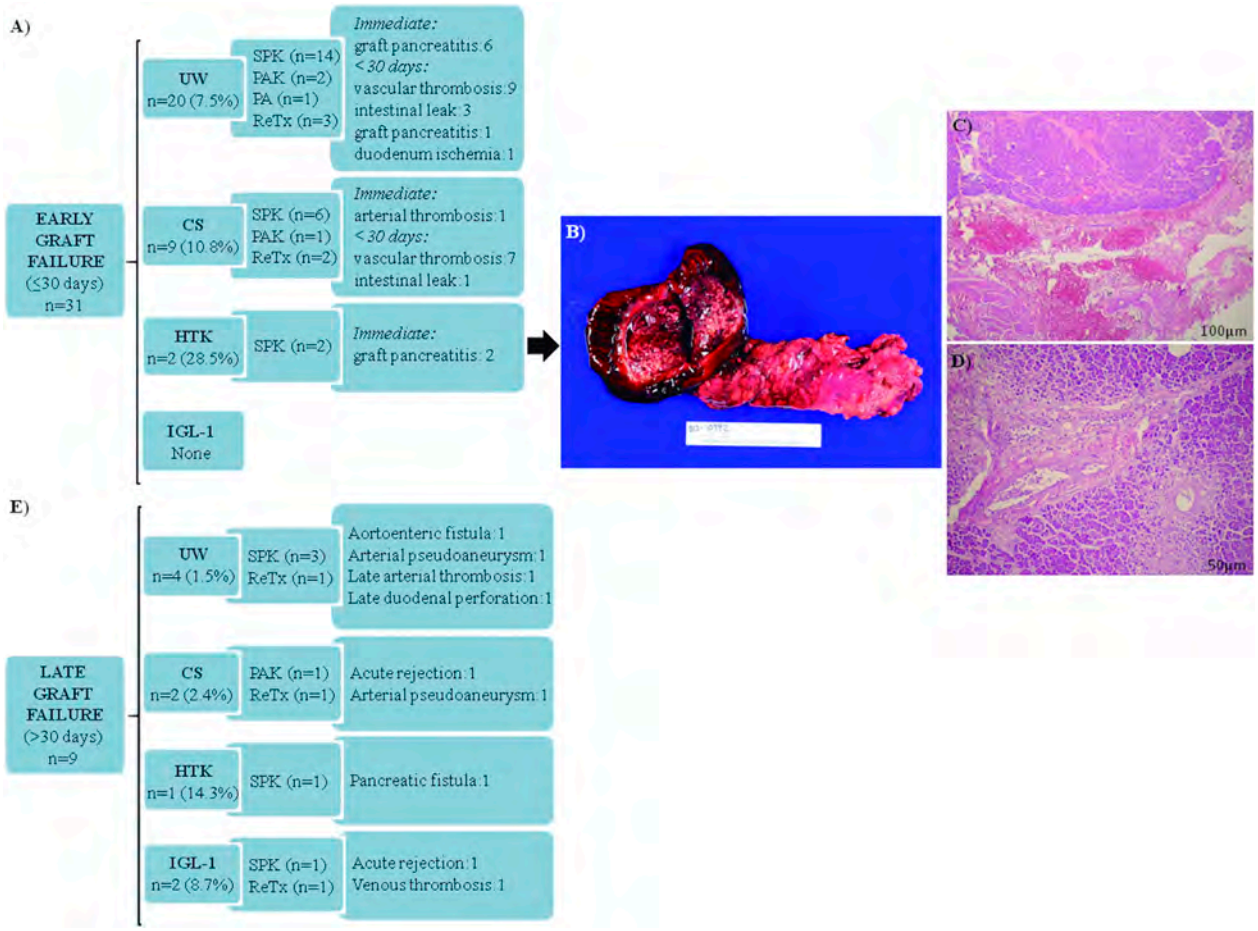


FIGURE 2 | Pancreas graft failure requiring early (≤30 days) and late (>30 days) transplantectomy in transplanted patients. **(A)** Main causes of transplantectomy within 30 days after pancreas transplantation. Vascular thrombosis for UW group includes: venous thrombosis (n = 5); arterial thrombosis (n = 3); and venous + arterial thrombosis (n = 1). Vascular thrombosis for the CS group includes: venous thrombosis (n = 4); venous + arterial thrombosis (n = 3). **(B)** Macroscopic aspect of the graft perfused with HTK with areas of hemorrhage and necrosis in pancreatic tissue and duodenum. **(C)** Extensive hemorrhagic areas affecting the pancreatic parenchyma and peripancreatic soft tissue indicated by asterisks (H&E, scale bar 100 µm). **(D)** Pancreatic parenchyma with ischemic necrosis indicated by asterisks (H&E, scale bar 50 µm). **(E)** Main causes of transplantectomy after 30 days of pancreas transplantation. UW, University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1; SPK, Simultaneous Pancreas-Kidney; PAK, Pancreas After Kidney; PA, Pancreas Transplant Alone; DM, Diabetes Mellitus; ReTx, Retransplant. H&E, Hematoxylin and eosin.

Transplant Outcomes

Early Graft Function

On the immediate postoperative days (24h–48 h), serum amylase levels were the following: UW, 198 (IQR 127–341) IU/L; CS, 148 (IQR 100–295) IU/L; HTK, 206 (IQR 62–2821.5) IU/L and IGL-1, 193 (IQR 89–375) IU/L. Statistical differences were found between UW and CS (**Figure 1A**). The serum lipase levels were as follows: UW, 212 (IQR 114–420) IU/L; CS, 135 (IQR 80.5–350) IU/L; HTK, 76 (IQR 74.5–1738) IU/L and IGL-1, 136 (IQR 66–343.66) IU/L. The highest values for lipase peak were observed in the UW group, compared with CS and IGL-1 (**Figure 1B**). Despite the fact that those patients that required immediate transplantectomy were excluded from the amylase/lipase postoperative analysis, functioning pancreatic allografts perfused and subsequently preserved in HTK solution had an elevated serum amylase and lipase peak as demonstrated by IQR-75% compared to those preserved using other solutions. Nevertheless, the differences were not statistically significant.

Interestingly, a total of 30 patients presented kidney delayed graft function (DGF): UW (7.1%); CS (7.2%), HTK (57.1%); IGL-1 (4.3%), ($p < 0.001$, HTK vs. others). Hemodialysis was required in 15 of them in the immediate postoperative period, with progressive normalization of renal function at the moment of discharge.

Graft Transplantectomy

Early graft failure requiring transplantectomy within 30 days post-transplant occurred in 31 (8.1%) patients (**Figure 2A**), being more frequent in the case of HTK solution (28.5%). None of the IGL-1-preserved allografts required transplantectomy before 30 days. Vascular thrombosis was the main cause of early graft

TABLE 4 | Surgical postoperative complications.

	Total *n* = 380	UW *n* = 267	CS *n* = 83	HTK *n* = 7	IGL-1 *n* = 23	*P*
Pancreas						
Abdominal hemorrhage	32 (8.4)	24 (8.9)	7 (8.4)	1 (14.3)		0.133[a]
Clavien-Dindo						
I	1 (3.1)	1 (4.2)				0.150[b]
II	3 (9.4)	1 (4.2)	2 (28.6)			0.876[c]
IIIa						
IIIb	28 (87.5)	22 (91.6)	5 (71.4)	1 (100)		
IV						
Graft pancreatitis	14 (3.7)*	8 (3)	2 (2.4)	3 (43)	1 (4.3)	
Clavien-Dindo						
I	1 (7.1)				1 (100)	0.720[a]
II						0.620[b]
IIIa	5 (35.7)	2 (25)	2 (100)	1 (100)		0.779[c]
IIIb						
IV						
Abdominal fluid collection	10 (2.6)	7 (2.6)	3 (3.6)			
Clavien-Dindo						
I	2 (20)	1 (14.3)	1 (33.3)			0.432[a]
II	2 (20)	2 (28.6)				0.355[b]
IIIa	1 (10)		1 (33.3)			0.635[c]
IIIb	5 (50)	4 (57.1)	1 (33.3)			
IV						
Intestinal complication	25 (6.6)	15 (5.6)	8 (9.6)	1 (14.3)	1 (4.3)	
Clavien-Dindo						
I	3 (12)	1 (6.7)	2 (25)			0.798[a]
II	2 (8)		1 (12.5)	1 (100)		0.421[b]
IIIa						0.197[c]
IIIb	14 (56)	10 (66.7)	3 (37.5)		1 (100)	
IV	6 (24)	4 (26–7)	2 (25)			
Vascular thrombosis**	78 (20.5)	57 (21.3)	17 (20.5)		4 (17.4)	0.655[a]
Anticoagulation protocol	23 (29.5)	20 (35.1)	3 (17.6)			0.742[b]
Conservative	11 (14.1)	5 (8.8)	4 (23.5)		2 (50%)	0.866[c]
anticoagulation	19 (24.4)	17 (29.8)	2 (11.8)			
Interventional radiology	25 (32.1)	15 (26.3)	8 (47.1)		2 (50%)	
Relaparotomy						
Pancreas graft (no patients)	83 (21.8)	58 (21.7)	21 (25.3)	1 (14.3)	3 (13)	0.327[a]
Time after transplant (days)	6 (2–15)	6.5 (1.7–15)	4 (1–12.5)	2	19 (3–36)	0.214[b]
—						0.496[c]
Hospital stay	15 (11–22)	14 (11–21)	15 (12–24)	30 (11–34)	13 (11–19)	0.475[a]
						0.257[b]
						0.384[c]

Categorical variables are expressed as frequencies (%) and percentages and continuous variables as median and interquartile range (IQR).
Comparison of the analysed variables have been made between UW, CS, and IGL-1, groups. For HTK, group only a descriptive analysis is displayed.
[a]*IGL-1, vs. UW*
[b]*IGL-1, vs. CS*
[c]*UW, vs. CS.*
Include hemoperitoneum, intra-abdominal/subcutaneous hematoma.
**In 8 of the cases an immediate transplantectomy was required, not included in Clavien-Dindo classification.*
***Venous and arterial thrombosis.*
Anticoagulation protocol (enoxaparin + aspirin).
Conservative Anticoagulation (systemic heparin/acenocoumarol).
UW, indicates University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1.

failure in UW and CS-preserved allografts, while graft pancreatitis was the leading cause of pancreatic failure in HTK-preserved allografts. **Figure 2B** illustrates the appearance of one of the HTK-perfused grafts, presenting an immediate severe macroscopic hemorrhagic reperfusion pancreatitis, and confirmed by histopathological data (**Figures 2C,D**).

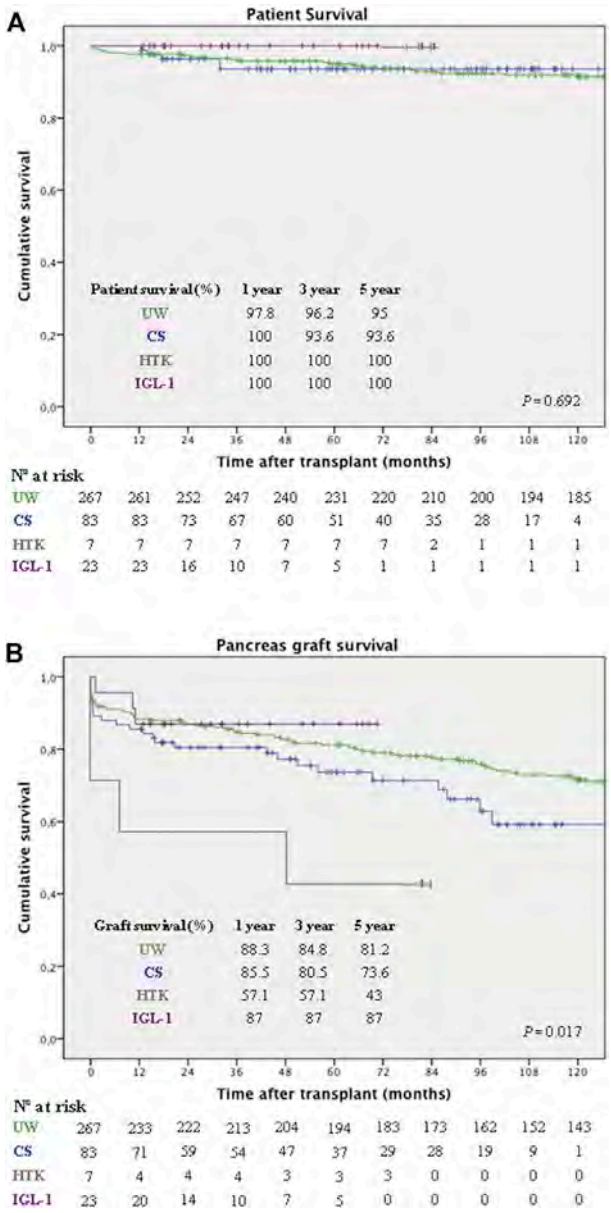


FIGURE 3 | Pancreas graft and patient survival in 380 consecutive pancreas transplants according to preservation solution. **(A)** Patient survival rates at 1, 3, and 5 years were: 97.8%, 96.2%, and 95%, respectively for the UW group (green line); 100%, 93.6%, and 93.6%, respectively for the CS group (blue line); 100% for the HTK group (grey line) and 100% for the IGL-1 group (purple line), with no significant differences ($p = 0.692$, LR). **(B)** Death-censored pancreas graft survival rates at 1, 3, and 5 years were: 88.3%, 84.8%, and 81.2%, respectively for the UW group (green line); 85.5%, 80.5%, and 73.6%, respectively for the CS group (blue line); 57.1%, 57.1%, and 43%, respectively for the HTK (grey line) group; and 87%, 87%, and 87%, respectively in the IGL-1 group (purple line) ($p = 0.017$, LR). UW, University of Wisconsin; CS, Celsior; HTK, Histidine-Tryptophan-Ketoglutarate; IGL-1, Institut Georges Lopez-1; LR, Log-rank test.

Late causes of graft failure requiring pancreas transplantectomy beyond 30-day post-transplant accounted for 2.4% of the cases (**Figure 2E**).

Surgical Complications After Transplant

Technical failures in the total cohort amounted to 37.4% (n = 142). The Clavien-Dindo grading system for the classification of surgical complications was as follow: grade I (5.3%), grade II (6%), grade IIIa (3.9%), grade IIIb (20.5%), grade IVa (1.6%).

Focusing on the most relevant postoperative events, as depicted in **Table 4**, abdominal hemorrhage was identified in 8.4% of the cases (Grade A ISGPS (3.1%) and Grade B ISGPS (96.9%)), being similar between groups, except for IGL-1 group, which had none.

In most cases, a surgical reintervention was required due to: hemoperitoneum (n = 24); intra-abdominal hematoma (n = 3); and subcutaneous hemorrhage (n = 1). Graft pancreatitis was diagnosed in a total of 14 patients. There were numerical differences based on the preservation solution type ($p < 0.001$), with a significantly high rate for the HTK group (43%, thymoglobulin (n = 2), basiliximab (n = 1)). Regarding the whole series, some 8 cases required an immediate transplantectomy because of a severe graft necro-hemorrhagic pancreatitis after reperfusion (**Figures 2A–D**). In those situations, the surgeon considered the graft not viable after checking the tightness and absence of thrombi of the vascular anastomoses. Another HTK case presented a less severe heterogeneous reperfusion with areas of intra-parenchymal hemorrhage (amylase/lipase at 24 h: 5250/3369 IU/L). In that situation, it was decided to salvage the graft, although a pancreas transplantectomy was mandatory 7 months later because of an infected persistent pancreatic fistula. The remaining 5 cases of pancreatitis, presented with a median 24 h serum values of amylase and lipase of 1017 IU/L (796.25–2007) and 776.5 IU/L (495.1–1968.7) respectively, evolved as peripancreatic fluid collection, requiring relaparotomy (n = 4) at a median of 12 days (6.5–15) post-transplant and percutaneous abscess drainage (n = 1). Other intra-abdominal fluid collections were diagnosed in 10 patients, without impact on graft survival. A relaparotomy was needed in half of them, performed in most cases when the patient was readmitted after discharge because of fever and abdominal pain at a median of 28 days (18–43.5) after transplant. Intestinal complications (6.6%) included post-transplant duodenal-enteric leaks and those related to small-bowel obstruction. A total of 5 patients required early transplantectomy because of: anastomotic leak (n = 2); a leak of the duodenal stump site (n = 2), and ischemia of the duodenum (n = 1). The UW, CS and IGL-1 preservation solutions presented similar rates of vascular thrombosis (venous (77%), arterial (6.4%), both (16.6%). Note that, in a total of 25 out of 78 patients, surgery was applied as treatment. Other therapeutic options used for thrombosed pancreas grafts are also described in **Table 4**.

Patient and Graft Survival

After a median follow-up of 118.4 months (IQR: 63.2–168.9), overall patient survival for the whole cohort at 1, 3, and 5 years was 98.4%, 96%, and 95%, respectively. Patient survival rates at 1, 3, and 5 years for the studied groups are depicted in **Figure 3A**, with no significant differences between them (p = 0.692).

Overall death-censored pancreas graft survival for the whole cohort at 1, 3, and 5 years was 87.1%, 83.4%, and 79%, respectively. **Figure 3B** represents the pancreas graft survival rates at 1, 3, and 5 years for the different preservation solution groups. Overall UW, IGL-1 and CS were associated with better pancreas graft survival, compared to HTK ($p = 0.017$).

Regarding pre-procedure variables related to donor and recipient, a significantly increased risk of graft loss on univariate analysis was associated with the following: CIT (>10 h), [hazard ratio (HR) 1.51, 95% CI 1.02–2.23; $p = 0.035$], HTK as preservation solution (HR 3.48, 95% CI 1.27–9.52; $p = 0.009$), pretransplant creatinine (>5.9 mg/dl) (HR 0.66, 95% CI 0.44–0.98; $p = 0.039$), type of transplant (other than SPK) (HR 2.12, 95% CI 1.38–3.25; $p < 0.001$), recipient gender (female) (HR 1.52, 95% CI 1.03–2.23; $p = 0.031$). Other variables with no statistical significance yet presented a tendency to influence graft survival were: donor BMI >27 kg/m^2 ($p = 0.057$) and donor cause death other than trauma ($p = 0.06$). In a multivariate Cox regression model for graft survival, the variables associated with an increased risk for graft failure were: type of transplant (other than SPK) (HR 5.46 CI 1.63–18.28; $p = 0.005$) and recipient gender (female) (HR 1.97, 95% CI 1.00–3.86; $p = 0.04$).

DISCUSSION

Of all solid organ transplant types, pancreas transplants are most susceptible to non-immunologic failure, with a reported graft loss rate of 5%–20% during the first year after transplantation (18-20). Because of the high vulnerability of the pancreas, an appropriate preservation solution could make a difference on graft and patient outcome. However, there is no universal consensus concerning the optimal preservation fluid in PTx (12).

Herein, we present the first retrospective single-center study comparing the effects of the four most commonly used preservation solutions in PTx, i.e. UW, CS, HTK, and IGL-1, on early pancreatic graft function as well as long-term patient and graft survival. By analyzing a large cohort of pancreas transplants in a 20–year period, this study shows that, although similar rates of graft survival were observed during the first year when comparing IGL-1, CS and UW, better results for IGL-1 were observed over the long term. Conversely, the HTK-preserved pancreas had the lowest graft survival in comparison to the other preservation solutions employed, supporting the findings of Hameed AM et al. (12) when comparing UW, HTK and CS preservation solution in a meta-analysis study.

Of note, out of the total 31 cases with early graft failure requiring transplantectomy within 30 days post-transplant, none were associated with the use of IGL-1 preservation solution. However, even though this result seems promising, they need to be interpreted cautiously because of the small sample size of IGL-1 cohort in comparison with UW or CS. When analyzing the intraoperative events, severe reperfusion pancreatitis with immediate graft removal was present in 28.5% of preserved-graft with HTK, a higher percentage when compared to other solutions. Clinical experience with HTK solution still generates controversy. It is known that its low viscosity necessitates larger solution volumes, as initially recommended by the manufacturers. However, it has been demonstrated that this factor may also be detrimental for optimal pancreas preservation, and that abdominal organs can be adequately preserved with a total volume of 5–7 L of HTK (21). In the majority of clinical studies, the HTK-flushed grafts had a higher risk of graft loss due to acute pancreatitis and thrombosis when experiencing ischemic times in excess of 12 h (22-24). In our cohort, the median of HTK-perfused solution used was 7 L. Despite the fact that HTK was used in grafts with shorter CIT, and that no changes were made in organ recovery practices, transplant techniques, or transplanting surgeons, a significant increase in the rate of pancreatitis in recipients was observed ($p < 0.001$). These findings are in contrast to a larger series published by Fridell et al. (25), who found no differences in outcomes of 308 pancreas transplants with the use of UW and HTK, suggesting that the observed differences in other studies may have been attributed to long ischemic times (19) and larger flush volumes.

A study from Ngheim et al. suggested that dual perfusion may alter pancreatic function during pancreas procurement in comparison to the aortic-only vascular perfusion (26). The authors found that the 6 pancreas retrieved by dual aortic and portal flush had higher serum amylase and lipase levels and lower levels of urine bicarbonate and pH. However, due to the lack of larger studies, both single and dual perfusion are currently considered as effective methods when procuring the pancreas for transplantation (12, 27). The impact of this factor could not be evaluated in the present series as aortic-only perfusion was not investigated. However, this method could be a source of future research to assess whether or not dual perfusion is a possible risk factor for increased graft injury resulting from venous congestion and graft edema.

Although vascular thrombosis has been shown to be a risk factor for graft loss (28-34), in this series no differences have been observed in relation to the preservation solution used. The same applies to intestinal-related morbidity.

Another important consideration when analyzing the results of our series is the quality of the pancreatic donor. Examination of the records showed no statistically significant differences regarding donor characteristics and preservation solutions used, with the exception of older pancreatic grafts in the HTK and CS groups, and longer CIT for UW and CS cases. Studied groups were also similar regarding recipient characteristics, with the exception of older patients for IGL-1 group, and longer DM *vintage* for HTK group.

No active interventions among pre-procedure factors with influence on graft survival, such as the recipient gender or type of transplant, are possible as they are unchangeable variables. Moreover, and taking into account the heterogeneous population and the long-time study period, neither the era of study (before and after 2010, as it was the midpoint of the period (2000–2019)), the type of vascular reconstruction nor the intestinal anastomosis had an impact on the early graft functioning.

In general, our findings are consistent with the scant published information in PTx using IGL-1. At the clinical level, one preliminary study suggests that IGL-1 is a safe preservation solution since it provides up to 17 h of cold ischemia. The five human pancreases preserved with IGL-1 acquired normal function immediately after reperfusion, without loss of the graft (35). Similar results were observed in a more recent study comprising a series of 47 consecutive PTx (36). Conversely, IGL-1 has been proven to be equivalent to UW or CS solutions for pancreas perfusion and cold storage before islet transplantation (37). Nevertheless, in a model of PTx in pigs, IGL-1 offered greater protection in membrane fluidity after reperfusion (38).

To the best of our knowledge, this is the only study exploring the effect of the four preservation solutions currently used for clinical PTx. We are aware that the suboptimal number of patients (mainly in the HTK group) limit the conclusions of the study, even though this factor is mitigated when evaluating the results from the point of view of "intention to treat". A low number of HTK-flushed pancreases has arisen due to an unexpected increase in the rate of immediate transplantectomy due to acute pancreatitis following reperfusion, as the latter is also an independent risk factor for impaired graft survival. This fact limited HTK's use in PTx and did not allow us to recruit an optimal number of cases for comparison with a suitable sample size. Furthermore, no hard conclusion could be obtained on the influence of induction therapy on technical failure as two out of the three cases with adverse effect were treated with thymoglobulin, which has potential broad anti-inflammatory properties that have been shown to reduce ischemia-reperfusion injury (39, 40). A long period time study carries with it inherent improvements in perioperative patient care, surgical technique and postoperative management, but the present series transplant era in question did not have statistically significant influence on the graft outcomes. Finally, the fact that surgical technique was changed in 2016 to duodenoduodenostomy does not affect immediate reperfusion injury rates, as vascular anastomoses were performed with the same technique throughout the time period in question. Despite numerous techniques to minimize exocrine pancreatic drainage complications, no universal technique has been standardized (41,42). To date, it is unclear whether duodenojejunostomy or duodenoduodenostomy provides the best long-term survival of the grafts (43). A prospective multicentre registry analysis may resolve this.

In conclusion, the fruits of this study indicate a trend towards a better graft and patient survival among IGL-1 recipients. Besides, IGL-1 composition is similar to that of the UW solution, currently considered as the "gold standard" in the reduction ischemia-reperfusion injury of the pancreas. Hence, successful PTx can be safely performed using IGL-1 solution. Further multicenter studies are still required to identify the "holy grail" of preservation solutions, especially in the current scenario of using marginal donors, including donors following circulatory death, in which the graft is exposed to a warm ischemia insult before cold storage, raising susceptibility to graft dysfunction.

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 Hospital Clinic of Barcelona Institutional Review Board (HCB/2020/0499). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JF-F, EF-P, JL, JF, and JG-V conceived and designed the study. JF-F, EF-P, PV-A, GC, DP, AG-C, JB, RG-P, ML-B, RR, EE, MR, FD, CF, LF-C, JF, and JG-V contributed to the acquisition of the data or analysis and interpretation of the data. JF-F, EF-P, JL, JF, and JG-V drafted the manuscript. All authors revised the manuscript critically for essential intellectual content. All authors read and approved the final version to be published.

FUNDING

This work was supported by grant from Ministerio de Ciencia, Innovación y Universidades, reference PID2019-104130RB-I00 awarded to EF-P.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We thank Steve Illing, Cambridge RSA CELTA, for English language assistance.

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ORIGINAL RESEARCH
published: 16 March 2022
doi: 10.3389/ti.2021.10158

Complement-Binding Donor-Specific Anti-HLA Antibodies: Biomarker for Immunologic Risk Stratification in Pediatric Kidney Transplantation Recipients

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Antibody-mediated rejection is a common cause of early kidney allograft loss but the specifics of antibody measurement, therapies and endpoints have not been universally defined. In this retrospective study, we assessed the performance of risk stratification using systematic donor-specific antibody (DSA) monitoring. Included in the study were children who underwent kidney transplantation between January 1, 2010 and March 1, 2018 at Stanford, with at least 12-months follow-up. A total of 233 patients were included with a mean follow-up time of 45 (range, 9–108) months. Median age at transplant was 12.3 years, 46.8% were female, and 76% had a deceased donor transplant. Fifty-two (22%) formed C1q-binding *de novo* donor-specific antibodies (C1q-*dn*DSA). After a standardized augmented immunosuppressive protocol was implemented, C1q-*dn*DSA disappeared in 31 (58.5%). Graft failure occurred in 16 patients at a median of 54 (range, 5–83) months, of whom 14 formed *dn*DSA. The 14 patients who lost their graft due to rejection, all had persistent C1q-*dn*DSA. C1q-binding status improved the individual risk assessment, with persistent; C1q binding yielding the strongest independent association of graft failure (hazard ratio, 45.5; 95% confidence interval, 11.7–177.4). C1q-*dn*DSA is more useful than standard *dn*DSA as a noninvasive biomarker for identifying patients at the highest risk of graft failure.

Keywords: antibody-mediated rejection, kidney allograft, children, transplant outcomes, immunosuppression

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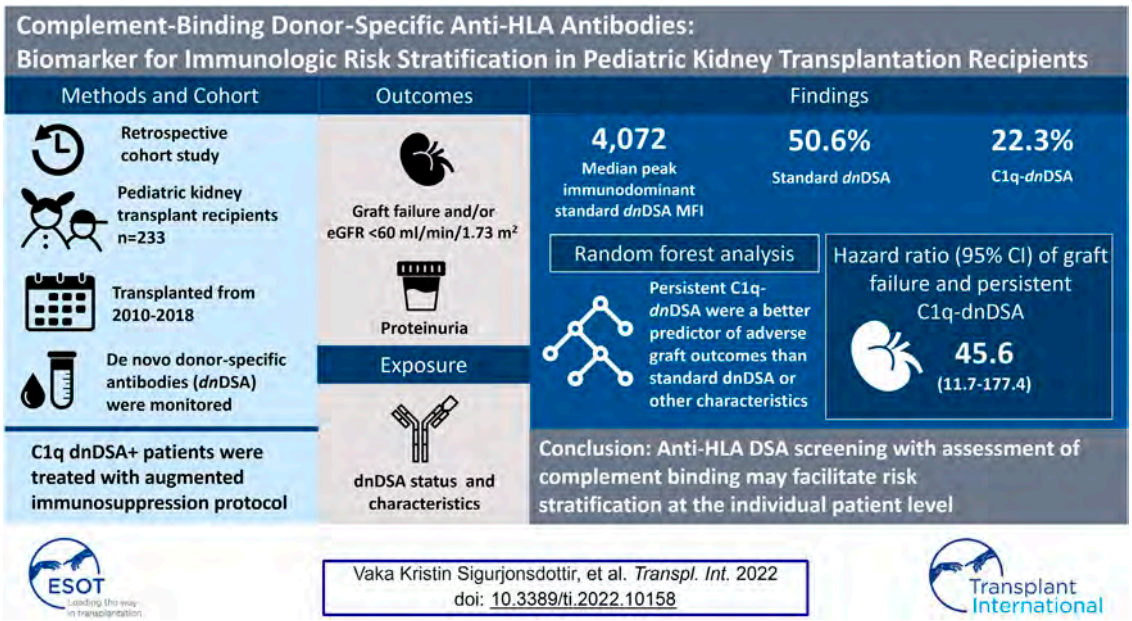
***Received:** 29 October 2021*
***Accepted:** 27 December 2021*
***Published:** 16 March 2022*

Citation:
Sigurjonsdottir VK, Purington N, Chaudhuri A, Zhang BM, Fernandez-Vina M, Palsson R, Kambham N, Charu V, Piburn K, Maestretti L, Shah A, Gallo A, Concepcion W and Grimm PC (2022) Complement-Binding Donor-Specific Anti-HLA Antibodies: Biomarker for Immunologic Risk Stratification in Pediatric Kidney Transplantation Recipients. Transpl Int 35:10158. doi: 10.3389/ti.2021.10158

Abbreviations: C1q-*dn*DSA, C1q-binding *de novo* donor-specific antibodies; *dn*DSA, De novo donor-specific antibody; DSA, Donor-specific antibody; GFR, Glomerular filtration rate; iDSA, Immunodominant donor-specific antibody; IVIG, Intravenous immunoglobulin; LPCH, Lucile Packard Children's Hospital; MFI, Mean fluorescence intensity; MMF, Mycophenolate mofetil; HLA, Polymorphic human leukocyte antigen; pPRA, Peak panel-reactive antibodies; RIS, Relative intensity score; SAB, Single antigen bead.



GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney allograft survival is typically between 10 and 15 years, reflecting an area of unmet need in pediatric kidney transplantation since these children are destined to require more than one transplant during their lifetime. The most common cause of graft failure is induced alloimmune response and rejection (1). Antibodies formed against polymorphic human leukocyte antigen (HLA) molecules on the donor endothelium are central to the pathogenesis of antibody-mediated rejection (ABMR). In kidney allograft recipients, the presence of donor-specific antibodies (DSA) both before and after transplantation correlates with poor graft survival (2–5). DSA that activate the complement system appear to cause particularly severe injury to the allograft, and new complement blood tests (C4d, C1q, C3d) have been developed as tools to stratify the immunologic risk (6–9). Studies link complement-binding *de novo* DSA (C1q-*dn*DSA) to inferior graft outcomes (3, 4, 10–16). The value of using complement-activating antibody testing in clinical care is currently a subject of substantial debate (6, 17–20). In a multicenter study comprising more than 1,000 DSA-positive kidney allograft recipients, Loupy et al. (3) reported that 24% of detected antibodies bound to C1q. More importantly, the glomerular filtration rate (GFR) was lower at 1-year posttransplant, and 5-years graft survival was significantly worse among patients whose DSA-bound C1q as compared to those with DSA that did not demonstrate C1q binding. In a retrospective cohort study of 193 patients at Lucile Packard Children's Hospital (LPCH) who received a kidney transplant in 2000–2008, a C1q solid-phase assay was employed in parallel to the standard immunoglobulin G (IgG) assay to identify C1q-*dn*DSA (12). Patients with C1q-*dn*DSA (n = 15) were almost 6 times more likely to suffer graft failure than those without such antibodies. In fact, 47% of the C1q-*dn*DSA-positive patients suffered premature graft failure at a mean of 33.0 ± 17 months posttransplant. A shortcoming of that study was that C1q-*dn*DSA were analyzed retrospectively in a small cohort using a single blood sample from each case.

A recent study of adult kidney allograft recipients with ABMR showed that persistence of C1q-*dn*DSA after augmented immunosuppressive treatment was an independent determinant of allograft loss (21). We wanted to better characterize the immunologic risk and tailor the treatment to reduce the risk of allograft complications, such as infection, in our pediatric kidney transplant population (22). After reporting that C1q-*dn*DSA are associated with increased risk of premature graft failure in this group of patients (12), we implemented a DSA monitoring and treatment protocol after transplant, using the DSA characteristics and kinetics as a biomarker in an individualized risk stratification for graft failure. Intensified immunosuppressive treatment was guided by the C1q kinetics. The purpose of this study was to assess the performance of our individualized risk stratification using systematic DSA monitoring, including their complement-binding capacity, in addition to the standard approach. We hypothesized that the presence, and in particular persistence, of C1q is a key biomarker for risk stratification.

MATERIALS AND METHODS

Ethical Approval

The present study was approved by the Stanford University Institutional Review Board (49,338). The clinical and research

TABLE 1 | Augmented immunosuppressive therapy directed at C1q-*dn*DSA.

- Treatment initiated if complement-binding donor-specific antibodies with MFI ≥1,000 and/or if positive C4d staining on biopsy
- Concurrent cellular rejection treated with corticosteroid if Banff borderline or 1a. Thymoglobulin considered for Banff 1b or 2
- Management
  - Tacrolimus target trough levels increased to 7–10 ng/ml and MMF to 4–6 mcg/ml for 2–3 months
  - Intravenous immunoglobulin (IVIG) 2 g/kg, administered initially and then every month for minimum of 3 months. Discontinued when C1q--*dn*DSA MFI <1,000 (defined as disappeared)
  - DSA levels are obtained prior to IVIG infusion (data used in study) and immediately after infusion is complete
  - If C1q-*dn*DSA persist, can consider continuing IVIG monthly
  - If the C1q-*dn*DSA do not respond to IVIG at all after 8 months, discontinuation of treatment should be considered. If MFI levels are decreasing, treatment is continued until disappearance
  - Rituximab 500 mg/m^2 administered within 2 weeks of first dose of IVIG.
  - Plasmapheresis added if severe graft dysfunction
  - Bortezomib if graft dysfunction is resistant to IVIG and/or plasmapheresis
- After C1q-binding *dn*DSA detection, DSA levels are monitored at a minimum of every 3 months until eliminated. If they do not disappear, DSA levels are followed at least every 3 months for 12 months. C1q-binding *dn*DSA then is monitored based on risk, but at least every 12 months

activities reported herein are consistent with the principles of the Declarations of Helsinki and Istanbul.

Study Population

Pediatric patients who underwent kidney transplantation at Stanford University's LPCH from January 1st, 2010 to March 1st, 2018 were eligible for the study. Patients with less than 12 months of follow-up, multiorgan transplants, or inconclusive DSA data were excluded. If a patient received more than one transplant during the study period, only data on the first allograft were used.

Clinical Data

Data were retrospectively extracted from the electronic medical record system, UNOS® and the Stanford Histocompatibility, Immunogenetics, and Disease Profiling Laboratory electronic database at LPCH. Information on patient characteristics, such as cause of end-stage kidney disease, donor information, HLA matching, and age at transplant, immunosuppressive treatment and allograft function were collected. Induction of immunosuppression protocol at LPCH included a rabbit antithymocyte globulin. Maintenance immunosuppression consisted of tacrolimus and mycophenolate mofetil (MMF) for all patients, with or without prednisone, based on immunologic risk. Serum creatinine and urine protein/creatinine ratio were measured at least every 3 months.

Monitoring, Scoring and Definition of DSA

De novo DSA formation was defined as donor-specific anti-HLA antibodies that were initially identified after the kidney transplant. During the study period, all patients in our kidney transplant program were tested for both standard *dn*DSA and C1q-*dn*DSA at 0 (time of transplant), 1, 2, 3, 6, and 12 months following transplant surgery, at least annually thereafter, and as clinically indicated (e.g., in the case of allograft dysfunction). Information was collected on all *dn*DSA, including A, B, C, DR, DQ and DP specificities. At the Stanford HLA laboratory, commercially available Single Antigen Bead (SAB) assay kits (LAB Screen; One Lambda, Inc., Canoga Park, CA, United States) were used for the detection of antibodies. Standard *dn*DSA were defined as HLA IgG antibodies identified by the solid phase assay on a Luminex platform (23).

The presence of C1q-*dn*DSA was determined using a SAB assay according to the manufacturer's protocol (C1qScreen™, One Lambda Inc.) on a Luminex platform. In brief, patients' sera were mixed with polystyrene beads, each uniquely distinguishable by subtle differences in fluorochromes and each coated with a different purified, single-cloned HLA class I or class II antigen. Data were analyzed using the HLA Fusion™ software (One Lambda Inc.), and interpretations were made using normalized (baseline) mean fluorescence intensity (MFI) values. Cutoffs for positive reactions were >1,000 MFI. DSA were considered to have disappeared if such antibodies were <1,000 MFI at the end of follow-up or at the time of graft failure. Persistence of DSA was defined as any DSA with MFI >1,000 at the end of follow-up or at the time of graft failure, even if they had transiently become negative at any point. Immunodominant DSA (iDSA) was defined as the highest MFI value of standard *dn*DSA. An important methodological issue is how to analyze and report the various patterns of antibody response in a heterogeneous patient group. Some patients may generate an antibody response to 1 or 2 HLA antigens, but with a very high MFI. Other patients might generate antibodies to multiple, even dozens of HLA antigens, but with intermediate or lower MFI. As an approach to this issue we used Jordan's previously published Relative Intensity Score (RIS) (24–26), in addition to MFI levels. To calculate RIS, we scored combined MFI of all DSA in the following manner: Each DSA with MFI <1,000 received 0 points; MFI 1000–5,000 (weak intensity) received 2 points; MFI 5,000–10,000 (moderate intensity) received 5 points; and MFI >10,000 (strong intensity) received 10 points. The points were summed to form the RIS score.

Kidney Allograft Biopsies, Definition of Antibody-Mediated Rejection and Immunosuppressive Protocols

During the period of the study, protocol biopsies were performed at 6, 12, and 24 months after kidney transplantation and as clinically indicated (i.e., if graft dysfunction and/or *dn*DSA

TABLE 2 | Baseline characteristics of pediatric kidney transplant recipients.

	N = 233
Age at transplant, years, median [IQR]	12.3 [11.4]
Sex, *n* (%)	
Female	109 (46.8%)
Male	124 (53.2%)
Race/ethnicity, *n* (%)	
Asian/Pacific Islander	34 (14.6%)
Black or African American	6 (2.6%)
Hispanic/Latino	90 (38.6%)
White	86 (36.9%)
Multiracial	6 (2.6%)
Other	11 (4.7%)
HLA match, *n* (%)	
0–1	125 (53.6%)
2–3	93 (39.9%)
4–6	15 (6.4%)
pPRA, median [IQR]	7.0 [33.0]
Donor status, *n* (%)	
Deceased	176 (75.5%)
Living	57 (24.5%)
Cause of ESKD, *n* (%)	
Renal aplasia/hypoplasia/dysplasia	52 (22.3%)
Glomerulonephritis	33 (14.2%)
Congenital obstructive uropathy	26 (11.2%)
Chronic pyelonephritis (reflux nephropathy)	20 (8.6%)
FSGS	14 (6.0%)
Polycystic kidney disease	11 (4.7%)
Medullary cystic kidney disease	9 (3.9%)
Cortical necrosis	8 (3.4%)
Hemolytic uremic syndrome	5 (2.1%)
Cystinosis	4 (1.7%)
Familial nephritis	4 (1.7%)
Congenital nephrotic syndrome	14 (6.0%)
Other	16 (6.9%)
Unknown	17 (7.3%)

ESKD, end-stage kidney disease; FSGS, focal segmental glomerulosclerosis; HLA, human leukocyte antigen; pPRA, historic peak panel-reactive antibodies; IQR, interquartile range.

appearance). To ensure consistency, transplant biopsy specimens were gathered and scored for analysis by an expert in kidney transplant pathology according to the consensus rules of the most recent international Banff Classification criteria (27). Baseline immunosuppression and treatment protocol for ABMR after detection of C1q-*dn*DSA, with or without biopsy, remained unchanged throughout the study period. With the initiation of standardized DSA monitoring, including complement-biding capacity, we implemented an augmented immunosuppressive treatment protocol for all patients with C1q-*dn*DSA MFI >1,000 shown in **Table 1**. This protocol was unchanged throughout the study period. Patients who formed standard *dn*DSA only were not treated.

Definition of Antibody Status, Risk Factors of C1q--*dn*DSA Formation and Outcomes

Donor-specific antibody status was considered as the exposure. C1q-*dn*DSA was a time-fixed variable of C1q status, categorized as negative, eliminated or persistent. If C1q-*dn*DSA disappeared after detection at some point and the MFI was <1,000 for the remainder of the study, the C1q-*dn*DSA were considered "eliminated." In those who continued to have C1q-*dn*DSA throughout the study or at the time of allograft failure, the C1q-*dn*DSA were categorized as "persistent." Standard *dn*DSA were either negative, present without ever binding complement or detected together with C1q-*dn*DSA. The persistence and elimination of standard *dn*DSA was followed in patients with C1q-*dn*DSA and defined in the same manner as above.

Decreased immunosuppressive therapy as a risk factor for *dn*DSA formation was defined as purposeful reduction of immunosuppression in response to side effects, malignancy or infectious concerns. Nonadherence to medical care was defined when documented in the medical chart, when patients had undetectable tacrolimus levels, missed clinic visits, missed blood draws, or by patient report. Allograft failure was defined as return to dialysis or preemptive re-transplantation. Decreased GFR was defined as estimated GFR <60 ml/min/1.73 m^2 that persisted over at least 3 months. The creatinine-based "Bedside Schwartz" equation (2009) and/or Chronic Kidney Disease Epidemiology Collaboration equation were used to calculate GFR. Proteinuria cutoff was set at urine protein/creatinine ratio of 0.5 mg/mg that persisted over 3 months. Patients who did not have adverse graft outcome were censored at their last follow up. No patient died before the endpoints of interest were reached.

Statistical Considerations

Baseline demographic and clinical characteristics of the cohort were summarized descriptively using means (range), medians (interquartile range, IQR), and counts (percentages) as appropriate. Median time-to-allograft failure was reported using Kaplan-Meier estimates. The Kruskal-Wallis, Mann-Whitney, Chi-squared, and Fisher's exact tests were used to compare clinical characteristics of patients with persistent C1q-*dn*DSA to those of patients with eliminated C1q-*dn*DSA as appropriate based on underlying statistical assumptions. A conditional inference forest analysis was employed to assess the hierarchy of the characteristics of *de novo* anti-HLA DSA based on their ability to predict adverse graft outcome, defined by decreased GFR or allograft failure (28). Included in the model were iDSA MFI, RIS of standard DSA, C1q-binding status, and standard *dn*DSA status. The variables included in the random forest approach were set to zero for patients who did not form anti-HLA *dn*DSA. The conditional forest was fit to 1,000 trees. Given the collinearity among features, conditional variable importance measures were computed in order to quantify the contribution of each variable, using the integrated Brier score as a risk measure (29). Out-of-bag model performance statistics were expressed for the binary outcome of adverse graft event.

Outcomes of graft failure and proteinuria were analyzed based on time-varying C1q-*dn*DSA-binding status. An unadjusted Cox proportional hazards regression model was fit to time-to-allograft failure as a function of time-varying C1q binding. The proportional hazards assumption was assessed for each model and found to hold in all cases (30). Hazard ratios (HR), 95% confidence intervals (CI), and model concordance (using the

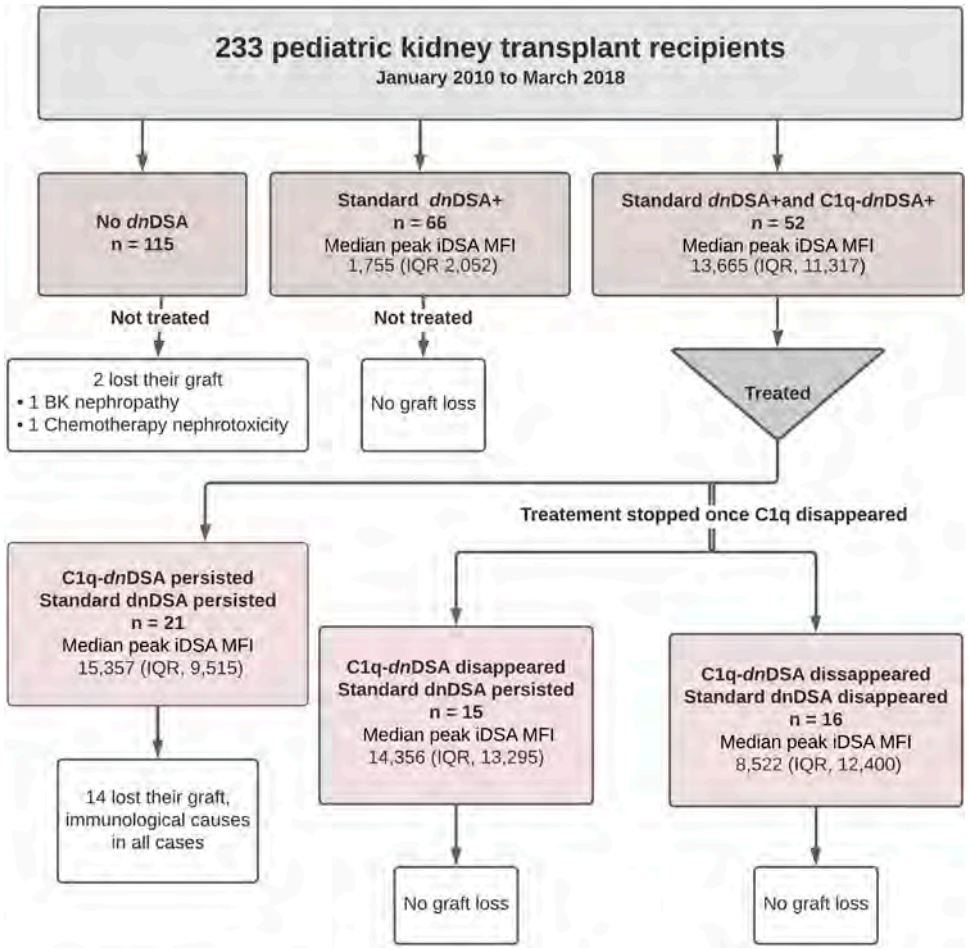


FIGURE 1 | Flow diagram of the study population.

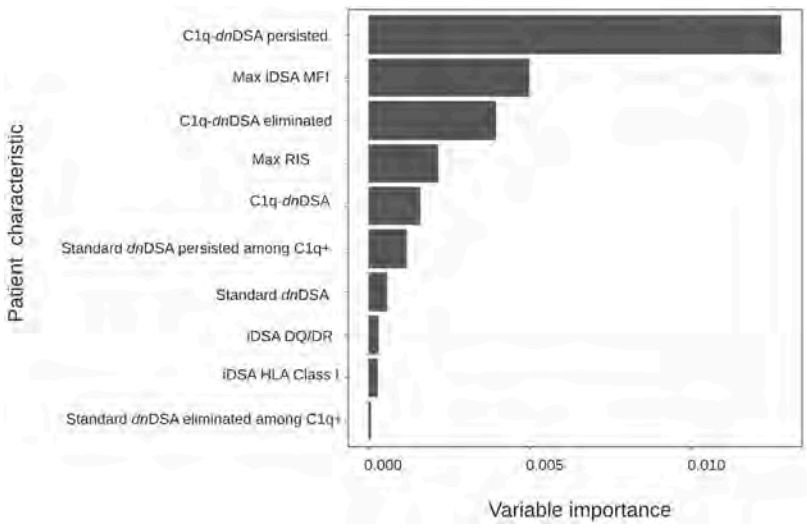


FIGURE 2 | Variable importance plot for time to adverse graft outcome. Hierarchical order of anti–HLA antibody characteristics based on their ability to classify patients according to risk of allograft loss using conditional random forest modeling (n = 233).

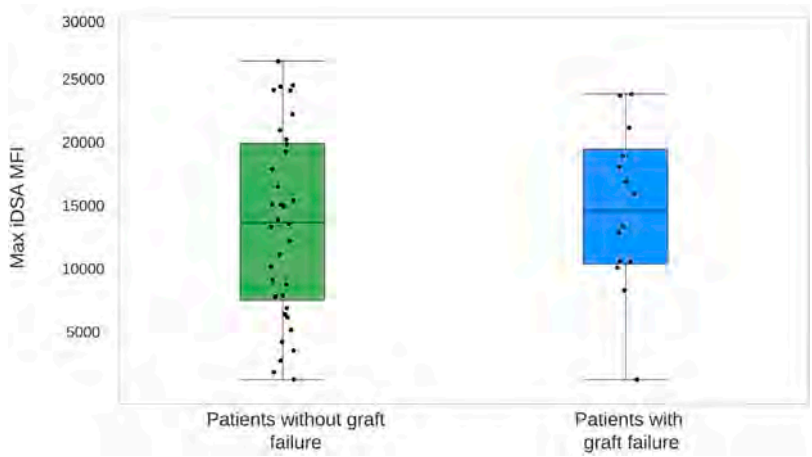


FIGURE 3 | Distribution of iDSA MFI levels in patients forming *dn*DSA, with and without graft loss. The center line represents the median. Each dot represents a single patient. One outlier of max iDSA MFI of 62,344 is not shown among patients without graft failure. iDSA, Immunodominant donor-specific antibody; MFI, mean fluorescent intensity.

C-index) are reported (31). Covariates of interest included sex, historic peak panel-reactive antibodies (pPRA), age at transplant, type of donor and HLA match. A p-value <0.05 was considered statistically significant. All analyses were conducted using R version 3.5.2.

RESULTS

Patient Characteristics

Out of 288 patients who underwent kidney transplantation at LPCH, 233 met the inclusion criteria. One patient who experienced graft failure before 12 months posttransplant due to BK nephropathy was included. Mean follow-up time was 45 (range, 9–108) months. The median age at transplant was 12.3 years, and the majority of the transplant recipients were of Hispanic/Latino origin (39%) and male sex (53%) (**Table 2**). A little over half (54%) had an HLA match of 0 or 1 out of 6, with only 6% having an HLA match >3/6. The median pPRA was 7%, and 76% of donors were deceased. The most common cause of end-stage kidney disease was renal aplasia/hypoplasia/dysplasia (22%).

Anti-HLA *dn*DSA Characteristics

Among the 233 study subjects, 118 formed standard *dn*DSA and of those, 52 also had complement-binding activity triggering interventions at initial detection of C1q-*dn*DSA. A flowchart illustrating the study cohort is provided in **Figure 1**. C1q-*dn*DSA-directed treatment was individualized based on MFI strength, clinical factors, and biopsy results if available, according to the augmented immunosuppressive therapy protocol. The iDSA were of HLA class 1 in 19 (16.1%) patients and HLA class 2 in 99 (83.8%) patients. The median peak MFI of iDSA in all patients forming anti-HLA *dn*DSA was 4,072 (IQR, 11,304). The iDSA were of DQ or DR specificity in 94 (79.6%) patients. Sixty-six (28.3%) patients formed standard *dn*DSA without C1q binding with a median peak iDSA MFI of 1755 (IQR, 2052), and 52 (22.3%) patients formed C1q-*dn*DSA with the median peak iDSA MFI of 13,665 (IQR, 11,317). The median peak RIS of standard *dn*DSA, calculated for patients who also had C1q-DSA, was 20 (IQR, 22).

All 233 patients were included in the random forest analysis. The most important characteristic for an adverse graft outcome (n = 46) was the persistence of C1q binding (**Figure 2**). Seventeen out of 21 (80.9%) patients with C1q persistence had an adverse graft outcome, 14 of whom lost their graft. In patients without DSA formation, 16 (13.9%) had an adverse graft outcome, including two who lost their graft. Eight (12.1%) of the patients with standard *dn*DSA without complement binding and 5 (16.1%) of those with standard *dn*DSA and disappearance of C1q after treatment experienced an adverse graft outcome, although none lost their graft. Adverse graft outcomes were significantly more frequent among patients with C1q persistence compared to others (p <0.0001), but were not significantly different between non-DSA formers, standard *dn*DSA formers only and C1q-*dn*DSA eliminated groups (p = 0.86). The random forest model correctly classified only 17 of the 46 (37%) patients as having an adverse graft outcome, while 184 of 187 (98.3%) were correctly classified as not having an event. Of 14 patients who lost their graft due to immunological reasons, 13 had HLA class 2 *dn*DSA. Of those with *dn*DSA and graft failure12 of 14 had DQ- or DR-specific iDSA. Maximum iDSA MFI was more useful to predict graft outcome than maximum RIS. Peak iDSA MFI was correlated with adverse outcome. Looking at the peak iDSA MFI among the 118 patients who formed standard *dn*DSA, patients with adverse graft outcome had significantly higher peak of 10,861 (IQR, 12,394) compared with 2,987 (IQR, 6,891) in those without adverse graft outcomes (p = 0.01). If we only analyze the C1q formers, there was no difference between peak

TABLE 3 | Clinical and renal histological features of pediatric kidney transplant recipients who formed C1q-*dn*DSA.

Clinical characteristics	C1q disappeared (*n* = 31)	C1q persistent (*n* = 21)	*p*-value
Age at first detection, mean (range)	10 (1–22)	16 (1–24)	0.009
Nonadherence, *n* (%)	18 (58.1)	21 (100)	0.002
Decreased immunosuppressive therapy[a], *n* (%)	12 (38.7)	1[b] (4.7)	0.008
Persistence of standard *dn*DSA	15 (48.4)	21 (100)	
Biopsy at the time of first C1q detection, *n* (%)	24 (77.4)	20 (95.2)	
Graft loss, *n* (%)	0 (0)	14 (66.7)	
Age at graft loss, mean (range)	NA	19 (7–24)	
Histological findings (Banff scores)			
Histological diagnosis of ABMR, *n* (%)	14 (58.3)	19 (95)	0.006
C4d	0.6	1.9	0.002
Total inflammation (%)	45.2	66.2	ns
Interstitial inflammation	1.5	2.3	0.04
Tubulitis	1.5	2.1	ns
Interstitial fibrosis	0.9	1.3	ns
Peritubular capillaritis	0.8	1.4	ns
Intimal arteritis	0.2	0.4	ns
Glomerulitis	0.4	0.7	ns
Transplant glomerulopathy	0.21	0.16	ns

[a]*Decreased immunosuppression prescribed by a physician due to side effects, infection or malignancy at the time of C1q detection.*
[b]*One patient had both infections and history of medication nonadherence. Scores are presented as means. Banff scores were not significantly different between groups.*

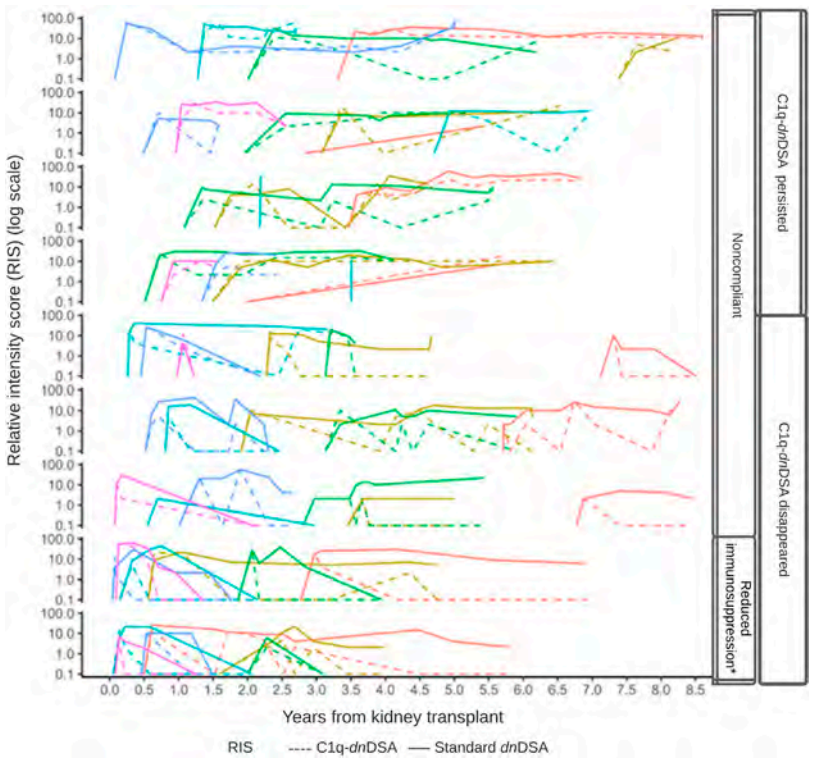


FIGURE 4 | DSA relative intensity score (RIS) over time by C1q status. Line plots of the patients (*n* = 52) with C1q-binding *dn*DSA during the study period. Within each row panel, the colored line corresponds to an individual patient's RIS trajectory, and the line type corresponds to either C1q-*dn*DSA (dashed) or standard *dn*DSA (solid) RIS. An individual patient's trajectory does not commence until the first C1q-binding *dn*DSA is detected.

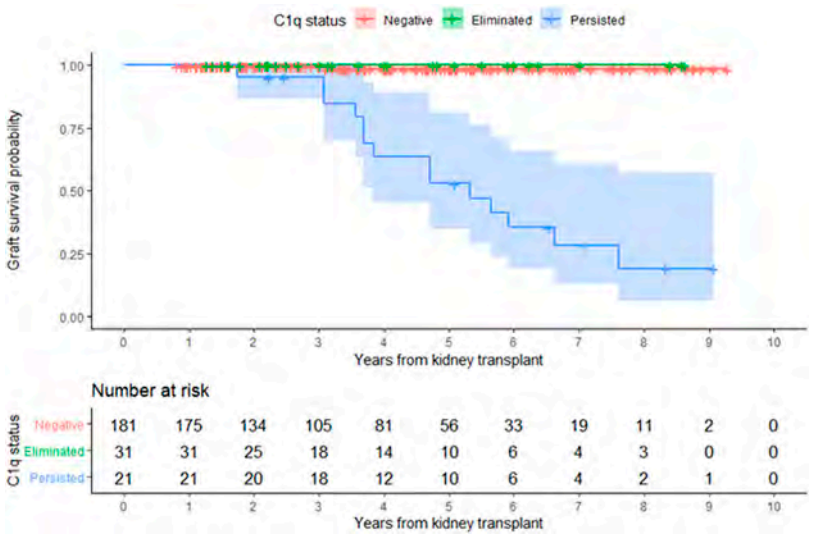


FIGURE 5 | Kidney allograft survival according to overall C1q status. Kaplan-Meier estimate of time to allograft loss.

iDSA MFI levels in patients with or without graft failure, 13,019 (IQR, 13,665) versus 13,569 (IQR, 7,421), respectively (**Figure 3**).

Formation of C1q-*dn*DSA and Antibody-Mediated Rejection

The 52 patients who formed C1q-*dn*DSA had a median time of 3.8 years (IQR, 1.3) until their first detection. C1q-*dn*DSA disappeared in 31 (59.6%) patients and persisted in 21 (40.4%). The time to detection was not different between patients with persistent C1q-*dn*DSA and those who eliminated the antibodies. Forty-four (84.6%) patients had a kidney allograft biopsy carried out at the time of detection, of those 33 (75%) had ABMR according to histological findings. Eight patients were not biopsied, of these four were treated according to protocol without a biopsy, two had contraindications to biopsy, 1 patient refused to have the biopsy performed, and 1 patient started dialysis shortly after detection of C1q. The clinical characteristics and histological findings of patients who formed C1q-*dn*DSA are shown in **Table 3**. The C1q persistent was more likely to have histological signs of ABMR on biopsy at the time of detection, including positive C4d and interstitial inflammation, compared with the C1q disappearance group. They were also older at kidney transplantation and more likely to be nonadherent to immunosuppressive treatment compared with patients who eliminated C1q. The latter group was younger at transplantation and at first detection of C1q; they were also more likely to have purposeful immunosuppressive therapy reduction as a risk factor for C1q formation. The evolution of DSA over time in the cohort is shown in **Figure 4**. Standard *dn*DSA RIS and C1q-*dn*DSA RIS were moderately correlated (Pearson correlation coefficient of 0.61). C1q-binding status varied and sometimes reappeared after a period of being undetectable. In the group where C1q disappeared, five patients had at least one C1q reappearance, whereas this was observed for nine patients in the group where C1q persisted. Two patients with C1q-*dn*DSA persistence were treated with plasmapheresis. All patients with persistent C1q had documented medication non-compliance. One patient with persistent C1q had both infections and history of non-compliance at the time of C1q detection. Two patients in whom C1q disappeared did not have an identifiable risk for C1q binding. Comparing the evolution of C1q-*dn*DSA and standard *dn*DSA, the latter stayed elevated longer and did not respond to enhanced immunosuppressive treatment in the same way as C1q-*dn*DSA. Patients with purposeful reduction of immunosuppression appeared to have a steeper decrease of C1q-*dn*DSA. In the patients with C1q-*dn*DSA, persistence of standard *dn*DSA was not strongly correlated with graft failure, unlike C1q persistence. Out of 31 patients who eliminated C1q, 15 (48.4%) had persistent standard-*dn*DSA compared with all 21 patients with persistent C1q-*dn*DSA. Among patients with persistent C1q-*dn*DSA, older age at transplant (HR, 3.69; 95% CI, 1.58–8.63) was significantly associated with graft failure. Younger age at first detection of C1q decreased the risk of graft failure (HR, 0.33; 95% CI, 0.15–0.72). Severe complications after treatment of *dn*DSA, such as malignancies, infections requiring admission to the intensive care unit, or hypogammaglobulinemia requiring intravenous immunoglobulin (IVIG), were not observed.

*dn*DSA Characteristics, Graft Failure and Proteinuria

In the entire cohort, graft failure occurred in 16 (6.9%) patients after a median of 45 months (IQR, 28). Fourteen of these patients experienced allograft rejection and two had other potential causes of graft failure; one had BK nephropathy and the other cancer chemotherapy nephrotoxicity (**Figure 5**). All patients whose grafts failed due to immunological causes had persistent C1q-*dn*DSA in

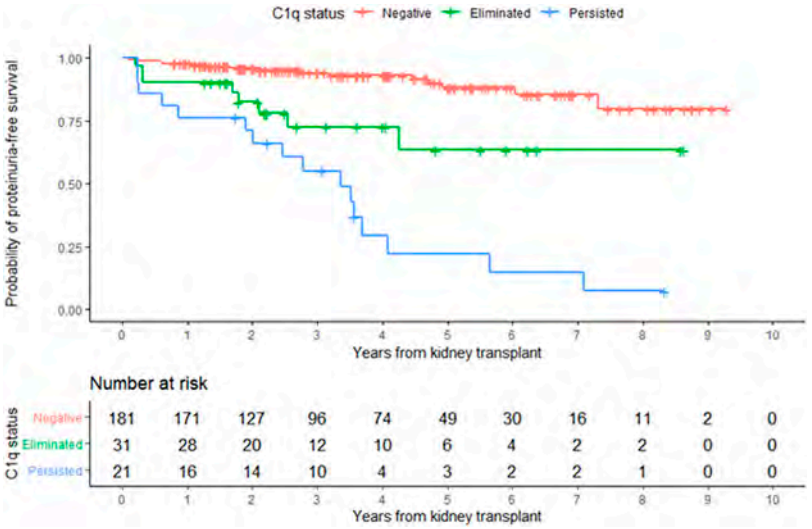


FIGURE 6 | Development of proteinuria according to overall C1q status. Kaplan-Meier estimates of time to proteinuria.

spite of augmented immunosuppressive treatment and lost their graft at a median of 51 months (IQR, 25). Persistent C1q-*dn*DSA were significantly associated with higher risk of graft failure (adjusted HR, 45.46; 95% CI, 11.7 to 177.4). In both the unadjusted and adjusted analysis, elimination of C1q-*dn*DSA was significantly associated with lower risk of graft failure (adjusted HR, 0.02; 95% CI, 0.01–0.09). Among patients with persistent C1q-*dn*DSA, older age at transplant (HR, 3.69; 95% CI, 1.58–8.63) and younger age at first detection of C1q (HR, 0.33; 95% CI, 0.15–0.72) were significantly associated with graft failure. No other demographic or clinical features were associated with graft failure in both groups. There was no correlation between peak iDSA and graft outcome or C1q persistence. The frequency of adverse graft outcome was not significantly different in patients with no *dn*DSA formation, standard-*dn*DSA only and eliminated C1q-*dn*DSA. The risk of proteinuria was highest in patients with persistent C1q-*dn*DSA, intermediate in those with transient C1q, and lowest in those who never developed C1q-*dn*DSA (**Figure 6**).

DISCUSSION

In this retrospective study of 233 pediatric kidney transplant recipients, we evaluated the value of systematic anti-HLA DSA screening in predicting risk of adverse graft outcomes and show that compliment binding capacity improved individual risk assessment and is potentially a useful guide of ABMR treatment. Detection of new complement-binding DSA simultaneously with standard *dn*DSA triggered intensified immunosuppression. Patients forming standard *dn*DSA without C1q binding were not treated and had non-inferior outcomes compared with those who did not form *dn*DSA. While higher standard *dn*DSA strength was associated with adverse graft outcomes, no patient experienced graft failure from immunological causes without persistence of C1q-*dn*DSA which was the strongest independent predictor of kidney allograft failure. Furthermore, there was no difference between peak iDSA MFI levels in patients with complement binding DSA with or without graft failure.

The appearance of both new standard *dn*DSA and complement binding serves as a decision point triggering a diagnostic kidney biopsy or a change in therapeutic immunosuppression. The present study shows that C1q-*dn*DSA assessment after interventions may reflect treatment effectiveness and may be a promising approach to guide the intensity and duration of immunosuppressive treatment. C1q-*dn*DSA status after treatment outperformed standard *dn*DSA as a predictor of adverse graft outcomes. Post-treatment assessment of complement binding guides therapy by informing when to stop interventions in case of C1q-*dn*DSA disappearance and justifies continued interventions as long as the C1q-*dn*DSA titer is responding. Additionally, the absence of C1q-*dn*DSA response is a useful sign that should prompt a change or discontinuation of therapeutic interventions. The disappearance of C1q-*dn*DSA is reassuring in our cohort as no patient in that group experienced graft failure. This is in contrast to 66.7% graft failure when C1q-*dn*DSA persists. Our study is in agreement with prior studies demonstrating the association of C1q-*dn*DSA persistence and allograft survival, irrespective of enhanced immunosuppressive therapy (3, 4, 12, 21). Our results, demonstrating that C1q-*dn*DSA response to treatment (persistence or disappearance) is a better predictor of outcome than that of standard *dn*DSA, are similar to those of Ramon et al. in adults treated for severe ABMR with plasmapheresis (16). More severe inflammation and C4d staining at the time of diagnostic biopsy was more common in patients with C1q-*dn*DSA persistence. Our findings expand the results of past research showing that patients with treatment resistant C1q-*dn*DSA were more likely to have a histological

diagnosis of ABMR, C4d deposition in peritubular capillaries and interstitial inflammation of the allograft (32, 33). Patients who have resolved their C1q-*dn*DSA might still be at risk for premature graft failure and should be monitored closely. Although this was not apparent in the current study with an average of 45 months of follow-up, graft injury that occurs during the presence of C1q-*dn*DSA may be progressive. The increased frequency of proteinuria in this group supports this notion (**Figure 6**). We hypothesize that there is a cumulative, dose-dependent effect of nonadherence to immunosuppressive medications on graft survival. Late detection of C1-*dn*DSA or ongoing medication nonadherence after detection may sabotage even the most effective C1q-*dn*DSA elimination therapy.

The strength of anti-HLA antibodies measured with the Luminex technology in the present study correlated moderately with C1q-binding status like most of studies included in a recent meta-analysis (13). The analysis had sufficient power to show that the association of C1q binding and allograft outcomes were independent of the level of standard anti-HLA DSA MFI (13, 32, 34), and that incorporation of complement-binding capacity increased the accuracy of risk prediction above that of the standard anti-HLA DSA MFI levels alone (32). These findings are consistent with the results of the current study. Observing the evolution of MFI of standard *dn*DSA and C1q-*dn*DSA, we noted that standard *dn*DSA MFI tends to stay elevated longer after initiation of therapy. In nearly half of patients with C1q-*dn*DSA disappearance, standard *dn*DSA were not eliminated with none of the patients losing their graft. It has been hypothesized that a difference in the pathogenicity of IgG subclasses may exist in patients with ABMR, explaining variable outcomes in patients with high MFI and rejection (35, 36). IgG is thought to follow a sequential subclass switching after the initial immune response in ABMR (37). The association of persistently elevated titers of standard *dn*DSA with good outcomes in our cohort possibly represent less injurious subclasses of IgG. Peak iDSA or clearance of standard *dn*DSA are not as useful for guiding treatment as C1q-*dn*DSA and would possibly lead to longer and more aggressive treatment regimen, carrying a risk of infection and impacting the cost of therapy.

The study has a several limitations which are largely inherent in the retrospective design. As such, the study was not originally designed for the purpose of addressing biomarker risk prediction or treatment response and therefore weakens the conclusions that can be drawn. Our treatment response has to be interpreted with caution since we did not have a concurrent control group and the two groups might be inherently different. In addition, follow-up allograft biopsy was not obtained in most of the patients. An appropriately powered randomized controlled trial is needed to confirm our observations. One of the strengths of this study is that C1q-*dn*DSA and standard *dn*DSA were simultaneously monitored over a long period of time, allowing us to characterize the kinetics of these antibodies.

In conclusion, this retrospective study of children with a kidney transplant demonstrates that systematic anti-HLA DSA screening together with complement-binding status and allograft biopsies improves risk stratification at the individual patient level and may assist the clinician in determining timing and duration of therapeutic interventions of AMBR. Timely treatment of C1q-*dn*DSA associated ABMR or C1q-*dn*DSA in the absence of kidney allograft biopsy, with early detection and elimination of C1q-*dn*DSA, may be associated with improved outcomes, whereas inability to clear C1q-*dn*DSA identifies the subset of patients with poor graft survival.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data cannot be shared due to protection of participant privacy. Requests to access the datasets should be directed to vakaks@gmail.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Stanford University Institutional Review Board (49338). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Study design: VS, PG. Data collection: VS, LM, VC, NK, KP. Data analysis: VS, NP, KP. Data verification: VS, KP, NP. Data interpretation: VS, NP, AC, RP, NK, WC, AG, PG. Manuscript writing: VS, RP, PG. Writing of the first draft of the manuscript: VS. Final approval of the version to be published: VS, NP, AC, RP, NK, VC, KP, LM, WC, AG, PG. The authors certify that neither this article nor any part of its essential substance, tables, or figures has been or will be published or submitted elsewhere.

FUNDING

VS was a Tashia and John Morgridge Endowed Postdoctoral Fellow of the Stanford Maternal and Child Health Research Institute. VS also received Young Scientist Award at Landspitali–The National University Hospital.

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
published: 23 March 2022
doi: 10.3389/ti.2022.10248

A Novel Method of CD31-Combined ABO Carbohydrate Antigen Microarray Predicts Acute Antibody-Mediated Rejection in ABO-Incompatible Kidney Transplantation

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Received: 17 November 2021
Accepted: 16 February 2022
Published: 23 March 2022

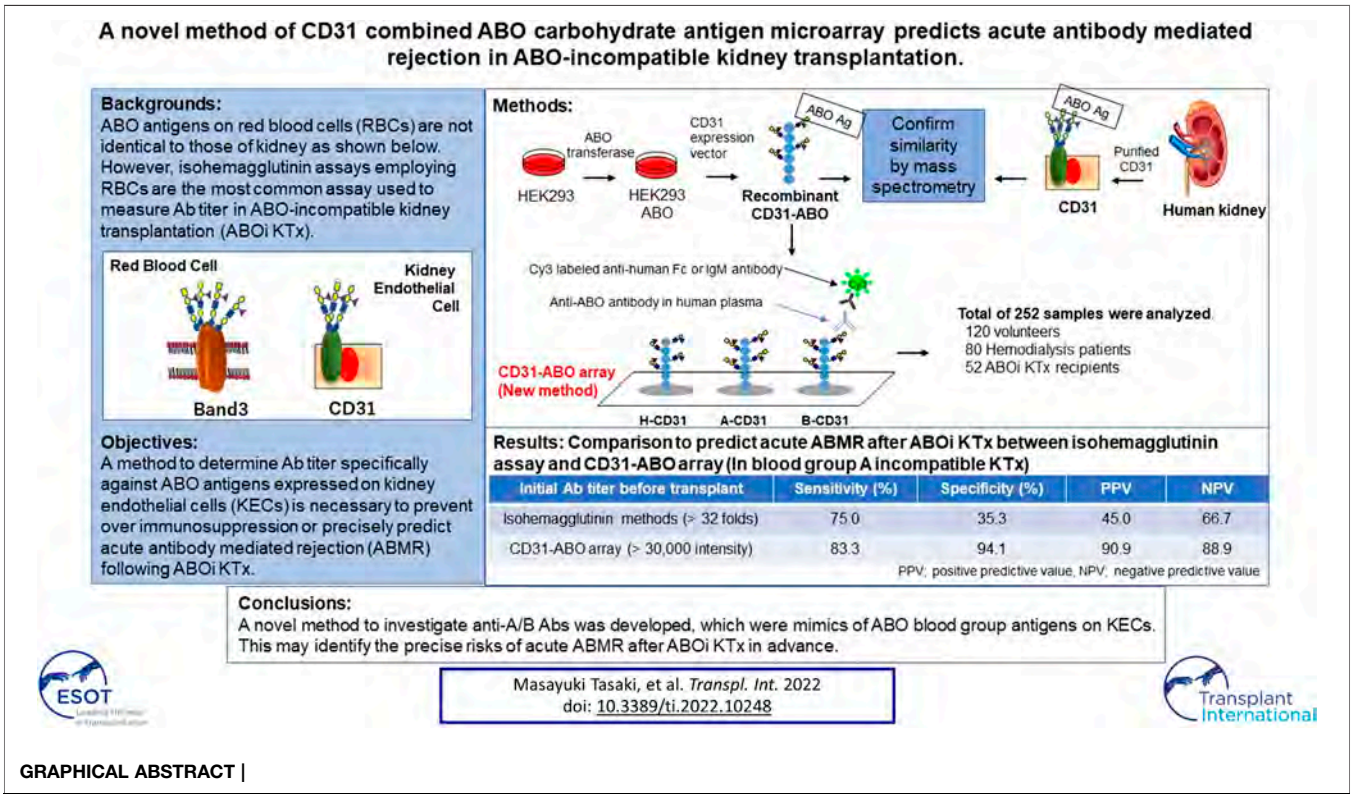
Citation:
Tasaki M, Tateno H, Sato T, Tomioka A, Kaji H, Narimatsu H, Saito K, Nakagawa Y, Aoki T, Kamimura M, Ushiki T, Okada M, Miwa Y, Hotta K, Yoshida Y, Takahashi K and Tomita Y (2022) A Novel Method of CD31-Combined ABO Carbohydrate Antigen Microarray Predicts Acute Antibody-Mediated Rejection in ABO-Incompatible Kidney Transplantation. Transpl Int 35:10248. doi: 10.3389/ti.2022.10248

Isohemagglutinin assays employing red blood cells (RBCs) are the most common assays used to measure antibody titer in ABO-incompatible kidney transplantation (ABOi KTx). However, ABO antigens expressed on RBCs are not identical to those of kidney and antibody titers do not always correlate with clinical outcome. We previously reported that CD31 was the main protein linked to ABO antigens on kidney endothelial cells (KECs), which was different from those on RBCs. We developed a new method to measure antibody titer using a microarray of recombinant CD31 (rCD31) linked to ABO antigens (CD31-ABO microarray). Mass spectrometry analysis suggested that rCD31 and native CD31 purified from human kidney had similar ABO glycan. To confirm clinical use of CD31-ABO microarray, a total of 252 plasma samples including volunteers, hemodialysis patients, and transplant recipients were examined. In transplant recipients, any initial IgG or IgM antibody intensity >30,000 against the donor blood type in the CD31-ABO microarray showed higher sensitivity, specificity, positive predictive value, and negative predictive value of AABMR, compared to isohemagglutinin assays. Use of a CD31-ABO microarray to determine antibody titer specifically against ABO antigens expressed on

Abbreviations: AABMR, acute antibody-mediated rejection; ABOi, ABO-incompatible; ABOc, ABO-compatible; HEK, human embryonic kidney; KEC, kidney endothelial cell; KTx, kidney transplantation; MS, mass spectrometry; NPV, negative predictive value; PPV, positive predictive value; RBC, red blood cell; rCD31, recombinant CD31 protein; ROC, receiver operating characteristic.

KECs will contribute to precisely predicting AABMR or preventing over immunosuppression following ABOi KTx.

Keywords: antibody-mediated rejection, ABO-incompatible kidney transplantation, antibody titer, CD31, microarray



GRAPHICAL ABSTRACT |

INTRODUCTION

In most countries, a paired donation program to circumvent the immunological challenge of ABO incompatibility is precluded by law. Therefore, a kidney transplant candidate with an ABO-incompatible (ABOi) living donor has a valuable option to wait for a deceased ABO-compatible donor with long-term dialysis therapy. Recent cohort studies have shown no significant difference in patient and graft survival in ABOi kidney transplantation (KTx) compared to ABO-compatible (ABOc) KTx (1–5). However, recent meta-analysis has shown lower patient and graft survival in ABOi KTx than ABOc KTx (6, 7). In ABOi KTx, over immunosuppression, leading to life-threatening infections, may cause lower patient survival (6, 7). In addition, acute antibody-mediated rejection (AABMR), due to anti-A or -B antibodies (Abs), contributes to lower graft survival (6, 7). Ab titers against donor blood group antigen may be an AABMR predictor following ABOi KTx, and tailored desensitization therapy according to Ab titer may avoid over immunosuppression (8). However, an acceptable Ab titer against donor blood group antigen to prevent AABMR has not been defined in ABOi KTx. In addition, the desensitization therapy protocol varies from institution to institution and the method to measure Ab titer is not unified.

Technological advances in HLA laboratory testing undoubtedly improved the sensitivity and specificity of HLA Ab assessment. Multiple methodologies such as complement-depending cytotoxicity test, flow cytometry, and Luminex-based technology can be available for HLA Abs test. The understanding of complement (C1q and C3d, etc) fixing Abs and IgG subclass in HLA Abs has become widespread. In contrast, Ab test against ABO antigens in ABOi organ transplantation is still primitive. Isohemagglutinin assays employing red blood cells (RBCs) are the most common assay used to measure Ab titer in ABOi KTx. However, ABO blood group antigens expressed on RBCs are not identical to those of the kidney due to different proteins linked to ABO carbohydrate antigens (9). Ab epitopes against ABO blood group antigens may differ between RBCs and endothelial cells (10). In some cases, Ab titers do not correlate with clinical outcome; AABMR does not occur in some patients with high Ab titers, and vice versa (11–13). A method to determine Ab titer specifically against ABO blood group antigens expressed on kidney endothelial cells (KECs) is necessary to prevent over immunosuppression or precisely predict AABMR following ABOi KTx.

Pecam1 (CD31) is the most abundant protein linked to ABO blood group antigens on KECs, which is different from Band3 mainly expressed on RBCs (9). Here, a new method was

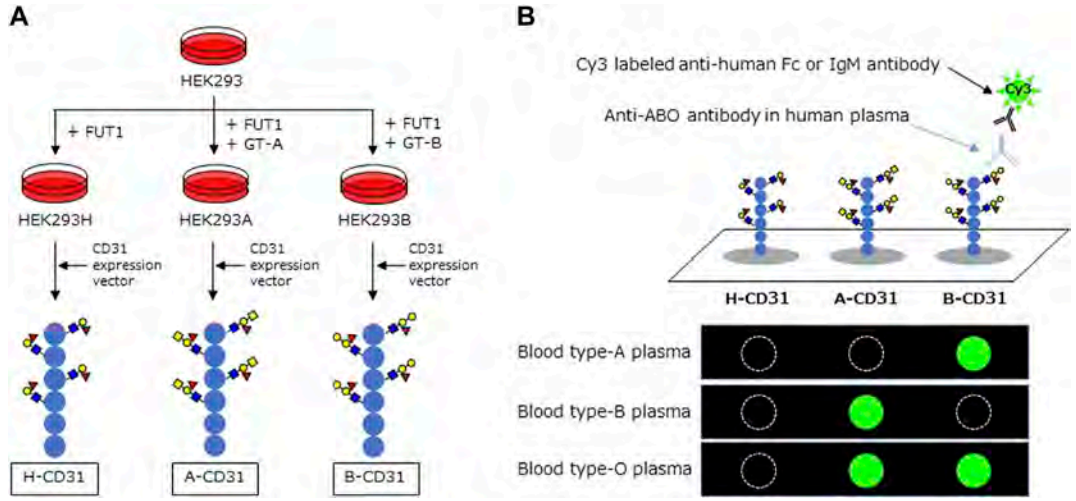


FIGURE 1 | Schema of CD31 linked to ABO carbohydrate antigen microarray. **(A)** The development of recombinant CD31 proteins containing ABO carbohydrate antigen. **(B)** Analyzing anti-A and B antibodies levels in CD31-ABO microarray. HEK: human embryonic kidney, FUT1: α1,2 fucosyltransferase, GT-A: α1,3 *N*-acetylgalactosaminyltransferase, GT-B: α1,3 galactosyltransferase.

developed to measure Ab titer using a microarray of CD31 linked to ABO carbohydrate antigens (CD31-ABO microarray) which is a mimic of ABO blood group antigens on KECs. This novel method may precisely predict AABMR following ABOi KTx.

MATERIALS AND METHODS

Sample and Data Collection

A total of 252 plasma samples were collected. Volunteers (n = 120) donated blood samples at the Japan Red Cross blood center. Approval for this study was obtained from the Japanese Red Cross Institutional Review Board (authorization number 28J0001). Samples were donated without personal identifiers. The only available demographic factor was ABO blood type for these samples. Other plasma samples were collected from patients undergoing hemodialysis (n = 80) and recipients (n = 52) who received ABOi KTx at the Niigata University Medical and Dental Hospital, Nagoya Daini Red Cross Hospital, and Hokkaido University Hospital, Japan. All participants in this study were Japanese. All transplantations were living-donor KTx. Clinical and laboratory information was extracted from electronic databases and patients' medical records. Transplant recipients were divided into two groups: patients without AABMR (-) and with AABMR (+) due to anti-A or B Abs after ABOi KTx. The study was performed in accordance with the guidelines of the Declaration of Helsinki, subsequent to approval from the hospital's Institutional Ethical committee (authorization number 2018-0311).

Anti-ABO Ab Isohemagglutinin Titers

Titration of anti-A and anti-B Abs were performed using the test tube method, as described in detail in the **Supplementary Methods**.

Immunosuppression for ABOi KTx

Immunosuppression therapy was performed according to the protocol at each institution, as described in detail in the **Supplementary Methods**. Plasma exchange or double-filtration plasmapheresis was performed before ABOi KTx to decrease Ab titers. Splenectomy was performed on the day of ABOi KTx before 2003, and rituximab was used after 2004, instead of splenectomy. Calcineurin inhibitors, methylprednisolone, mycophenolate mofetil, and basiliximab were given for induction therapy, with the exception of a few cases.

AABMR Diagnosis

There were no recipients who had donor human leukocyte antigen (HLA) specific performed Abs in this cohort. Whenever a rejection was clinically suspected, an episode biopsy was performed. A rejection diagnosis was made by the pathologist at each institution. AABMR due to anti-A or B Abs was diagnosed using pathological findings of ABMR (Banff19) when anti-donor HLA Abs were not detected at the time of rejection.

Preparation of Recombinant CD31 Containing ABO Carbohydrate Antigens

Recombinant CD31 proteins (rCD31) containing ABO carbohydrate antigens were produced in glycogene-modified human embryonic kidney (HEK293) cells. H-type glycan-expressing cells were established by overexpression of α1,2 fucosyltransferase (*FUT1*) into HEK293 cells; the resulting cells were designated HEK293H. A-type glycan- and B-type glycan-expressing cells were established by overexpression of α1,3 *N*-acetylgalactosaminyltransferase (*GT-A*), and α1,3 galactosyltransferase (*GT-B*) into HEK293H, respectively, and

designated HEK293A and HEK293B. The cDNA encoding the extracellular domain of CD31 was amplified by polymerase chain reaction using the following primers: Forward: 5′-aagcttcaggATGCAGCCGAGGTGGGCCCA-3′, including the HindIII site and Reverse: 5′-gcggccgcTTCTTCCATGGGGCAAGAATGA-3′, including the NotI site, and cDNA derived from human umbilical vein endothelial cells as a template. An approximately 1.8 kb DNA fragment was amplified and subcloned into the pCRII-blunt vector (Life Technologies). After confirmation of the correct sequence using a Genetic Analyzer 3130xl (Applied Biosystems), the HindIII and NotI fragment was inserted into the pcDNA3.1n-F expression vector, which was modified from pcDNA3.1n(+) (Life Technologies) by introducing the sequence encoding DYKDDDDK and a termination codon. The resulting plasmid, designated pcDNA3.1n-CD31-F, was transfected into HEK293H, HEK293A, and HEK293B cells using Lipofectamine LTX (Life Technologies), to produce rCD31 with a FLAG tag at the C-terminus in culture medium. After 48–72 h incubation at 37°C, each medium was collected and rCD31 was purified using an anti-FLAG M2 agarose affinity gel (Sigma-Aldrich). The culture medium (300 ml) was mixed with 500 µL suspension of anti-FLAG M2 agarose affinity gel and rotated slowly at 4°C for several hours. After centrifugation, the gel was washed 2–5× with PBS containing 0.01% Tween-20 and rCD31 was eluted from the affinity gel using a FLAG peptide (Sigma-Aldrich). The protein concentration of purified rCD31 was determined using a NanoDrop LITE spectrophotometer (Thermo Scientific) and was designated H-CD31, A-CD31, and B-CD31, respectively (**Figure 1A**).

Preparation of CD31 Proteins From Human Kidneys

Kidney tissues were obtained from patients, with their informed consent, who underwent surgical nephrectomy due to renal carcinoma at the Niigata University Medical and Dental Hospital. Proteins were extracted from normal kidney cortices of patients with different ABO blood types and CD31 proteins were purified, as described in detail in the supplementary materials and methods. Protein extracts were incubated with Dynabeads protein G (VERITAS) pre-bound to anti-CD31 Ab (Santa Cruz Biotechnology). Dynabeads were thoroughly washed with lysis solution and eluted with sodium dodecyl sulfate (SDS) sample buffer. The eluates with SDS sample buffer were separated by SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE gel pieces containing CD31 protein with molecular mass approximately 130 kDa were excised for mass spectrometry (MS).

Mass Spectrometry Analyses of CD31 Glycopeptides

Identification of *N*-glycosylated Asn sites and site-specific analysis of glycan compositions and structures for both the rCD31 and CD31 proteins from human kidneys were conducted using the IGOT (14) and Glyco-RIDGE (15) methods, respectively. CD31 proteins were digested with Lysyl endopeptidase and trypsin. The

TABLE 1 | The results of anti-A antibodies in A-CD31 microarray compared to isohemagglutinin assay, median (range).

		Anti-A Ab (IgG)			Anti-A Ab (IgM)		
		Healthy volunteers (*n* = 120)	Hemodialysis patients (*n* = 80)	*p*-value	Healthy volunteers (*n* = 120)	Hemodialysis patients (*n* = 80)	*p*-value
O	microarray	58199 (3235–62235)	62146 (5810–65535)	0.596	14666 (1572–59954)	12344 (1819–29384)	0.268
	isohemagglutinin	48 (4–512)	64 (8–1024)	0.344	24 (4–64)	16 (4–64)	0.128
A	microarray	0 (0–224)	0 (0–1548)	0.382	0 (0–0)	0 (0–445)	0.314
	isohemagglutinin	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A
B	microarray	4924 (0–22223)	6687.5 (1436–54723)	0.243	12967 (489–55322)	17157.5 (2859–59642)	0.403
	isohemagglutinin	1 (1–16)	2 (1–32)	0.101	16 (4–128)	8 (4–64)	0.639
AB	microarray	0 (0–891)	0 (0–98)	0.571	0 (0–878)	0 (0–0)	0.791
	isohemagglutinin	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A

N/A, not applicable.

TABLE 2 | The results of anti-B antibodies in B-CD31 microarray compared to isohemagglutinin assay, median (range).

		Anti-B Ab (IgG)			Anti-B Ab (IgM)		
		Healthy volunteers (*n* = 120)	Hemodialysis patients (*n* = 80)	*p*-value	Healthy volunteers (*n* = 120)	Hemodialysis patients (*n* = 80)	*p*-value
O	microarray	32564 (0–65416)	17311 (1677–65244)	0.575	4877 (201–61871)	3466 (341–62727)	0.339
	isohemagglutinin	48 (1–512)	32 (1–512)	0.923	16 (4–64)	16 (2–64)	0.862
A	Microarray	173 (0–7421)	89 (0–10772)	0.883	4346 (0–28812)	4422 (0–31820)	0.634
	isohemagglutinin	1 (1–4)	1 (1–8)	0.164	12 (2–32)	8 (2–64)	0.672
B	Microarray	0 (0–614)	0 (0–1614)	0.791	0 (0–0)	0 (0–2762)	0.134
	isohemagglutinin	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A
AB	Microarray	0 (0–1271)	0 (0–1)	0.382	0 (0–2893)	0 (0–572)	0.837
	isohemagglutinin	0 (0)	0 (0)	N/A	0 (0)	0 (0)	N/A

N/A, not applicable.

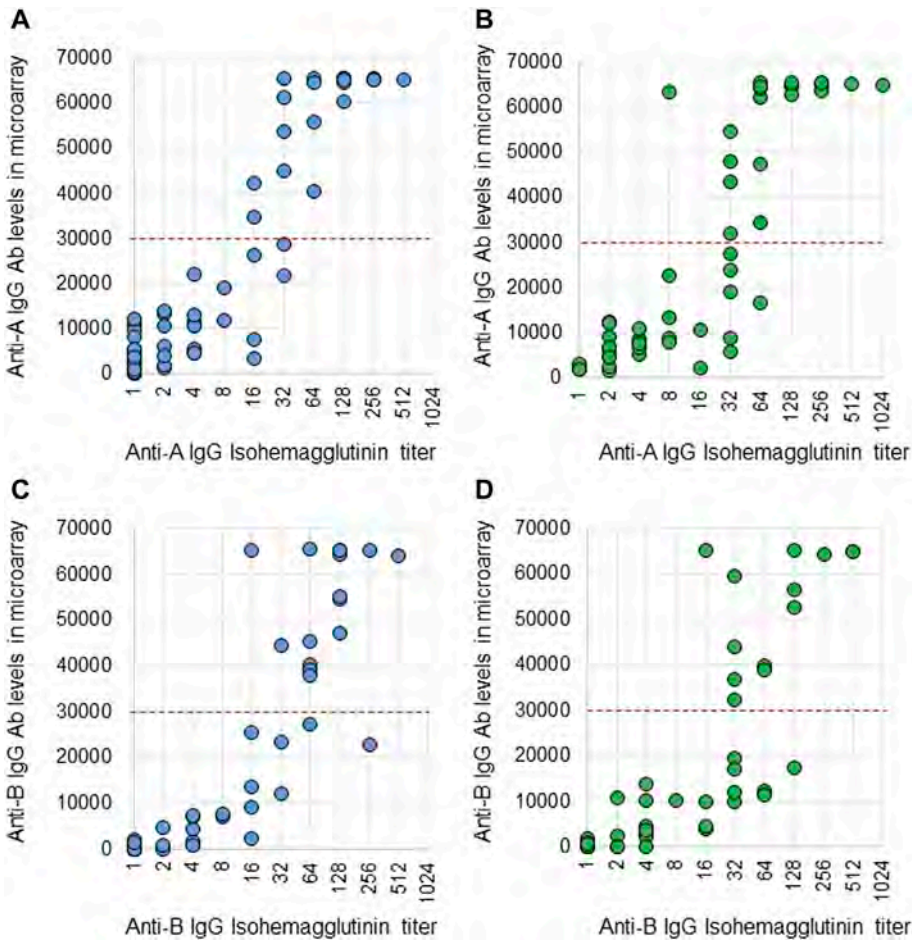


FIGURE 2 | Comparison of anti-A and B IgG Abs between the isohemagglutinin and CD31-ABO microarray methods in volunteers and hemodialysis patients. **(A)** Anti-A IgG Ab in volunteers. **(B)** Anti-A IgG Ab in hemodialysis patients. **(C)** Anti-B IgG Ab in volunteers. **(D)** Anti-B IgG Ab in hemodialysis patients. The x-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph. The y-axis is antibody level on the CD31-ABO microarray. The red dot-line shows the cut-off levels of the CD31-ABO microarray for predicting AABMR.

digests were separated by hydrophilic interaction chromatography on Amide-80 column (TOSOH) to collect glycopeptides. An aliquot of glycopeptide was treated with peptide-*N*-glycanase in $H_2{}^{18}O$ to remove *N*-glycan and label the glycosylated Asn residues with ^{18}O for identification of *N*-glycosylated sites (IGOT method). Another aliquot of glycopeptides was heated in 0.1% trifluoroacetic acid to

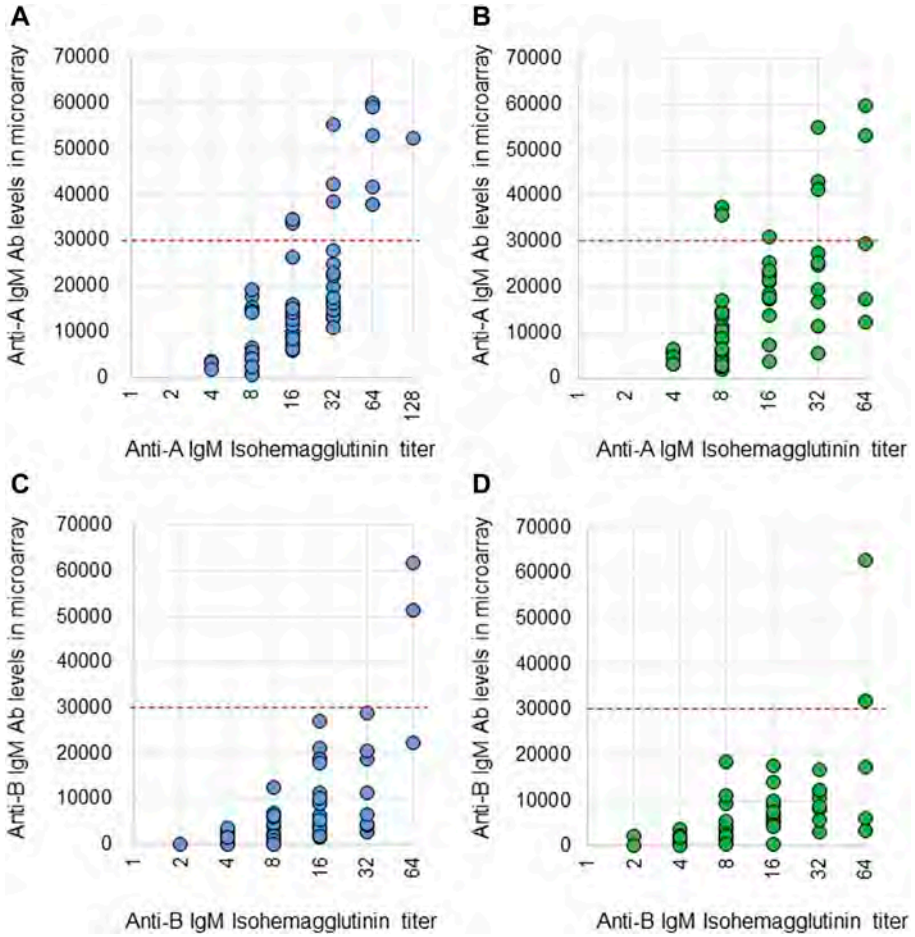


FIGURE 3 | Comparison of anti-A and B IgM Abs between the isohemagglutinin and CD31-ABO microarray methods in volunteers and hemodialysis patients. **(A)** Anti-A IgM Ab in volunteers. **(B)** Anti-A IgM Ab in hemodialysis patients. **(C)** Anti-B IgM Ab in volunteers. **(D)** Anti-B IgM Ab in hemodialysis patients. The x-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph. The y-axis is antibody level on the CD31-ABO microarray. The red dot-line shows the cut-off levels of the CD31-ABO microarray for predicting AABMR.

remove sialic acids. The deglycosylated or desialylated glycopeptides were analyzed using a nano-flow liquid chromatography-coupled Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Desialylated glycopeptides were analyzed for their site-specific glycan compositions and partial structure using the Glyco-RIDGE method. For more experimental details, refer to the supplementary methods.

Microarray of CD31 Linked to ABO Carbohydrate Antigens (CD31-ABO Microarray)

The CD31-ABO microarray was produced as described previously (detail in the **Supplementary Methods**) (16). rCD31 containing ABO carbohydrate antigens (H-CD31, A-CD31, and B-CD31) were dissolved at a concentration of 0.1 mg/ml in a spotting solution (Matsunami Glass) and spotted onto epoxysilane-coated glass slides (Schott) in triplicate using a non-contact microarray printing robot (Microsys4000, Genomic Solutions). The glass slides were incubated at 25°C overnight to allow immobilization, washed with probing buffer, and incubated with the blocking reagent at 20°C for 1 h. Finally, glass slides were washed with TBS containing 0.02% NaN3 and stored at 4°C until use.

Human plasma (80 µL/well) was diluted 100-fold with probing buffer and incubated with the CD31-ABO microarray at 20°C overnight. After washing twice with 100 µL/well probing buffer, 1 µg/ml Cy3-conjugated goat anti-human Fc (Jackson ImmunoResearch: 109-165-098) or Cy3-conjugated goat anti-human IgM (Jackson ImmunoResearch: 109-165-043) were added and incubated at 20°C for 1 h. Fluorescence images were acquired using an evanescent field-activated fluorescence scanner Bio-REX Scan200 (Rexxam). The fluorescence signal of each spot was quantified using Array Pro Analyzer version 4.5 (Media Cybernetics), and background values were subtracted. Background values were obtained from an area without immobilized samples (**Figure 1B**). Anti-A and B Ab levels

TABLE 3 | Patients' demographics and clinic characteristics in ABOi KTx patients.

	ABOi KTx w/o AABMR (*n* = 31)	ABOi KTx with AABMR (*n* = 21)	*p*-value
Male, n (%)	22 (71.0)	13 (61.9)	0.556
Age, y.o, median (range)	44 (23–63)	54.0 (14–76)	0.066
Duration of dialysis (M), median (range)	14 (0–213)	3 (0–119)	0.896
Donor age, y.o, median (range)	55 (30–69)	58 (38–74)	0.111
ABO incompatible transplantation			
A-incompatible, n (%)	21 (67.7)	15 (71.4)	0.785
B-incompatible, n (%)	7 (22.6)	6 (28.6)	0.842
AB-incompatible, n (%)	3 (9.7)	0 (0.0)	0.060
HLA mismatch[a], mean ± SD	3.2 ± 1.3	3.8 ± 1.5	0.118
Preemptive KTx, n (%)	8 (25.8)	7 (33.3)	0.756
WIT (min), mean ± SD	3.2 ± 1.8	2.7 ± 1.6	0.404
TIT (min), mean ± SD	98.7 ± 50.2	93.6 ± 34.2	0.692
Immunosuppression			
FK, n (%)	20 (71.0)	11 (52.4)	0.405
CyA, n (%)	11 (29.0)	10 (47.6)	0.405
MMF, n (%)	29 (93.5)	20 (95.2)	1.000
AZ, n (%)	2 (6.5)	1 (4.8)	1.000
CPA, n (%)	0 (0.0)	3 (14.3)	0.060
Rituximab, n (%)	28 (90.3)	14 (66.7)	0.069
Splenectomy, n (%)	3 (9.7)	3 (14.3)	0.675
Antibody removal before KTx[b], n (%)	15 (48.4)	21 (100.0)	<0.001
POD at diagnosis of AABMR, median (range)	N/A	5 (0–19)	N/A

ABOi, ABO-incompatible; KTx, kidney transplantation; AABMR, acute antibody mediated rejection; HLA, human leukocyte antigen; WIT, worm ischemic time; TIT, total ischemic time; FK, tacrolimus; CyA, cyclosporine A; MMF, mycophenolate mofeti; AZ, azathioprine; CPA, cyclophosphamide; POD, post-operative days; N/A, not applicable.

[a]*Average number of HLA mismatches for each recipient.*

[b]*The number of patients who received antibody removal before KTx.*

(relative intensity) were calculated as the subtraction of the H-CD31 reaction from the A-CD31 or B-CD31 reaction in each sample.

Statistical Analysis

The continuous variables are expressed as the mean ± standard deviation, and the categorical variables are expressed as N and percentages. A Mann-Whitney *U*-test or student's *t*-test was used to compare two groups of continuous variables, and a chi-square test was used to compare categorical data. The diagnostic potential of the CD31-ABO microarray was determined by calculating the receiver operating characteristic (ROC) curve plotted to evaluate the sensitivity and specificity for predicting AABMR after ABOi KTx. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were used to investigate its accuracy as a diagnostic tool for AABMR after ABOi KTx.

RESULTS

ABO Glycan Analysis of rCD31 and Human Kidney CD31 by MS

We successfully produced rCD31-contained ABH glycans by using HEK293 cells *in vitro* and used them for microarray. We needed to know whether rCD31 in this microarray had similar ABH glycans to those of native human kidney. Using the Glyco-RIDGE method, glycan compositions of two core glycopeptides (VLENSTK, including Asn-453, and EGKPFYQMTSNATQAFWTK, including Asn-551) derived from rCD31 proteins purified from culture media of HEK193H, HEK293A, and HEK293B cells were assigned and compared (**Supplementary Figures S1, S2**). The glycan composition of each signal is shown in **Supplementary Figure S1** as XYZ corresponding to the numbers of Hex, HexNAc, and dHex (Fucose) on the tri-mannosyl core (Man3GlcNAc2 = 000). The compositions containing multiple fucoses are shown in blue. These glycopeptides are presumed to have blood type glycans, since one fucose at least is attached on non-reducing terminus. Characteristic or increased compositions in blood type A or B are shown in each spectrum with triangles. In **Supplementary Figure S2**, generation of blood type glycans is suggested clearly. For example, 232, significant in type H, seemed to be shifting to 242 of type A, and to 332 of type B, suggesting the generation of type A and type B antigens, respectively. CD31 prepared by immunoprecipitation from the normal parts of human kidney extract followed by SDS-PAGE was analyzed by the same way as rCD31 (**Supplementary Figure S3**). Accumulated spectra of the glycopeptide (VLENSTK containing Asn-453) of each blood type are compared. Signals assigned to the CD31 glycopeptide are marked with their compositions and the MS/MS spectra of signals marked with red triangle were compared (**Supplementary Figures S4–S6**). All MS/MS spectra show the presence of core fucose and glycan-derived signals such as Hex(1)HexNAc(1) Fuc(1), Hex(1)HexNAc(2)Fuc(1), and Hex(2)HexNAc(1) Fuc(1), suggesting the presence of blood group antigens of blood type H, A, and B (for more details see "**Supplementary**

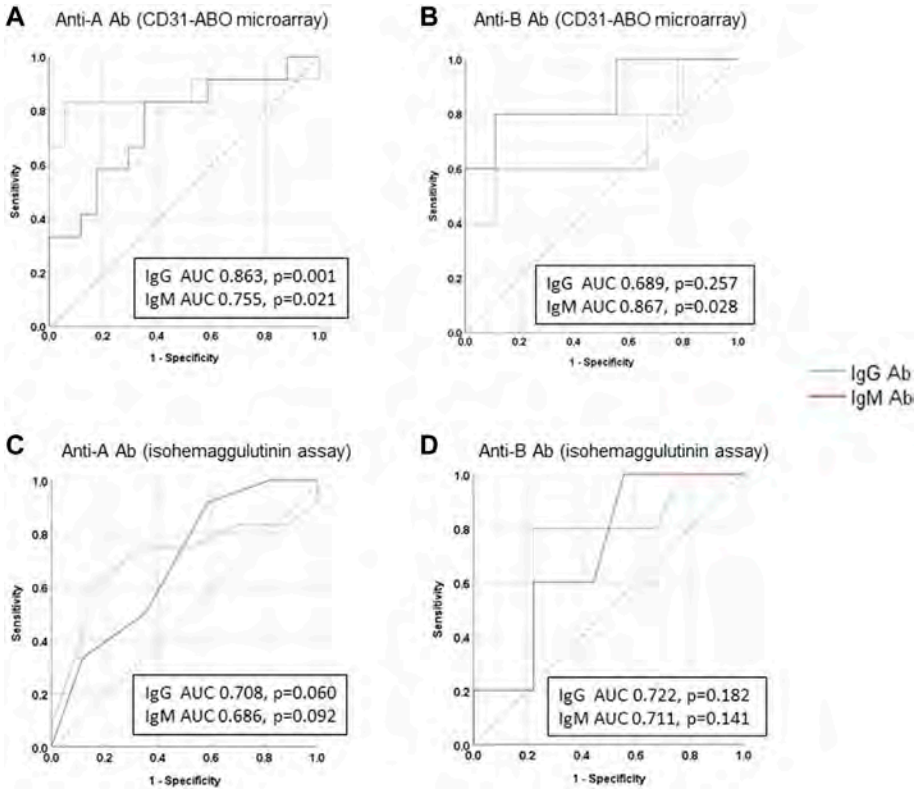


FIGURE 4 | Receiver operating characteristics (ROC) curve analysis for predicting AABMR based on initial anti-A and B Ab. **(A)** anti-A Ab in CD31-ABO microarray, **(B)** anti-B Ab CD31-ABO microarray, **(C)**: anti-A Ab in isohemaggulutinin method, **(D)** anti-B Ab in isohemaggulutinin method.

TABLE 4 | Comparison of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of AABMR after ABOi KT.

Initial Ab titers or levels against donor blood type	Sensitivity (%)	Specificity (%)	PPV	NPV
Anti-A Ab (any of IgG or IgM) in all cases				
≧16 folds by isohemagglutinin	91.7	17.7	44.0	75.0
≧32 folds by isohemagglutinin	75.0	35.3	45.0	66.7
≧64 folds by isohemagglutinin	75.0	58.8	56.3	76.9
≧15,000 by microarray	83.3	52.9	55.6	81.8
≧30,000 by microarray	83.3	94.1	90.9	88.9
Anti-B Ab (any of IgG or IgM) in All cases				
≧16 folds by isohemagglutinin	80.0	33.3	40.0	75.0
≧32 folds by isohemagglutinin	80.0	66.7	57.1	85.7
≧64 folds by isohemagglutinin	60.0	66.7	75.0	66.7
≧15,000 by microarray	80.0	66.7	57.1	85.7
≧30,000 by microarray	60.0	100.0	100.0	81.8
Anti-A Ab (any of IgG or IgM) in Rituximab-use patients				
≧16 folds by isohemagglutinin	100	17.7	35.3	100
≧32 folds by isohemagglutinin	100	35.3	54.6	100
≧64 folds by isohemagglutinin	100	58.8	46.2	100
≧15,000 by microarray	100	52.9	42.9	100
≧30,000 by microarray	100	94.1	85.7	100
Anti-B Ab (any of IgG or IgM) in Rituximab-use patients				
≧16 folds by isohemagglutinin	100	33.3	40.0	100
≧32 folds by isohemagglutinin	100	66.7	57.1	100
≧64 folds by isohemagglutinin	75	66.7	57.1	100
≧15,000 by microarray	100	66.7	57.1	100
≧30,000 by microarray	75	100	100	90.0

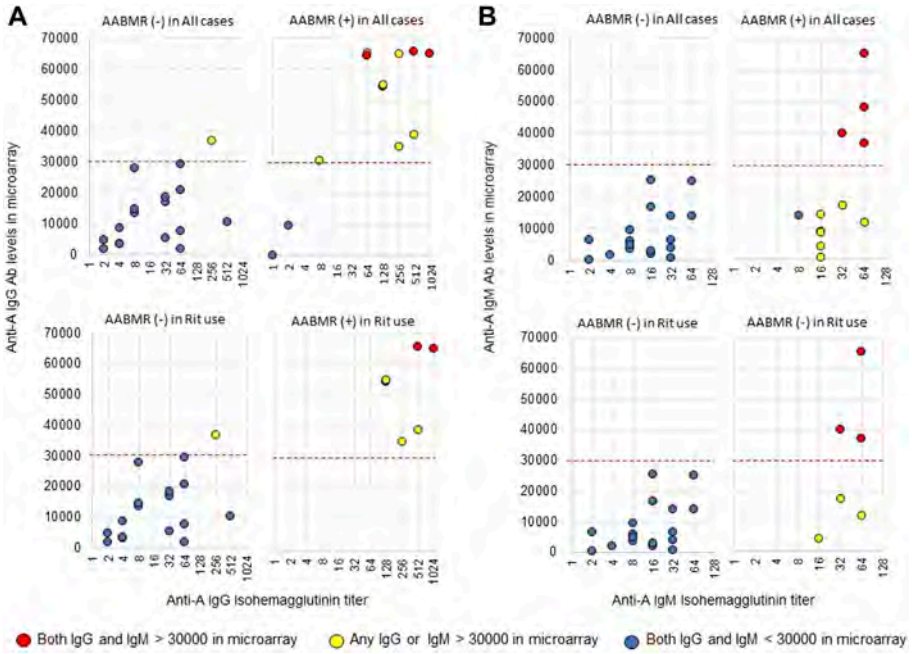


FIGURE 5 | Comparison of anti-A IgG and IgM Abs between the isohemagglutinin and CD31-ABO microarray methods in blood group A-incompatible KTx patients. All samples were collected before desensitization therapy for ABOi KTx. **(A)** Anti-A IgG Ab. **(B)** Anti-A IgM Ab. Upper and lower figures are the results from all patients and rituximab (Rit)-used patients, respectively. Red circles are the results in patients who had both IgG and IgM antibody levels >30000 in the CD31-ABO microarray. Yellow circles are the results in patients who had any IgG or IgM antibody levels >30000 in the CD31-ABO microarray. Blue circles are the results in patients who had both IgG and IgM antibody levels <30000 in the CD31-ABO microarray. The x-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph. The y-axis is antibody level on the CD31-ABO microarray. AABMR, acute antibody-mediated rejection.

Results"). Taken together, rCD31 used for the CD31-ABO microarray had the glycopeptide (VLENSTK) conjugated to the blood group H, A, and B glycan, and CD31 derived from human kidney had the same glycopeptide, which was strongly suggested to have blood group H, A, and B glycans.

Anti-A and B Abs in Volunteers and Hemodialysis Populations

The results of Ab levels measured using the CD31-ABO microarray are shown in **Tables 1**, **2**. These microarrays specifically detected anti-A and anti-B Abs. Anti-A and B Ab levels were not significantly different between volunteers and hemodialysis populations. Both anti-A and B IgG Ab levels were significantly higher in the type O population than those in the type B and A populations, respectively ($p < 0.01$). However, anti-A and B IgM Ab levels were not significantly different between the type O and type B, and type O and type A populations, respectively. We analyzed the same samples by using the isohemagglutinin method (**Tables 1**, **2**), showing a similar trend as CD31-ABO microarray.

Anti-A and B Abs were compared between these two methods. Ab titers in the isohemagglutinin method and Ab levels in the CD31-ABO microarray were roughly correlated in volunteers and the hemodialysis population (**Figure 2**, **3**). However, Ab levels in the CD31-ABO microarray varied even in samples with the same isohemagglutinin titers.

Patient Characteristics

Table 3 shows the patient characteristics in the two groups divided by the existence of AABMR after ABOi KTx. There were no significant differences, except for Ab removal therapy before ABOi KTx. Ab titers before desensitization therapy and on the day of ABOi KTx against donor blood type measured using the isohemagglutinin method were not significantly different between the two groups (data not shown). The median post-operative day at diagnosis of AABMR was 5 (range; 0–19).

Prediction of AABMR After ABOi KTx Using anti-A and B Abs by the CD31-ABO Microarray

The area under the receiver operating characteristic (ROC) curve (AUC) indicated the significant prognostic power for AABMR after ABOi KTx using initial Ab levels measured by the CD31-ABO microarray, except for anti-B IgG Ab (**Figures 4A,B**). The prognostic power in the CD31-ABO array was better than those of isohemaggulutinin assay (**Figures 4C,D**). **Table 4** shows the comparison of the prognostic power for AABMR with several cut-offs, suggesting the CD31-ABO microarray had higher prognostic power for AABMR than isohemagglutinin method. Any initial IgG or IgM Ab levels against donor blood type >30,000 in the CD31-ABO microarray showed high sensitivity,

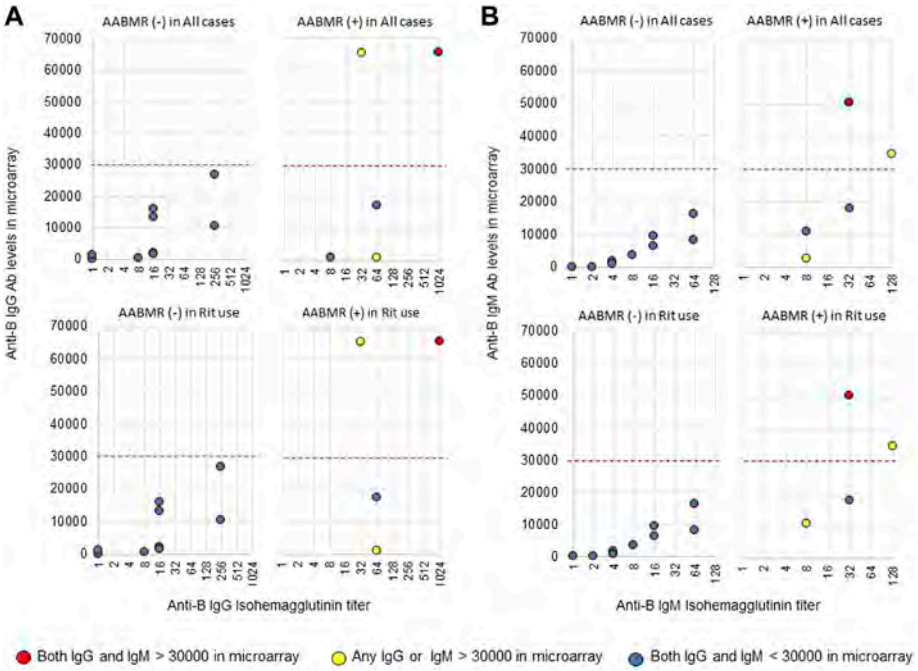


FIGURE 6 | Comparison of anti-B IgG and IgM Abs between the isohemagglutinin and CD31-ABO microarray methods in blood group B-incompatible KTx patients. All samples were collected before desensitization therapy for ABOi KTx. **(A)** Anti-B IgG Ab. **(B)** Anti-B IgM Ab. Upper and lower figures are the results from all patients and rituximab (Rit)-used patients, respectively. Red circles are the results in patients who had both IgG and IgM antibody levels >30000 in the CD31-ABO microarray. Yellow circles are the results in patients who had any IgG or IgM antibody levels >30000 in the CD31-ABO microarray. Blue circles are the results in patients who had both of IgG and IgM antibody levels <30000 in the CD31-ABO microarray. The x-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph. The y-axis is antibody level on the CD31-ABO microarray. AABMR, acute antibody-mediated rejection.

specificity, positive predictive value (PPV), and negative predictive value (NPV) in both anti-A and anti-B Abs. After excluding the patients whose rituximab was not used, these significant results could be seen in rituximab-based protocol patients (**Table 4**). To investigate whether Ab levels in the CD31-ABO microarray would more accurately predict AABMR after ABOi KTx than isohemagglutinin method, initial anti-A and B Abs of the samples obtained before desensitization therapy were compared (Upper **Figures 5**, **6**). In A-incompatible KTx, anti-A IgG Ab levels by microarray were significantly higher in the AABMR (+) group than those in the AABMR (-) group (median: 54721 vs. 10211, $p < 0.001$). Ten out of 12 patients with AABMR (83.3%) had anti-A IgG Ab levels >30,000 in the CD31-ABO microarray; in contrast, only 1 out of 17 patients without AABMR (5.9%) had anti-A IgG Ab levels >30,000 (upper **Figure 5A**). Anti-A IgM Ab levels in the CD31-ABO microarray were significantly higher in the AABMR (+) group than those in the AABMR (-) group (median: 14277.5 vs. 5887, $p = 0.03$). No one had anti-A IgM Ab levels >30,000 in the AABMR (-) group, in contrast, 4 out of 12 patients with AABMR had anti-A IgM Ab levels >30,000 in the microarray (upper **Figure 5B**). Eight out of 12 patients with AABMR had anti-A IgM Ab levels <30,000 in the CD31-ABO microarray. However, six of these samples had anti-A IgG Ab levels >30,000 by microarray; probably, these anti-A IgG Abs induced AABMR in these patients (yellow circles in upper **Figure 5B**). Taken together, 10 out of 12 patients with AABMR (83.3%) had initial anti-A IgG or IgM Ab levels >30,000 in A-incompatible KTx, as shown by the CD31-ABO microarray. When we analyzed the predictive value of AABMR in the rituximab-based protocol patients, 6 out of 6 patients (100%) had anti-A IgG or IgM Ab levels >30,000 in A-incompatible KTx (Lower **Figures 5A,B**). **Figure 6** shows anti-B Abs in patients undergoing B-incompatible KTx. Anti-B IgG Ab levels in the CD31-ABO microarray were significantly higher in the AABMR (+) group than those in the AABMR (-) group (median: 16378 vs. 1970, $p = 0.047$). Anti-B IgM Ab levels in the CD31-ABO microarray were significantly higher in the AABMR (+) group than those in the AABMR (-) group (median: 18058 vs. 3481, $p = 0.021$). No one had anti-B IgG and IgM Ab levels >30,000 using the CD31-ABO microarray in the AABMR (-) group (Upper **Figures 6A,B**). Three out of 5 patients with AABMR (60.0%) had initial anti-B IgG or IgM Ab levels >30,000 in B-incompatible KTx, as shown by the CD31-ABO microarray. When we analyzed the predictive value of AABMR in the rituximab-based protocol patients, 3 out of 4 patients (75%) had anti-B IgG or IgM Ab levels >30,000 in B-incompatible KTx (Lower **Figures 6A,B**).

Samples obtained after ABOi KTx were also investigated (**Supplementary Figures S7, S8**). The timing of plasma sample collection was different in each case. In patients without AABMR, the samples were collected within 1 month after ABOi KTx. Plasma samples were collected when AABMR

was clinically suspected, but before treatment. Four out of 16 cases (25%) had anti-A IgG or IgM Abs >30,000 in A-incompatible KTx, as shown by the CD31-ABO microarray (**Supplementary Figure S7**). One out of 5 cases (20%) had anti-B IgG or IgM Abs >30,000 in B-incompatible KTx, as shown by the CD31-ABO microarray (**Supplementary Figure S8**). However, there were no significant differences between the AABMR (+) and AABMR (-) groups in levels of anti-A and -B Abs examined by both the isohemagglutinin and CD31-ABO microarray methods. We showed how Ab levels changed before and after ABOi KTx in **Supplementary Figure S9**. Ab titers by isohemagglutinin method before desensitization were not significantly different between AABMR (+) and AABMR (-). However, CD31-ABO microarray could show that they were significantly higher in AABMR (+) than ABMR (-) before desensitization therapy. As described above, Ab titers after ABOi KTx were not significantly different between AABMR (+) and AABMR (-) in either of the two methods.

DISCUSSION

To evaluate the risk of AABMR in patients undergoing ABOi transplants, anti-A or -B Ab titers are required. There are several methods to measure anti-A and -B Ab titers, such as the tube test assay (17), the column agglutination technique (18), flow cytometry (19, 20), and the solid phase red cell adherence technique (21). In these methods, the reaction of Abs against RBCs is used to determine anti-A or -B Ab titers. ABO blood group antigens are expressed on both RBCs and KECs. RBCs are used as targets to investigate anti-A or -B Ab titers from the convenience of use and obtainability before ABOi KTx. Initial anti-A or -B Ab titers against RBCs are a good predictor of AABMR in ABOi KTx (22), suggesting ABO blood group antigens are similar between RBCs and KECs. However, CD31 is major protein linked to ABO carbohydrate antigens in human KECs, and is different from those expressed on RBCs (9). Anti-blood group Ab epitopes against ABO blood group antigens are thought to be different between RBCs and endothelial cells (10). The Ab removal-free protocol has been reported in ABOi KTx when anti-A or -B Ab titers are below 64-fold, resulting in no AABMR (23). In contrast, anti-A or -B Ab induced AABMR and thrombotic microangiopathy in ABOi-KTx remain critical issues (24), and heavier immunosuppression is required. To clarify the risk of AABMR and avoid infectious events due to over immunosuppression after ABOi KTx, the real reaction of anti-A or -B Ab against ABO blood group antigens on KECs needs to be known.

In the present study, a method to evaluate anti-A and -B Abs that react to ABO blood group antigens expressed on KECs was developed. rCD31 proteins containing ABO carbohydrate antigens were used to form the CD31-ABO microarray. ABO glycans were compared between rCD31 used for the CD31-ABO microarray and CD31 derived from normal human kidney by MS analysis, which suggested that the CD31-ABO microarray was a mimic of ABO blood group antigens on human KECs. Anti-A and -B Abs titers were roughly correlated between the isohemagglutinin and CD31-ABO microarray methods. However, there was great variability in anti-A and -B Abs levels in the CD31-ABO microarray among patients who had the same Ab titer using the isohemagglutinin method. The desensitization therapy contents were not significantly different between the two groups of patients with and without AABMR, except for Ab removal. In spite of isohemagglutinin Ab titers using RBCs that were not significantly different between the two groups, the patients who suffered from AABMR had significantly higher Ab levels of the CD31-ABO microarray in AABMR (+). The sensitivity of predicting AABMR in the CD31-ABO microarray was not high in B-incompatible KTx when the cut-off Ab level was >30,000. However, there were no patients who had anti-B Ab levels >30,000 in B-incompatible KTx without AABMR, using the CD31-ABO microarray (the specificity of predicting AABMR was 100%). In this study, we found that Ab levels measured by the CD31-ABO microarray was the most important to predict AABMR after ABOi-KTx.

Ab levels examined by the CD31-ABO microarray were low in the samples obtained when AABMR was clinically suspected. After ABOi KTx, it is possible that anti-A or -B Ab reacted to ABO antigens on graft endothelial cells and were absorbed. The absorption of anti-A or -B Abs could affect plasma Ab levels determined using the CD31-ABO microarray more than the isohemagglutinin method because of the specificity to KECs. Thus, the CD31-ABO microarray might not be a significant tool to predict AABMR after ABOi KTx.

There are limitations to the present study. We do not routinely examine blood group A subtype. However, 99.8% of Japanese people of blood type A belong to A1 (25, 26). The cohort of ABOi KTx consisted of a heterogeneous population who received different immunosuppressive protocols in this study. However, the desensitization therapy protocol for ABOi KTx varies from institution to institution. In a real situation for ABOi KTx, we tried to examine the new method of CD31-ABO microarray on various patients in the present study. The number of samples obtained from patients with ABOi KTx was small, especially B-incompatible KTx. To elucidate the value of the CD31-ABO microarray to predict AABMR in ABOi KTx, further examination using more samples is required. Samples from the day of the ABOi KTx were not stored and could not be investigated by CD31-ABO microarray. It is important to know Ab levels that should be decreased by desensitization therapy before ABOi KTx. A multi-center study using the CD31-ABO microarray is currently ongoing to determine if AABMR may be avoided after ABOi KTx and how much high Ab levels should be decreased before ABOi KTx.

In conclusion, a novel method to investigate anti-A and -B Abs was developed, which were mimics of ABO blood group antigens on KECs. This may identify the precise risks of AABMR after ABOi KTx in advance. As large meta-analysis of ABOi KTx has shown, graft and patient survival in ABOi KTx were significantly worse than those of ABOc KTx (6, 7). They suggest two issues of ABOi KTx: AABMR and infectious

events. According to the results of the CD31-ABO microarray, we will be able to strengthen or reduce desensitization therapy, resulting in decreased numbers of AABMR and infectious events.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethical committee of Niigata University (authorization number 2018-0311). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MT, KS, YN, MO, YM, KH, KT, and YT collected data and samples. HT, TS, and HN performed research (developed CD31-ABO array and analyzed antibody levels). AT and HK performed research and mass spectrometry analysis. TA, MK, and TU performed research (measured isohemagglutinin antibody titers). MT and YY performed research (purified CD31 from human kidney). MT, HT, TS, and HK analyzed data and prepared the manuscript.

FUNDING

This study was supported by JSPS KAKENHI Grant Number 17K11195.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We thank Dr. Yoshihiko Watarai of Nagoya Daini Red Cross Hospital and Dr. Takaaki Kobayashi of Aichi Medical University School of Medicine for donating plasma samples, and Ms. Masako Sukegawa and Dr. Mika Fujita of the National Institute of Advanced Industrial Science and Technology for sample preparation for mass spectrometry. We would like to thank Editage (www.editage.com) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10248/full#supplementary-material

Supplementary Figure S1 | Accumulated mass spectra of glycopeptides composed of a core peptide (VLENSTK including Asn-453) derived from recombinant CD31. Spectrum for **(A)** HEK293H, **(B)** for HEK293A, **(C)** for HEK293B. Glycan compositions (the numbers of Hex, HexNAc, and Fuc, on trimannosyl core) characteristic or increased against H are presented in each spectrum with triangles, and those containing multiple Fuc are in blue. Assignment of signals are summarized in **Supplementary Table S1**.

Supplementary Figure S2 | Accumulated mass spectra of glycopeptides composed of a core peptide (EGKPFYQMTSNATQAFWTK including Asn-551) derived from recombinant CD31. Spectrum for **(A)** HEK293H, **(B)** for HEK293A, **(C)** for HEK293B. Glycan compositions (the numbers of Hex, HexNAc, and Fuc on trimannosyl core) characteristic or increased against H are presented in each spectrum with triangles, and those containing multiple Fuc are in blue. Assignment of signals are summarized in **Supplementary Table S2**. Presumed addition of GalNAc and Gal on H antigen are shown with arrow and predicted glycan structures are shown.

Supplementary Figure S3 | Accumulated mass spectra containing human kidney CD31 glycopeptides; VLENSTK including Asn-453. **(A)** Spectrum for blood type O, **(B)** for blood type A, **(C)**, for blood type B. Signals indicated with glycan compositions (the numbers of Hex, HexNAc, and Fuc on trimannosyl core) are assigned to CD31 peptide having various glycans. MS/MS spectra were compared for signals shown with red triangle (**Supplementary Figures S4–S6**).

Supplementary Figure S4 | MS/MS spectra of glycopeptides derived from the normal parts of human kidney CD31 (type O). MS/MS spectra of glycopeptides assigned as compositions 221, 222, and 332, are compared. The core peptide of selected glycopeptides is common and found to be VLENSTK, from core peptide-related ions, Y0, Y1, and Y2, as indicated with green arrow heads. Y1+F (fucose) signals indicating the attached glycan has core-fucose are shown with orange arrow heads. Glycan-derived fragment ions are indicated with blue arrow heads. One (or two) putative glycan structures is shown on the right side of each spectrum.

Supplementary Figure S5 | MS/MS spectra of glycopeptides derived from the normal parts of human kidney CD31 (type A). MS/MS spectra of selected glycopeptides are compared. The core peptide of the selected glycopeptides is common and found to be the same for **Supplementary Figure S4**, VLENSTK, from core peptide-related ions, Y0, Y1, and Y2. Y1+F (fucose) signals indicating the attached glycan has core-fucose are shown with orange arrow heads. Glycan-derived fragment ions are indicated with blue arrow heads. One (or two) putative glycan structure is shown on the right side of each spectrum.

Supplementary Figure S6 | MS/MS spectra of glycopeptides derived from the normal parts of human kidney CD31 (type B). MS/MS spectra of selected glycopeptides are compared. The core peptide of the selected glycopeptides is common and found to be the same for **Supplementary Figures S4, S5**, VLENSTK, from core peptide-related ions, Y0, Y1, and Y2. Y1+F (fucose) signals indicating the attached glycan has core-fucose are shown with orange arrow heads. Glycan-derived fragment ions are indicated with blue arrow heads. One (or two) of putative glycan structure is shown on the right side of each spectrum.

Supplementary Figure S7 | Comparison of anti-A IgG and IgM Abs between the isohemagglutinin and CD31-ABO microarray methods in blood group A-incompatible KTx patients. All samples were collected after ABOi KTx. In patients with AABMR (+), the plasma samples were collected when AABMR was clinically suspected, but before treatment. **(A)**: Anti-A IgG Ab. **(B)**: Anti-A IgM Ab. Red circles are the results in patients who had both IgG and IgM antibody levels > 30000 in the CD31-ABO microarray. Yellow circles are the results in patients who had any IgG and IgM antibody levels > 30000 in the CD31-ABO microarray. Blue circles are the results in patients who had both IgG and IgM antibody levels < 30000 in the CD31-ABO microarray. The x-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph. The y-axis is antibody level on the CD31-ABO microarray. AABMR: acute antibody-mediated rejection.

Supplementary Figure S8 | Comparison of anti-B IgG and IgM Abs between the isohemagglutinin and CD31-ABO microarray methods in blood group B-incompatible KTx patients. All samples were collected after ABOi KTx. In

patients with AABMR (+), the plasma samples were collected when AABMR was clinically suspected, but before treatment. **(A)**: Anti-B IgG Ab. **(B)**: Anti-B IgM Ab. Blue circles are the results in patients who had both IgG and IgM antibody levels < 30000 in the CD31-ABO microarray. The x-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph. The y-axis is antibody level on the CD31-ABO microarray. AABMR, acute antibody-mediated rejection.

Supplementary Figure S9 | Ab titers' changes before and after ABOi KTx. **(A)**: anti-A Ab titer measured by isohemagglutinin assay, **(B)**: anti-B Ab titer measured by isohemagglutinin assay, **(C)**: anti-A Ab level measured by CD31-ABO microarray, **(D)**: anti-B Ab level measured by CD31-ABO microarray. pre: before desensitization therapy, Tx: on the day of transplantation (Ab levels weren't examined at this point by CD31-ABO microarray), post: on the day of suspicious AABMR [the samples were collected within 1 month after ABOi KTx in the patients of AABMR(-)]. The y-axis is isohemagglutinin titers on a log2 scale; titer values of zero are displayed as 1 on the graph **(A,B)**. The y-axis is antibody level on the CD31-ABO microarray **(C,D)**. AABMR, acute antibody-mediated rejection. $^{*}p < 0.05$.

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ESOT | Transplant International

ORIGINAL RESEARCH
published: 24 March 2022
doi: 10.3389/ti.2022.10344

Delayed Graft Function Under the Microscope: Surveillance Biopsies in Kidney Transplantation

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Delayed graft function (DGF) is a common complication of kidney transplantation and frequently leads to the necessity of surveillance biopsies. The purpose of this study is to describe the histological findings in surveillance biopsies of deceased donor kidney transplant recipients and evaluate the risk factors for graft outcomes. This is a monocentric, retrospective study including kidney transplant recipients that underwent a graft biopsy during the DGF period between January 2006 and July 2019. 356 biopsies were performed in 335 deceased donor transplant recipients. Biopsies were analyzed according to the Banff classification. The main histological findings were: acute tubular necrosis in 150 biopsies (42.1%), acute rejection in 96 biopsies (26.9%), and borderline findings in 91 biopsies (25.5%). In the multivariate analysis, recipient age (p = 0.028) and DGF duration (p = 0.005) were associated with rejection, antibody-induction with anti-thymocyte globulin (ATG) was protective (p = 0.001). The occurrence of rejection was associated with lower death-censored graft survival (log-rank; p = 0.009). Surveillance biopsies of kidney grafts experiencing DGF remain an essential tool for the care of kidney transplant recipients. The recipient's age and duration of DGF are independent risk factors for acute rejection, while antibody-induction therapy with ATG is associated with protection from its occurrence.

Keywords: renal transplantation, delayed graft function, renal biopsy, acute rejection, immunosuppression

OPEN ACCESS

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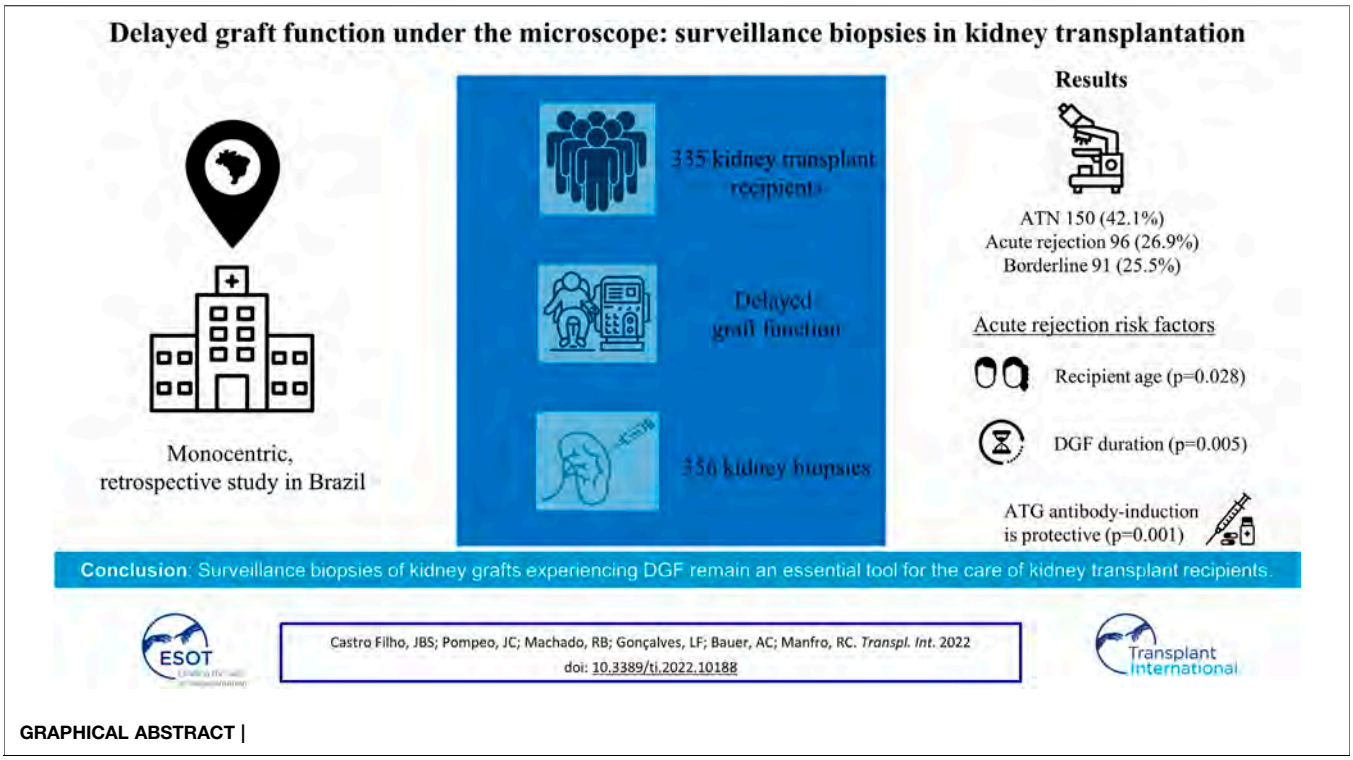
Received: *06 January 2022*
Accepted: *24 February 2022*
Published: *24 March 2022*

Citation:
Castro Filho JBSD, Pompeo JDC, Machado RB, Gonçalves LFS, Bauer AC and Manfro RC (2022) Delayed Graft Function Under the Microscope: Surveillance Biopsies in Kidney Transplantation. Transpl Int 35:10344. doi: 10.3389/ti.2022.10344

INTRODUCTION

Over the last decades, kidney transplantation became an effective lifesaving procedure for a substantial portion of patients with end-stage kidney diseases (1). Besides increasing life expectancy, successful renal transplants offer a better quality of life than renal replacement therapies (2). Between 2010 and 2019, the number of kidney transplants increased by 35% in Brazil. That occurred mostly due to the increment in deceased donor transplantation since the number of living donor transplants is progressively decreasing in this country (3). As compared to transplants from living donors, deceased donor kidney transplantation is associated with a higher incidence of delayed graft function (DGF), which by itself is associated with acute rejection, lower graft survival, and possibly lower patient survival (4, 5).

Delayed graft function is currently most frequently characterized by the need for dialysis within the first week after transplantation (6). It occurs in approximately one-fourth of kidney transplants in



GRAPHICAL ABSTRACT |

Europe and North America but in Brazil, its incidence is much higher (7-9). The increasing age of the deceased donors, the use of organs from expanded criteria donors (ECD), or with high kidney donor profile index (KDPI), which are usually allocated to older recipients, may contribute to increasing its incidence (10, 11). Other known risk factors include prolonged cold ischemia time, type of preservation solution, preservation technique (static versus pulsatile), and the immunosuppressive regimen (12).

During DGF graft injuries may go unnoticed due to the absence of graft functional parameters used for their monitoring and currently, the only reliable diagnostic tool in this setting is the graft surveillance biopsy. Moreover, the incidence of acute rejection is substantially higher in kidney grafts undergoing DGF (13, 14).

Current transplant guidelines recommend graft tissue histologic evaluation every 7–10 days until the graft acquires

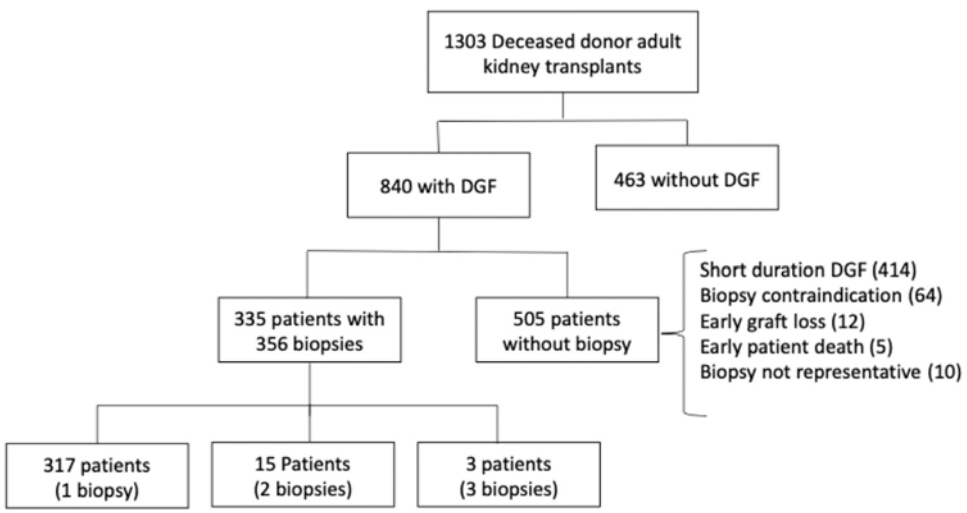


FIGURE 1 | Study outflow.

TABLE 1 | Demographic data of recipients, donors and transplants.

Patients/biopsies (number)	335/356
Donor age (years, mean ± SD)	43.7 ± 16.6
Donor creatinine (mg/dl, median; IQR)	1.40 [0.90–2.20]
Expanded criteria donors (number; %)	116 (34.4%)
Recipient age (years, mean ± SD)	46.1 ± 12.9
Male recipient (number, %)	205 (61.2%)
Recipient ethnicity (Caucasian, number, %)	251 (74.9%)
HLA mismatches (ABDR, median; IQR)	3.00 [3.00–4.00]
Panel reactive antibodies (PRA, I and/or II)	
PRA 0 (both class I and II)	164 (48.9%)
PRA 1–50 (either class I or II)	115 (34.3%)
PRA > 50 (either class I or II)	56 (16.7%)
Donor specific antibodies (yes/no)	57 (18.4%)/253 (81.6%)
Cold ischemia time (hours, mean ± SD)	25.6 ± 5.6
Vascular anastomosis time (minutes, mean ± SD)	27.7 ± 6.6
DGF duration (days, mean ± SD)	26.4 ± 19.9
Dialysis sessions (median; IQR)	8.0 [5.00–13.00]
Transplant number ([1, >1]; number, %)	308 (91.9%/27 (8.1%)
Biopsy postoperative day (mean ± SD)	14.7 ± 8.2
First biopsy postoperative day (*N* = 317; mean ± SD)	12.4 ± 6.1
Second biopsy postoperative day (*N* = 15; mean ± SD)	22.6 ± 7.7
Third biopsy postoperative day (*N* = 3; mean ± SD)	31.6 ± 14.3

DGF, delayed graft function; SD, standard deviation; 95% CI, 95% confidence interval; PRA, panel reactive antibodies; IQR, interquartile range.

function (15, 16). However, such recommendations were made in an era in which the effectiveness of immunosuppressive regimens for the prevention of acute rejection was substantially lower than nowadays (15-17).

The present study aimed to evaluate the utility of surveillance biopsies in uncovering graft injuries, other them those related to ischemia and reperfusion, that would lead to specific treatments, mainly acute cellular rejection and antibody-mediated rejection. We also evaluated the influence of the initial immunosuppressive regimen on the incidence of acute rejection in the surveillance biopsy and patient and graft survivals.

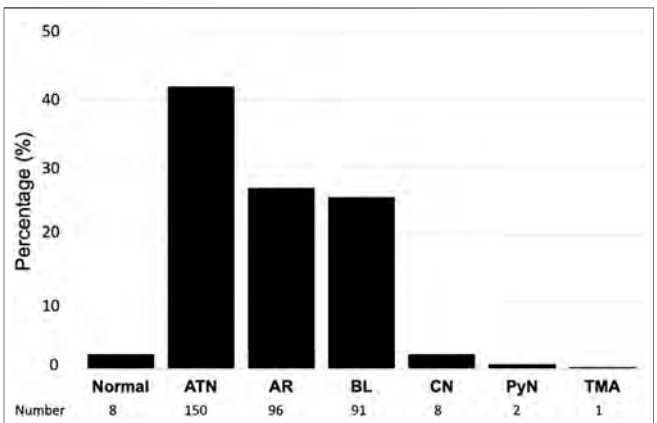


FIGURE 2 | Histological diagnosis in surveillance biopsies of kidney transplant recipients with delayed graft function. ATN, acute tubular necrosis; AR, acute rejection; BL, borderline findings; CN, cortical necrosis; PyN, pyelonephritis; TMA, thrombotic microangiopathy.

MATERIALS AND METHODS

Study Design, Biopsies and Definitions

The study included all adult kidney transplant recipients who received a deceased donor graft, developed DGF, and underwent a surveillance biopsy between January 2006 and July 2019 at Hospital de Clínicas de Porto Alegre, RS, Brazil. We excluded kidney-pancreas and kidney-liver transplant recipients and kidney transplants performed after another solid-organ transplantation. The study flowchart is shown in **Figure 1**. Data were collected through the review of transplant charts and electronic medical records. Donor, recipient, and transplant-related variables were included for analysis.

During the study period, 1,303 brain dead deceased donor kidney transplants were performed and the vast majority of these organs (1,300) were preserved by cold storage. Three hundred and thirty-five patients underwent 356 representative surveillance biopsies and were included in the study. Kidney allograft biopsies were performed at the attending team's discretion every 7–14 days during DGF. Biopsies occurred under real-time ultrasonography guidance, through a semiautomatic gun with a 16-G biopsy needle. A renal pathologist analyzed slides stained with hematoxylin-eosin, periodic acid shift, and Masson's trichrome and interpreted them according to the Banff classification in effect at the time of assessment.

All patients received corticosteroids, calcineurin inhibitors, and mycophenolate as immunosuppressive therapy. Patients that did not receive antibody induction and patients treated with Basiliximab received immunosuppressive drugs at the usual initial doses. Patients treated with anti-thymocyte globulin (ATG) induction, at standard immunological risk, did not receive calcineurin inhibitors until the graft achieved function. Those at high immunological risk received an initially reduced dose. Cellular rejections were treated with corticosteroid pulses or

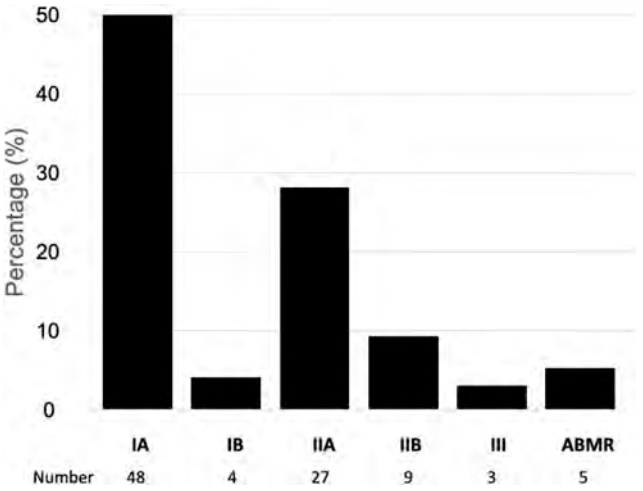


FIGURE 3 | Banff classification of rejection in kidney transplant recipients with delayed graft function. ABMR, antibody mediated acute rejection.

ATG if scored Banff 2A or higher, antibody-mediated rejections were treated with plasmapheresis and polyclonal IV immunoglobulins. Treatment of patients with borderline findings on the surveillance biopsy was decided by the attending team based on the estimated risk of rejection.

Delayed graft function was defined by the requirement of at least one dialysis session during the first week after transplantation (6, 13). DGF duration was recorded from the day of transplantation to the day of the last dialysis session. Expanded criteria donors were defined according to the UNOS criteria (18, 19).

The study was approved by the Ethics and Research Committee of the Hospital de Clínicas de Porto Alegre approved the study (protocol number 64239617.4.0000.5327). The clinical and research activities being reported are in accordance with the ethical standards of the Declaration Helsinki and Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Statistical Analysis

Data is presented in absolute numbers, percentages, and frequencies. Continuous variables are presented as mean ± standard deviation, compared using ANOVA, with Tukey post hoc test. Categorical variables are presented as frequencies and compared using Chi-square tests. Sixteen variables were included in the risk analysis for acute rejection. Namely, donor and recipient age, donor and recipient ethnicity, recipient gender, donor final serum creatinine >1.5 mg/dl, expanded criteria donor (ECD), previous transplantation, panel reactive antibodies (PRA) > zero, HLA mismatches, DSA, cold ischemia time, vascular anastomosis time, DGF duration (days), positive historic B cell and presence and type of antibody induction (no induction, Basiliximab, or ATG). In the univariable analysis, prevalence ratio (PR) and 95% confidence intervals (CI) were calculated and the Chi-square test was used to assess their significance. Variables with a p-value ≤ 0.2 in the univariable analysis were included in the multivariable analysis model. For the multivariable analysis, prevalence ratios and confidence intervals were estimated by Poison's regression with robust estimation of variance. We used Kaplan-Meier estimate tests for analyzing patients and grafts survivals and GraphPad Prism for data presentation (version 8; GraphPad Software, San Diego, CA, United States). A p-value lower than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, United States).

TABLE 2 | Univariate and multivariate analysis of acute rejection risk factors.

Univariate analysis	PR	95% CI	p-value
Donor related factors			
Age	1.004	0.993–1.015	0.474
Ethnicity (non-white)	1.040	0.659–1.641	0.865
Expanded criteria donor	1.053	0.731–1.518	0.781
Acute kidney injury	1.140	0.808–1.608	0.456
Recipient related factors			
Age	0.991	0.979–1.004	0.164
Ethnicity (non-white)	1.158	0.763–1.713	0.462
Gender (male)	1.172	0.808–1.699	0.402
Previous transplantation	0.545	0.217–1.371	0.298
Absence of induction therapy with ATG	2.140	1.422–3.221	0.000
PRA > 0	0.801	0.567–1.133	0.209
Presence of DSA	0.761	0.443–1.307	0.322
HLA mismatches	1.106	0.944–1.296	0.213
Positive historic B cell crossmatch	2.188	1.124–4.260	0.021
DGF duration	1.010	1.003–1.017	0.005
Surgery related factors			
Cold ischemia time	0.989	0.961–1.016	0.418
Vascular anastomosis time	1.018	1.009–1.027	0.000
Multivariate Analysis			
Recipient age	0.985	0.972–0.998	0.028
Absence of induction therapy with ATG	2.320	1.443–3.731	0.001
Positive historic B cell crossmatch	1.634	0.802–3.327	0.176
DGF duration	1.011	1.003–1.019	0.005
Vascular anastomosis time	1.010	0.999–1.022	0.080

PRA, panel reactive antibodies; DSA, donor specific antibody; DGF, delayed graft function; PR, prevalence ratio; 95% CI, 95% confidence interval.

RESULTS

Study Population Characteristics

In the study period, 356 representative allograft biopsies were performed in 335 transplant recipients. Data of the recipients, donors, and transplant-related variables are shown in **Table 1**.

The majority of the patients were male (61.2%), Caucasian (74.9%), and almost half were not HLA sensitized (48.9% zero PRA). All patients received an initial immunosuppressive regimen with steroids, calcineurin inhibitors (tacrolimus or cyclosporine), and anti-proliferative agents (azathioprine, mTOR inhibitors, or sodium/mofetil mycophenolate) with or without antibody induction. One hundred and forty-eight patients (44.1%) received monoclonal anti-α-chain IL-2 receptor antibodies (Simulect®) and underwent 157 biopsies, 151 (45.1%) patients received ATG (Thymoglobulin®) and underwent 157 biopsies. Thirty-six patients did not receive antibody induction and underwent 42 biopsies. The most

TABLE 3 | Frequency of histological findings from surveillance biopsies of kidney transplant recipients according to DGF duration.[a]

Histological finding (Number of biopsies)	ATN (150)	Borderline (91)	Acute rejection (96)	Other lesions (19)
DGF duration				
≤7 days (41)	22 (50%)[b]	9 (20.4%)[c]	8 (18.2%)	2 (11.4%)
8–14 days (90)	39 (41.9%)	28 (30.1%)	20 (21.5%)	3 (6.4%)
15–21 days (75)	33 (43.4%)	23 (30.3%)	19 (25.0%)	0 (0%)
≥22 days (142)	56 (39.2%)	31 (21.7%)	49 (34.3%)[d]	6 (4.9%)

[a]*Excluding normal biopsies.*
[b]*ATN significantly higher than the other groups (p < 0.05).*
[c]*Borderline findings significantly lower than the other groups (p < 0.05).*
[d]*Acute rejection significantly higher than the other groups (p < 0.05).*

TABLE 4 | Frequency of rejection in the biopsies according to the Banff classification and antibody-induction therapy status.

Patients/Biopsies	No Ab induction (36/42)	Basiliximab (148/157)	ATG (151/157)
Banff classification			
Borderline	10 (23.8%)	46 (29.3%)	35 (22.3%)
IA	4 (9.5%)	36 (22.9%)	8 (5.1%)
IB	2 (4.7%)	2 (1.3%)	0 (0%)
IIA	6 (14.3%)	11 (7.0%)	10 (6.4%)
IIB	0 (0%)	5 (3.2%)	4 (2.5%)
III	1 (2.4%)	2 (1.3%)	0 (0%)
ABMR	0 (0%)	0 (0%)	5 (3.2%)
All rejections[a]	13 (30.9%)	56 (35.7%)	27 (17.9%)**

Ab, antibody; ABMR, antibody-mediated acute rejection; ATG, Anti Thymocyte globulin.
[a]*Excluding borderline findings; ** = p < 0.05.*

frequent maintenance regimen was tacrolimus, sodium mycophenolate, and steroids in 288 (85.9%) patients.

Biopsy Results

The number of patients with one, two, and three biopsies was 317, 15, and 3 respectively. As shown in **Figure 2**, eight biopsies (2.2%) were classified as normal kidney transplant, 150 (42.1%) presented acute tubular necrosis (ATN), 91 (25.5%) presented borderline changes, 96 (26.9%) were acute rejections, either cellular (91 cases, 25.5%) or antibody-mediated (5 cases, 1.4%), 8 (2.2%) presented coagulation necrosis, 2 (0.5%) had acute pyelonephritis and one biopsy (0.2%) showed thrombotic microangiopathy (**Figure 2**).

Figure 3 shows the Banff grades of the biopsies interpreted as acute rejection. Most were cellular rejections, predominantly IA and IIA phenotypes, with a lower frequency of the more severe cellular phenotypes and antibody-mediated rejection. All biopsies with acute antibody-mediated rejection were from patients with increased immunological risk who received induction therapy with ATG (**Figure 3**).

Table 2 shows risk factors for acute rejection in the univariate and multivariate analysis. Positive historic B cell cross-matching ($p = 0.023$), vascular anastomosis time ($p = 0.0001$), DGF duration ($p < 0.05$), and absence of induction therapy with ATG ($p = 0.0001$) were associated with acute rejection. These risk factors along with the recipient's age ($p < 0.2$) were included in the multivariate analysis model, which showed that the recipient's age, DGF duration, and absence of induction therapy with ATG were significantly associated with the occurrence of acute rejection (**Table 2**).

Delayed Graft Function Duration and the Occurrence of Acute Rejection

For this analysis, we divided patients and biopsies into four groups according to DGF duration. In group 1, with DGF duration up to 7 days, 44 biopsies were performed in 43 patients with eight episodes of rejection identified (18.2%). In group 2, with DGF duration between 8 and 14 days, there were 93 biopsies in 83 patients with 20 rejection episodes (21.5%). In group 3, with DGF duration between 15 and 21 days, there were 76 biopsies in 70 patients with 19 rejection episodes (25.0%) and, in group 4, with DGF duration longer than 21 days, 143 biopsies

TABLE 5 | Incidence of acute rejection in unsensitized patients and patients without donor-specific HLA antibodies, receiving tacrolimus and sodium mycophenolate, according to antibody-induction therapy status.

Category	(Number of patients)	With/without rejection	% With rejection	p
0% PRA, no induction[a]	(18)	4/14	22.2	0.345 vs.[b]
0% PRA, Basiliximab[b]	(65)	25/40	38.5	0.009 vs.[c]
0% PRA, ATG[c]	(36)	4/32	11.1	0.652 vs.[a]
No DSA, no induction[d]	(19)	4/15	21.1	0.198 vs.[e]
No DSA, Basiliximab[e]	(107)	42/65	39.3	0.001 vs.[f]
No DSA, ATG[f]	(86)	10/76	11.6	0.655 vs.[d]

PRA, panel reactive antibodies; DSA, donor specific anti-HLA antibodies.
The small letters identify the groups of patients according to the presence and type of induction therapy and the respective group comparisons.

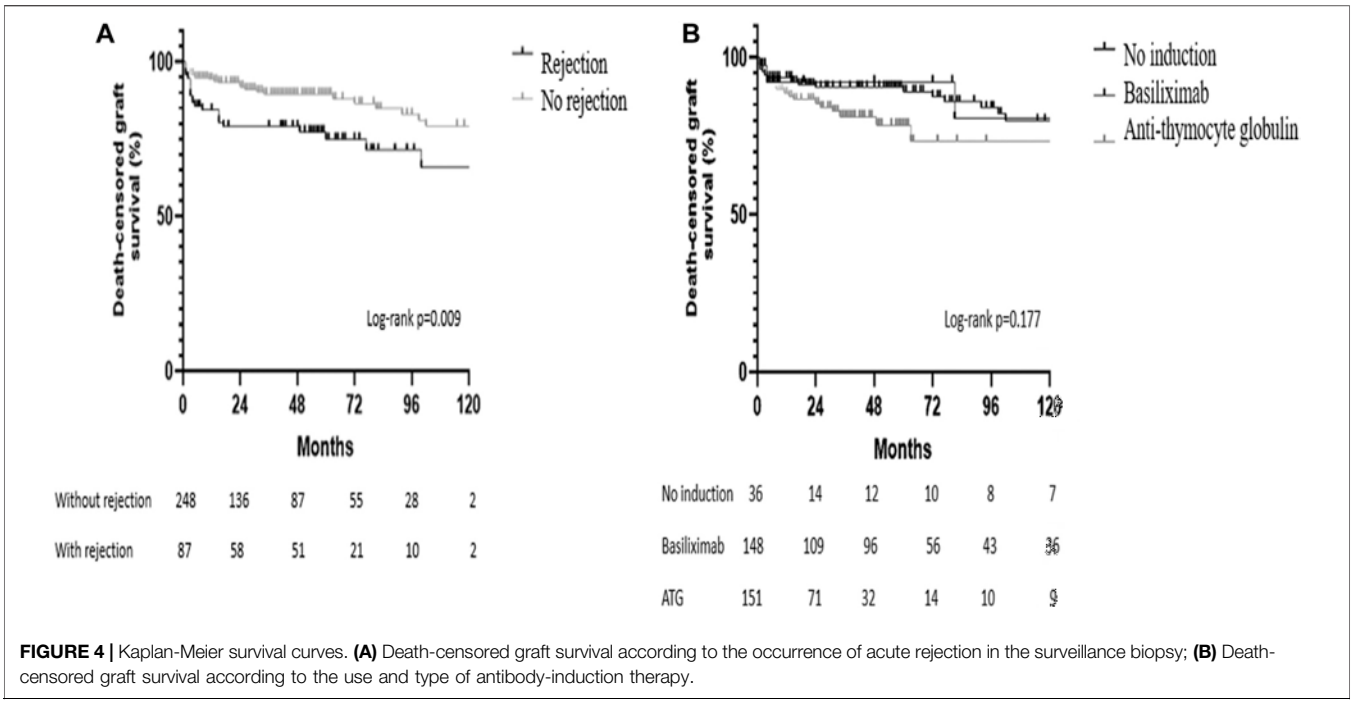


FIGURE 4 | Kaplan-Meier survival curves. **(A)** Death-censored graft survival according to the occurrence of acute rejection in the surveillance biopsy; **(B)** Death-censored graft survival according to the use and type of antibody-induction therapy.

TABLE 6 | Univariate and multivariate analysis of graft survival risk factors.

Univariate analysis	PR	95% CI	*p*-value
Donor related factors			
Age	0.998	0.994–1.002	0.348
Ethnicity (non-white)	0.882	0.732–1.063	0.187
Expanded criteria donor	0.998	0.835–1.193	0.982
Acute kidney injury	0.955	0.844–1.080	0.461
Recipient related factors			
Age	0.993	0.989–0.998	0.003
Ethnicity (non-white)	1.083	0.904–1.297	0.386
Gender (male)	1.136	1.003–1.287	0.045
Previous transplantation	0.873	0.676–1.128	0.299
Absence of induction therapy with ATG	1.014	0.758–1.354	0.839
PRA > 0	1.013	0.908–1.130	0.819
Presence of DSA	0.992	0.846–1.162	0.917
HLA mismatches	1.045	0.977–1.117	0.203
Positive historic B cell crossmatch	0.957	0.600–1.527	0.854
DGF duration	0.987	0.982–0.992	0.000
Surgery related factors			
Cold ischemia time	1.009	0.998–1.020	0.115
Vascular anastomosis time	0.999	0.992–1.006	0.738
Acute rejection	1.125	0.994–1.274	0.062
Multivariate Analysis			
Donor ethnicity (non-white)	0.933	0.809–1.077	0.344
Recipient age	0.993	0.989–0.997	0.001
Recipient gender (male)	1.149	1.021–1.294	0.021
DGF duration	0.987	0.983–0.991	0.000
Cold ischemia time	1.007	0.998–1.017	0.127
Acute rejection	1.158	1.041–1.287	0.007

PRA, panel reactive antibodies; DSA, donor specific antibody; DGF, delayed graft function; PR, prevalence ratio; 95% CI, 95% confidence interval.

were performed in 139 patients with 49 episodes of rejection (34.3%) (p = 0.005). **Table 3** presents the frequency of the histological diagnoses according to DGF duration. Noteworthy, the frequency of acute tubular necrosis decreased and the frequency of acute rejection increased as DGF lasted longer.

Immunosuppressive Regimen and Presence of Rejection in the Surveillance Biopsy

The type of immunosuppressive regimen was associated with the occurrence of acute rejection. In the group of 36 patients who did not receive antibody-induction therapy, acute rejection incidence was 36.1% (13 patients with cellular rejections). In the group of 148 patients treated with Basiliximab, the incidence was 37.8% (56 patients with cellular rejections), and, in the 151 patients who received ATG, an incidence of 17.9% of acute rejection was observed (27 patients with rejection, being 22 with cellular rejections and 5 with antibody-mediated rejections). No difference in acute rejection incidence was observed between patients treated with Baxiliximab and those without antibody-induction therapy (p = 0.126). However, patients treated with ATG had a significantly lower incidence of acute rejection than those in the other two groups (p = 0.0001).

Table 4 shows the frequency of acute rejection according to the presence and type of antibody induction therapy. The lower incidence of acute rejection in the group of patients treated with ATG occurred despite the higher risk of rejection present in this group who presented longer cold ischemia time (25:02 ± 5:54, 19:55 ± 4:57, 17:27 ± 5:13 h:min; p = 0.001), higher class I PRA (28.1 ± 35.6, 4.1 ± 9.7, 5.2 ± 18.2%; p = 0.001), higher class II PRA (24.2 ± 32.1, 2.0 ± 8.6, 5.9 ± 15.9%; p = 0.001), higher frequency of re-transplants (16.5, 1.3, 0%; p = 0.002), and more patients with donor-specific anti-HLA antibodies (30.5, 6.1, 5.5%; p = 0.003), respectively for the ATG, Basiliximab and no-induction groups.

Table 5 shows the analyzes of the impact of antibody induction on the incidence of acute rejection in patients at lower risk for rejection. The analysis included patients without anti-HLA sensitization and patients without anti-donor HLA antibodies receiving tacrolimus and sodium mycophenolate. A high incidence of rejection, remarkably in the groups of patients that did not receive antibody-induction or received Basiliximab, occurred despite the lower risk and use of this association of immunosuppressive agents. In comparison to the group of patients treated with Basiliximab, the group of patients that received ATG had a significant reduction in the incidence of rejection.

Antibody Induction Therapy, Acute Rejection, and Patient and Graft Survival

Neither acute rejection (p = 0.145) nor the use or type of antibody-induction therapy (p = 0.665) were associated with patient survival. Death censored graft survival was significantly lower in the group of patients with acute rejection (log-rank p = 0.009) and was not influenced by the use or type of antibody-induction therapy (log-rank p = 0.177) (**Figure 4**).

Table 6 shows risk factors for graft failure in the univariate and multivariate analysis. Recipient age (p = 0.003), male gender (p = 0.045), and DGF duration were associated with graft loss. These risk factors along with donor's ethnicity, cold ischemia time, and acute rejection were included in the multivariate analysis model, which showed that the recipient's age, recipient's male gender, DGF duration, and acute rejection were significantly associated with graft loss (**Table 6**). To further analyze the role of DGF duration as a risk factor for graft loss, we performed a sub-analysis including only the patients who presented ATN at the surveillance biopsies and found that both mean survival time and 1-year death censored graft survival decreased in parallel to DGF duration ($p < 0.0095$).

Grafts were lost due to chronic graft failure in 40 cases (2 in the no induction group, 18 in the ATG group, and 20 in the Basiliximab group), immunological causes (acute or chronic rejections) in 12 cases (none in the no induction group, 5 in the ATG group and 7 in the Basiliximab group), vascular causes in 6 cases (2 in the no induction group, none in the Basiliximab group and 4 in the ATG group) and recurrent focal and segmental glomerulosclerosis in 1 case.

DISCUSSION

In the present study, we analyzed the histological findings on surveillance graft biopsies of kidney transplant recipients experiencing DGF. The main findings were acute tubular necrosis and a high incidence of other graft injuries occurred, particularly acute cellular rejection and borderline findings. We also found that the occurrence of acute rejection during DGF leads to inferior graft survival.

Delayed graft function is a frequent complication of brain-dead deceased donor kidney transplantation and is even more frequent in kidney transplants from donors on circulatory death (20). Kidney transplantation from expanded criteria donors and/or high KDPI donors is also associated with a high incidence (13, 21). Clinically DGF presents as a post-transplant acute kidney injury with significantly increased serum creatinine and many times with decreased urinary output leading to the necessity of renal replacement therapy. Among other factors, it may be associated with organ procurement characteristics (inotropic support of the donor, cold ischemia time, and cold storage preservation), donor characteristics (age, renal function, and comorbidities), and recipient characteristics (hypovolemia, previous transplantation, preformed anti-donor antibodies and obesity). The transplant surgery itself and postoperative care (hydration, vascular anastomosis time, and hemodynamic support) can also influence the occurrence of DGF (12, 22-25).

The incidence of DGF is highly variable worldwide. It varies from around one-fourth to approximately two-thirds of kidney transplants from brain-dead deceased donors (26–29). In Brazil, for reasons that are not entirely understood, the reported incidence of DGF is consistently higher compared to other registries (7-9). In a recently published large Brazilian multicenter study, we found a high incidence of DGF and concluded that late referral and poor donor maintenance account for the high overall incidence while variability in donor and recipient selection, organ preservation method, and type of antibody induction may account for the wide variation observed among centers (7).

Two-thirds of the recipients in the present study developed DGF by the definition adopted in the present study. This frequency is similar to the one found in the Brazilian multicenter study (7) and to previous monocentric Brazilian studies (8, 9) but is much higher than that reported in other national registries (28, 29). Importantly, we observed a high incidence of acute rejection and borderline findings in the surveillance biopsies, which is in line with previous reports (8, 9). Additionally, in agreement with a large study in the Australian and New Zealand Dialysis and Transplant Registry database, we found that longer periods of DGF correlated with a higher incidence of rejection (29).

As expected, the largest percentage of the biopsies obtained during the DGF period presented with a histological diagnosis of acute tubular necrosis. However, Banff grade IA or higher acute rejections occurred in one-fourth of the biopsies. A noteworthy overtime change occurred in the frequency of the histological lesions. The frequency of acute tubular necrosis decreased, and the frequency of rejection increased, reflecting the healing of ischemia and reperfusion injuries partially replaced by alloimmune injury, possibly acting for maintaining graft dysfunction. Vascular anastomosis time was highly significant at the univariate analysis but presented borderline statistical value in the multivariate analysis. In line with our findings, a recent publication by Lim et al. reported an important association between DGF duration and acute rejection. They described that three-quarters of the acute rejection episodes occurred in kidney transplant recipients whose DGF lasted longer than 2 weeks (29). Moreover, borderline findings were diagnosed in another one-fourth of the surveillance biopsies. Such histological finding may be the expression of an initial T cell-mediated rejection but may also be due to non-alloimmune inflammation induced by different injuries, leaving its

significance uncertain. This often represents a treatment dilemma for the transplant physician, particularly in non-functioning allografts, as in DGF (14, 30). In our cohort half of the patients with borderline findings were treated for rejection.

Some controversy remains on whether or not DGF is associated with an increased incidence of acute rejection and reduced graft survival. Such may be due to a lack of homogeneity of the study cohorts and the non-uniform DGF definitions (5, 6, 14, 17). However, several studies describe a higher incidence of rejection in kidney transplant recipients experiencing DGF (5, 14, 17). Wu and collaborators reported that DGF is a major risk factor for acute rejection in the modern era of immunosuppression in deceased donor kidney transplants, showing that the cumulative probability for rejection was greater in patients undergoing DGF at all points during the follow-up period (17). Also, a recent study by Weber et al. demonstrated that the hazard ratio for developing acute rejection within the first year after transplantation was 71% higher in the group of patients with DGF (14). The impact of DGF on graft survival is well established, as shown in a recently published large multicenter study (7). However, controversy is still out on whether the worst graft survival is restricted only to recipients of kidneys from standard criteria donors (31, 32).

Perhaps the major hurdle of DGF is the inability to detect or even suspect the occurrence of acute rejection due to the lack of functional parameters usually used to monitor injuries. Importantly, in the absence of accurate non-invasive methods or biomarkers of rejection in this setting, the only reliable tool to uncover alloimmune graft injury is the surveillance graft biopsy.

In agreement with previous studies, our results demonstrated that the rejection rate decreases with age supporting the notion that immunosuppressive therapy may be reduced in elderly recipients due to the progressive decline in immune functions, leading to a lower risk of rejection and a higher risk of infectious complications (33–35).

Antibody induction therapy with polyclonal anti-T cell antibodies is often used to prevent acute rejection, particularly in recipients with high immunological risk. In our study, the group of patients who received ATG was at higher immunological risk and, despite this, had a substantially lower incidence of rejection in the surveillance biopsy as compared to patients that received monoclonal anti-IL2 receptor antibodies or patients who did not receive antibody-induction therapy. Recently, Alloway and collaborators reported a reanalysis of the data from prior trials comparing ATG with anti-IL-2 receptor monoclonal antibodies, showing the superiority of such polyclonal antibodies in preventing acute rejection (36). Moreover, in previous studies including patients with DGF, ATG was more efficient in preventing acute rejection (37, 38).

Interestingly a considerably high incidence of histological rejection occurred even in patients considered of lower immunological risk such as patients without anti-HLA sensitization, and patients without anti-donor HLA antibodies, receiving ATG induction and baseline immunosuppressive regimen with steroids, tacrolimus, and sodium mycophenolate. In the group of patients that did not receive antibody induction and in the group that received Basiliximab the incidence of rejection was very high despite the potent association of baseline immunosuppressive agents (39). These findings give support to the notion that ischemia and reperfusion injury, by overexposing graft antigens, elicits a strong alloimmune response (12, 40).

A study by Hatoum and collaborators evaluated the utility of surveillance allograft biopsies during DGF in patients receiving antibody-induction therapy with ATG or Basiliximab and baseline immunosuppression with steroids, mycophenolate, and tacrolimus. They concluded that rejection episodes during DGF are uncommon and, therefore, the usefulness of serial surveillance biopsies is limited. These results differ from ours in some ways, including a much lower incidence of DGF, the inclusion of kidney recipients of living donors, and a higher proportion of African-American recipients (38). Therefore, the differences found in the incidence of rejection are probably due to the study population, sample sizes, and severity of the ischemia-reperfusion injury.

It is conceivable that with a higher incidence of DGF and the use of organs from expanded criteria donors, the incidence of rejection would be higher (14). Importantly our study occurred within a period in which the donor acceptance policy and immunosuppressive regimen did not change substantially. However, current immunosuppressive regimens are different from those employed at the time of guidelines publications. Nevertheless, even under current immunosuppressive regimens a high frequency of histological lesions, mainly acute rejections, are uncovered by surveillance biopsies.

A recent study by Van Loon et al. also revealed a very high incidence of acute rejection in their subset of patients with DGF. In their study, the risk factors for rejection were HLA mismatches and pre-transplant HLA-DSA. The authors found that non-immune risk factors were not strong risk factors for early inflammation (41). This is in contrast with our findings where the recipient age, DGF duration, and absence of antibody-induction with polyclonal anti-T cell antibodies were the identified risk factors. We believe that the discrepancies may be related, at least in part, to the frequency and type of antibody-induction therapy since in our more patients received antibody-induction with ATG.

The association between prolonged vascular anastomosis time and DGF is long known and was confirmed in two recent studies (26, 27). However, a possible association between prolonged vascular anastomosis time and occurrence of acute rejection, as seen in this study, is new and deserves further investigation. Prolongation of warm ischemia time can lead to more intense ischemia and reperfusion injury. A well-known consequence of such injury is the activation of innate immunity signaling transcription factors that encode genes involved in the regulation of inflammation. In such an inflamed environment, graft antigens are more exposed, and therefore recognized and processed by antigen-presenting cells and presented to the host immune system, facilitating the occurrence of acute rejection (41, 42).

The present work, by selecting only biopsies of patients with DGF, does not allow the analysis of DGF risk factors, particularly cold ischemia time. However, in previous studies, cold ischemia time surfaced as an independent risk factor for acute rejection (24, 43). We did not find such correlation and believe that a possible effect may have been lost due to the usually prolonged cold ischemia time, observed in our region, and perhaps for sample size matters.

Our study has limitations intrinsic to its retrospective and monocentric design. Early protocol biopsies of function kidney

grafts, at a similar time as the surveillance biopsies in DGF, could also reveal unsuspected lesions. We did not analyze the outcomes of patients who had DGF but were not subjected to the surveillance biopsy. Also, caution is needed in the interpretation of the immunosuppressive regimen results due to the non-randomized design. Nevertheless, the timing of the biopsies was dictated by current guidelines and, a considerable incidence of acute cellular rejection occurred during the DGF period indicating that surveillance biopsies are instrumental for the clinical care of kidney transplant recipients.

In conclusion, the surveillance biopsy of kidney grafts with DGF remains an essential tool for the clinical care of the kidney transplant recipient. These biopsies are even more important in settings where kidneys from expanded criteria donors and/or high KPDI donors are frequently utilized, with prolonged cold ischemia time and high incidence of DGF.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics and Research Committee of the Hospital de Clínicas de Porto Alegre (protocol number 64239617.4.0000.5327). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JC—Performed study, collected and analyzed data, and wrote the paper. JP—Collected and analyzed data. RM—Collected and analyzed data. LG—Analyzed data, wrote the paper. AB—Analyzed data, wrote the paper. RM—Designed study, analyzed data and wrote the paper.

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
published: 17 March 2022
doi: 10.3389/ti.2022.10094

The Clinical Impact of Anti-HLA Donor Specific Antibody Detection Through First Year Screening on Stable Kidney Transplant Recipients

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Anti-HLA Donor Specific Antibody (DSA) detection post kidney transplant has been associated with adverse outcomes, though the impact of early DSA screening on stable patients remain unclear. We analyzed impact of DSA detection through screening in 1st year stable patients (n = 736) on subsequent estimated glomerular filtration rate (eGFR), death censored graft survival (DCGS), and graft failure (graft loss including return to dialysis or re-transplant, patient death, or eGFR < 20 ml/min at last follow up). Patients were grouped using 1st year screening into DSA+ (Class I, II; n = 131) or DSA- (n = 605). DSA+ group were more DR mismatched (p = 0.02), more sensitized (cPRA ≥90%, p = 0.002), less Caucasian (p = 0.04), and had less pre-emptive (p = 0.04) and more deceased donor transplants (p = 0.03). DSA+ patients had similar eGFR (54.8 vs. 53.8 ml/min/1.73 m^2, p = 0.56), DCGS (91% vs. 94%, p = 0.30), and graft failure free survival (76% vs. 82%, p = 0.11). DSA timing and type did not impact survival. Among those with a protocol biopsy (n = 515), DSA detected on 1st year screening was a predictor for graft failure on multivariate analysis (1.91, 95% CI 1.03–3.55, p = 0.04). Overall, early DSA detection in stable patients was an independent risk factor for graft failure, though only among those who underwent a protocol biopsy.

Keywords: donor-specific antibodies, screening, kidney transplant, graft survival, rejection

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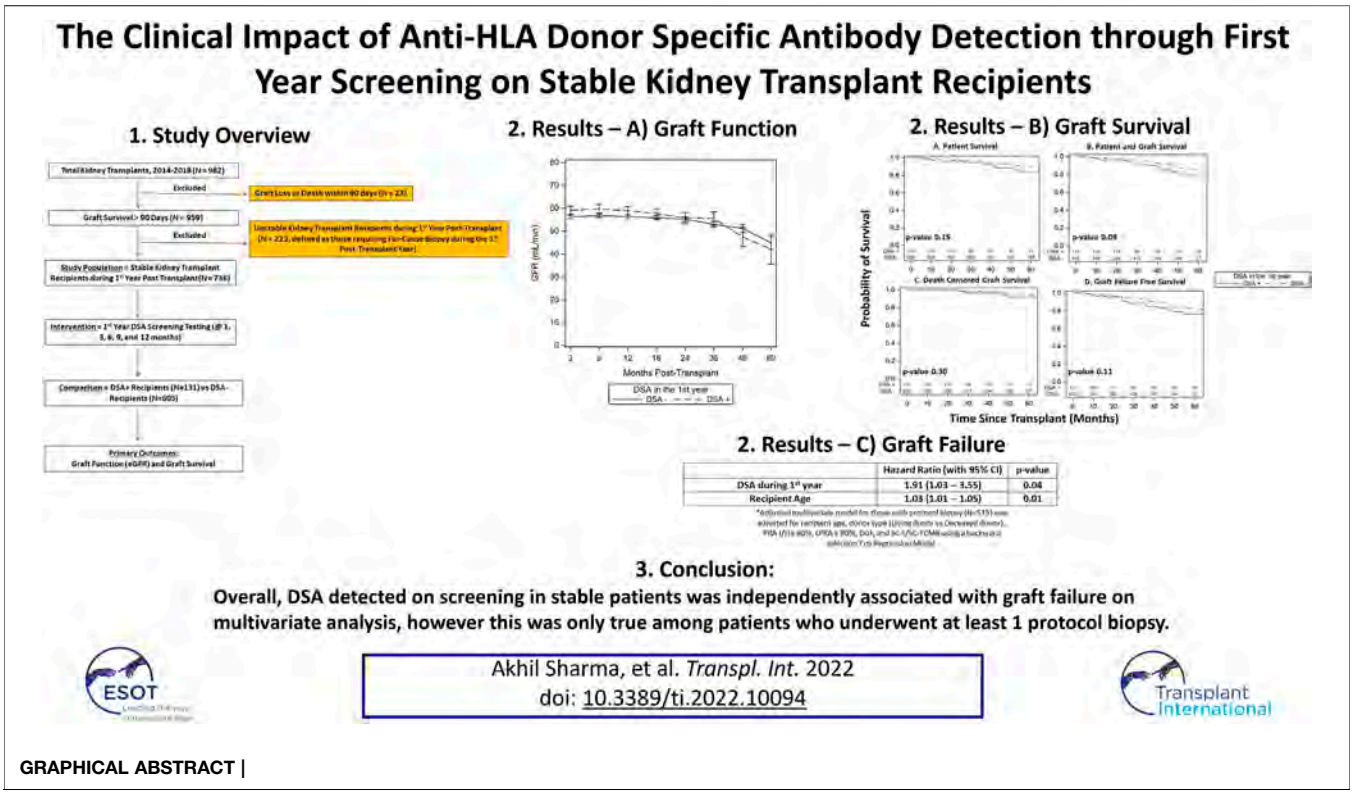
***Received:** 05 October 2021*
***Accepted:** 09 February 2022*
***Published:** 17 March 2022*

Citation:
Sharma A, Jorgensen DR, Mehta RB, Sood P, Puttarajappa CM, Wu CM, Tevar AD, Molinari M, Zeevi A and Hariharan S (2022) The Clinical Impact of Anti-HLA Donor Specific Antibody Detection Through First Year Screening on Stable Kidney Transplant Recipients. Transpl Int 35:10094. doi: 10.3389/ti.2022.10094

Abbreviations: ABMR, antibody mediated rejection; BBC, banff borderline changes; CIT, cold ischemia time; cPRA, calculated panel reactive antibody; DCGS, death censored graft loss; DD, deceased donor; DGF, delayed graft function; DSA, donor specific antibody; eGFR, estimated glomerular filtration rate; FC, flow crossmatch; ESKD, end stage kidney disease; HLA, human leukocyte antigen; IFTA, interstitial fibrosis and tubular atrophy; IRB, institutional review board; KDPI, kidney donor prognostic index; LD, living donor; MFI, mean fluorescence intensity; MI, minimal inflammation; NI, no inflammation; PRA, panel reactive antibody; SCI, subclinical inflammation; SC-TCMR, subclinical T cell mediated rejection; SC-ABMR, subclinical antibody mediated rejection; TCMR, T cell mediated rejection.



GRAPHICAL ABSTRACT |

INTRODUCTION

Anti-donor human leukocyte antigen (HLA) specific antibody (DSA) development after kidney transplant is associated with poor clinical outcomes (1–4). Specifically, DSA has been associated with Antibody Mediated Rejection (ABMR) and T-Cell Mediated Rejection (TCMR), with early rejection linked to inferior outcomes (5–11). DSA detection also has been correlated with worse transplant survival, though many with DSA will still have a functioning transplant at 5 years (~83%) (12). Studies assessing DSA have been varied in population and testing indication, often mixing both for-cause and screening testing. Further, DSA testing is not standardized resulting in variation between laboratories (13–15). These factors have limited assessment of DSA testing as a screening tool in stable patients. With increased efforts to curb health care costs, magnified by an ongoing pandemic, each test ordered and performed must add value to care provided (16–20).

The impact of early DSA screening on patients with stable kidney function without pre-existing DSA at transplant remains unclear and has been identified as a topic requiring study (21–23). To address the impact of early post-transplant DSA screening, we analyzed DSA detected on screening within the 1st year in stable kidney transplant patients and examined correlations with primary outcomes of kidney function and survival. We also analyzed secondary outcomes of subclinical events in the 1st year using protocol biopsies and clinical events beyond the 1st year using for-cause biopsies. We hypothesized that DSA detected on screening in stable 1st year patients would not be associated with inferior survival or function.

MATERIALS AND METHODS

Study Population

We studied 982 adult patients who underwent kidney transplant alone (ABO compatible and DSA absent, flow crossmatch (FC) negative at the time of transplant based on last serum within <30 days of transplant) from January 1, 2014 to December 31, 2018 at the Thomas E. Starzl Transplantation Institute—University of Pittsburgh. Repeat kidney and kidney after other solid organ transplant patients were included. We excluded those with early graft loss or death (<90 days, $n = 23$) or an unstable 1st year course (defined as those requiring for-cause biopsy in 1st year, $n = 223$) to limit for-cause DSA testing that often accompanies for-cause biopsies and graft dysfunction. The remaining 736 patients served as our primary study cohort (**Figure 1**).

DSA Monitoring

DSA was tested within the 1st year (1, 3, 6, 9, 12 months) per our center's screening protocol, at time of any biopsy, and annually until 5 years. DSA was considered newly detected as last serum sample available at time of transplant was DSA negative (prior serum was not analyzed). DSA was measured using One Lambda LABScreen™ single antigen bead assay and considered positive if adjusted mean fluorescent intensity (MFI) was ≥1,000 units based

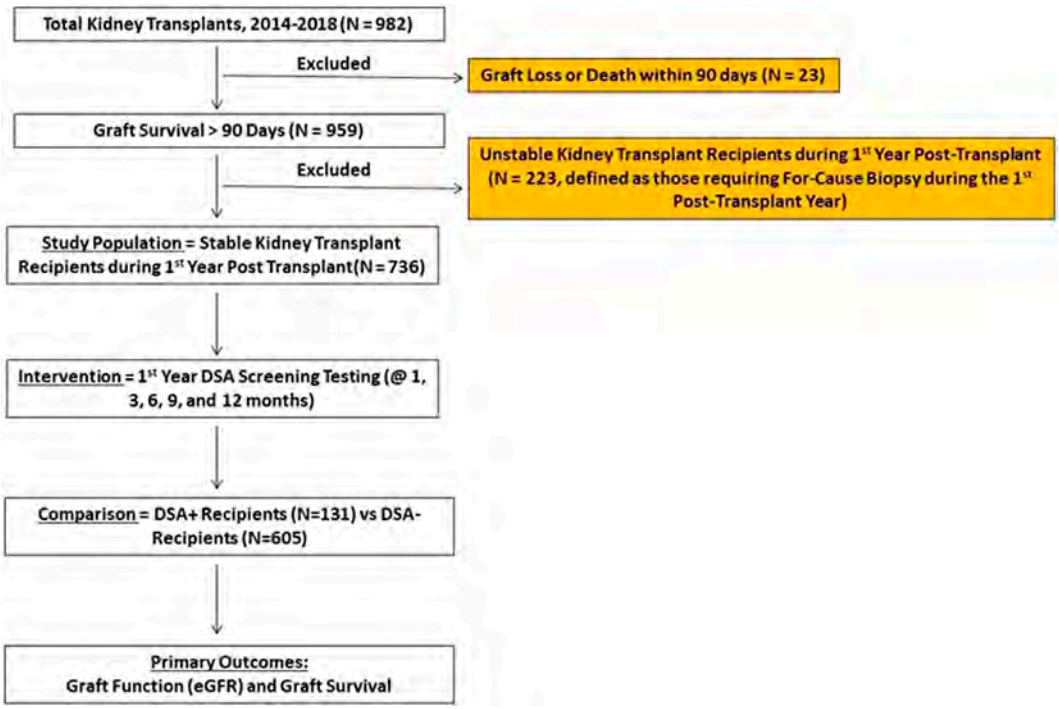


FIGURE 1 | Schematic representation of the study which shows 982 patients who had kidney alone transplant from 2014–2018 and exclusion of 246 patients. The remaining 736 patients with a stable 1st year kidney transplant formed the study cohort and were divided into DSA+ (N = 131, 18%) versus DSA- (N = 605, 82%) based on 1st year surveillance DSA testing. Primary clinical outcomes were assessed as noted.

on our HLA lab's designation. A single positive DSA reading (for either class) was considered as a single positive and multiple positive DSA tests for the same class separated in time were considered as multiple positive.

Immunosuppression

Induction was mainly with thymoglobulin and rarely with basiliximab (if 0% calculated panel reactive antibody [cPRA], 0 antigen mismatch, and a living donor [LD] transplant recipient). For maintenance, majority were on mycophenolate mofetil and calcineurin inhibitor (mainly Tacrolimus) with a minority also on prednisone (those with cPRA ≥ 90% or those on prednisone prior, 5 mg daily or their dose prior to transplant). Prednisone (5 mg daily) was subsequently added to maintenance regimen for any rejection episodes (clinical or subclinical). There was no systematic center protocol for adjusting maintenance immunosuppression based on DSA detection alone.

Biopsies

Protocol biopsies were recommended to all patients at ~3 and 12 months post-transplant unless contraindicated. Potential contraindications included those patients on systemic anticoagulation, those on dual anti-platelet therapy, those with intrabdominal kidney location, those who received *en bloc* kidneys, those with active malignancy or serious infection at time of scheduled protocol biopsy, or those lacking transportation. Additionally, as with any medical procedure, patients had the option to decline recommendation to undergo a protocol biopsy after risks and benefits were thoroughly discussed. Biopsies were scored using Banff 2013 and later 2017 classification (24, 25). For-cause biopsies were done for renal dysfunction (rise in serum creatinine >25% from baseline and/or new or worsening proteinuria [>1 g/day and/or >1 g/g urine protein to creatinine ratio]), but not for isolated DSA detection alone.

Allograft Histology

Protocol biopsy findings were defined as no inflammation (NI, Banff t score 0 + i/ti score 0), subclinical inflammation (SCI, minimal inflammation [MI] Banff t score <0 + i/ti score ≥0 or Banff Borderline Changes [BBC] Banff t score >0 + i/ti score ≥0 and <1A TCMR), and subclinical TCMR (SC-TCMR, ≥1A TCMR). Those with subclinical ABMR (SC-ABMR) were included within these three groups using associated findings (NI, SCI, or SC-TCMR) and were also analyzed separately. Protocol biopsies were also grouped based on timing and maximum grade (highest grade noted on any 1st year protocol biopsy). For-cause biopsies beyond the 1st year were defined as negative (no pathologic findings), inflammation (MI or BBC), rejection (≥1A TCMR and/or ABMR), and non-alloimmune events (urinary tract infection, BK virus nephropathy, acute tubular injury, glomerulonephritis, secondary oxalate nephropathy).

Follow-Up

The median follow up was ~3.3 years (**Table 1**).

TABLE 1 | Recipient and donor demographics and transplant characteristics of kidney transplant recipients and post-transplant events such as delayed graft function and biopsy rates among study recipients with who had a stable 1st year post-transplant course with DSA+ and DSA-.

	Total (N = 736)	DSA- (N = 605)	DSA+ (N = 131)	*p*-value
Recipient age (years, mean/SD)	52 (14)	53 (14)	51 (12)	0.12
Recipient gender (% male)	59	60	53	0.12
Recipient race (% caucasian)	76	78	68	**0.04**
Body mass index at transplant (kg/m^2, Mean/SD)	28.7 (5.7)	28.5 (5.7)	29.6 (5.9)	0.05
Preemptive transplant (%)	19	20	12	**0.04**
Cause of end stage kidney disease				
Diabetes mellitus %	23	22	25	0.43
Hypertension %	18	19	15	0.23
Polycystic kidney disease %	11	11	12	0.99
Glomerulonephritis %	5	5	3	0.35
Other/unknown %	43	43	45	0.78
Prior kidney transplant (%)	17	16	21	0.17
Any prior transplant (%)	25	25	24	0.35
Deceased donor %	68	67	76	**0.03**
Donor age (years, mean/SD)	40 (14)	40 (14)	39 (13)	0.66
Donor gender (% male)	55	55	56	0.86
Donor race (% Caucasian)	89	89	86	0.50
Cold ischemia time (minutes, median/IQR)	506 (88–792)	497 (85–792)	544 (204–782)	0.26
KDPI % (Mean/SD)	42 (25)	43 (25)	41 (26)	0.59
% with panel reactive antibody class I ≥ 90%	5	5	7	0.25
% with panel reactive antibody class II ≥ 90%	6	6	6	0.83
% with calculated panel reactive antibody ≥ 90%	15	13	24	**0.002**
Total HLA mismatches (median/IQR)	4 (3–5)	4 (3–5)	4 (4–5)	0.11
DR mismatches (median/IQR)	1 (1–2)	1 (1–2)	1 (1–2)	**0.02**
Cytomegalovirus D+/R- (%)	21	22	16	0.28
Epstein-barr virus D + R- (%)	5	5	7	0.50
Delayed graft function (%)	16	16	17	0.76
At least 1 protocol biopsy (%)	70	69	73	0.36
3 month protocol biopsy (%)	64	63	66	0.46
12 month protocol biopsy (%)	55	54	57	0.61
Biopsy anytime during study (%)	77	76	79	0.41
Any DSA detected beyond 1 year (%)	15	10	39	**<0.001**
Median follow up (days, median/IQR)	1,199 (808–1,640)	1,204 (805–1,646)	1,146 (832–1,523)	0.44

Bold values considered statistically significant with p*-value < 0.05.*

Outcome Measures

Primary outcomes were kidney function (estimated glomerular filtration rate [eGFR] using CKD-EPI formula) and survival (patient, combined patient and graft, death censored graft survival [DCGS], and graft failure [defined as graft loss with return to dialysis or re-transplant, death, or eGFR < 20 ml/min at last follow up] free survival). Secondary outcomes were subclinical events (SCI, SC-TCMR, SC-ABMR, mean cumulative acute scores [defined as sum of Banff i/ti, t, g, ptc, and v scores], and mean IFTA score) within 1st year and clinical events (rejection, inflammation, non-alloimmune events) beyond 1st year.

Ethical Guidelines

Patient information was obtained through specified personnel at Thomas E. Starzl Transplantation Institute as regulated by the institutional review board (IRB) at the University of Pittsburgh. The institution maintains a prospectively collected electronic database of all kidney transplant patients. The studies involving human participants were reviewed and approved by the University of Pittsburgh IRB and the patients were not required to provide written consent for this study per the University of Pittsburgh IRB. We collected data under IRB number PRO-13060220. The activities reported are consistent with the Principles of the Declaration of Istanbul as outlined in the "Declaration of Istanbul on organ trafficking and Transplant Tourism" and Declaration of Helsinki.

Statistical Methods

Analysis was completed using SAS 9.4 (SAS Institute Inc., Cary NC). Continuous variables are presented as mean ± standard deviation for normally distributed data and median with interquartile range for nonparametric data. Differences in baseline and transplant variables were assessed using analysis of variance and chi-square tests. We evaluated differences in recipient and donor demographics (age, race, gender), and other variables [body mass index (BMI) at transplant, preemptive transplant, End Stage Kidney Disease (ESKD) cause, cold ischemia time (CIT), Kidney Donor Prognostic Index (KDPI), donor type [deceased donor (DD) vs. LD], PRA (panel reactive antibody) Class I/II, cPRA, HLA mismatch (A, B, DR), Cytomegalovirus (CMV)/Epstein Barr Virus (EBV) serostatus, delayed graft function (DGF), biopsy accrual rates] between groups (**Table 1**). Linear mixed model was used to assess eGFR with a serum creatinine value of 8 mg/dl assigned for

TABLE 2 | Donor specific antibody (DSA) characteristics for DSA+ patients who underwent kidney transplant and had a stable 1st year post-transplant course.

DSA characteristic	DSA+ (N = 131)
# of Class I tests during 1st year[a]	8 (6–10)
# of Class II tests during 1st year[a]	8 (6–10)
# of + Class I tests during 1st year[a]	1 (0–2)
# of + Class II tests during 1st year[a]	1 (0–3)
Time to + Class I test (days)[a]	41 (30–108)
Time to + Class II test (days)[a]	38 (31–135)
Class I + % during 1st year	57
Class II + % during 1st year	62
DSA detected within 100 days (%)	77
Single positive DSA (%)	46
Multiple positive DSA (%)	60
DSA type	
Negative (%)	0
Class I (%)	38
Class II (%)	44
Class I and Class II (%)	18

[a]*Median and IQR, noted.*

graft loss. Covariates for multivariate analysis were identified and evaluated for inclusion before modeling. All multivariate analysis included recipient age, donor type (LD vs. DD), PRA I/II ≥ 90%, cPRA ≥ 90%, DGF, and SCI/SC-TCMR using a backward selection Cox Regression Model with variables with $p < 0.2$ included in the model. Survival (patient, graft, graft failure free) was examined by Kaplan Meier method with survival curves compared by Log rank test. Adjusted Bonferroni p-values were used with multiple log-rank comparisons. We examined relationship between DSA and 1st year protocol biopsy findings on eGFR in an exploratory analysis. A p-value <0.05 was considered statistically significant.

RESULTS

Patient Demographics

The DSA+ cohort included 131 patients (18%) with at least one positive screening DSA test (class I and/or II) during the 1st year and the remaining 605 patients (82%) were the DSA- cohort. DSA+ patients had less Caucasians (68% vs. 78%, $p = 0.04$), fewer were pre-emptive (12% vs. 20%, $p = 0.04$), more were deceased donor (76% vs. 67%, $p = 0.03$), more were anti-HLA sensitized (% cPRA ≥ 90%, 24% vs. 13%, $p = 0.002$), more were DR mismatched (1 [1–2] vs. 1 [1–2], $p = 0.02$), and more had DSA detected (persisting from 1st year or new) beyond 1 year (39% vs. 10%, $p < 0.001$) (**Table 1**). Other donor and recipient variables as well as protocol biopsy accrual rates were all similar. DSA detection was comparable for those with and without protocol biopsy (19% with vs. 16% without, $p = 0.36$), but those without protocol biopsy were more likely to have diabetic ESKD, prior transplant, increased anti-HLA sensitization, received DD transplant, longer CIT, and less likely to have had a preemptive transplant (**Supplementary Table S1**).

TABLE 3 | Summary of protocol biopsy findings during the 1st year post-transplant for study recipients who had a stable 1st year post-transplant course and had at least one protocol biopsy during the 1st year. Percentages are reflective of percentage of biopsies (not patients) falling within each category.

	Total (N = 515)	DSA- (N = 419)	DSA+ (N = 96)	*p*-value
Mean Acute Score Sum at 3 months (i + t + v + g)	1.7 (1.7)	1.6 (1.6)	1.9 (1.8)	0.26
Mean Acute Score Sum at 12 months (i + t + v + g)	2.1 (2.0)	2.1 (1.9)	2.5 (2.4)	0.16
Mean IFTA Score 3 months (ct + ci)	1.4 (1.1)	1.4 (1.1)	1.4 (0.9)	0.99
Mean IFTA Score 12 months (ct + ci)	2.0 (1.1)	2 (1.2)	2 (0.9)	0.78
Number of Protocol Biopsies during the 1st year	855 (100%)	696 (81%)	159 (19%)	
Biopsy Grade–Max during 1st Year				0.25
No Inflammation % (95% CI)	13 (11–17)	14 (11–18)	11 (5–19)	
Subclinical Inflammation % (95% CI)	56 (51–60)	55 (50–60)	58 (47–68)	
Subclinical TCMR % (95% CI)	31 (27–35)	31 (26–35)	31 (22–41)	
Biopsy Grade–3 months				0.91
No Inflammation % (95% CI)	27 (23–32)	28 (23–33)	26 (17–36)	
Subclinical Inflammation % (95% CI)	56 (51–60)	55 (50–60)	57 (46–67)	
Subclinical TCMR % (95% CI)	17 (13–20)	17 (13–21)	17 (10–27)	
Biopsy Grade–12 months				0.34
No Inflammation % (95% CI)	22 (18–26)	22 (18–27)	20 (12–32)	
Subclinical Inflammation % (95% CI)	52 (47–57)	52 (47–58)	52 (40–64)	
Subclinical TCMR % (95% CI)	26 (22–30)	26 (21–31)	28 (18–39)	
Subclinical ABMR				
Anytime % (95% CI)	0.8 (0.3–2)	0	4 (2–9)	< **0.001**
3 months % (95% CI)	0.9 (0.2–2)	0	5 (1–11)	< **0.001**
12 months % (95% CI)	0.8 (0.1–2)	0	4 (1–12)	< **0.001**
Type of Subclinical ABMR				
Sub-Clinical ABMR Alone % (95% CI)	0.1 (0–0.7)	0	0.6 (0–4)	**0.02**
Sub-Clinical ABMR + SCI % (95% CI)	0.4 (0.1–1)	0	2 (0.4–5)	< **0.001**
Sub-Clinical ABMR + SC-TCMR % (95% CI)	0.4 (0.1–1)	0	2 (0.4–5)	< **0.001**

Bold values considered statistically significant with p*-value < 0.05.*

Abbreviations are as follows: IFTA, interstitial fibrosis tubular atrophy; CI, Confidence interval; TCMR, T cell mediated rejection; ABMR, antibody mediated rejection; SCI, subclinical inflammation.

TABLE 4 | Summary of for-cause kidney biopsies performed beyond 1 year for patients who had a stable 1st year post-transplant course. Patients were grouped by whether Donor Specific Antibody (DSA) was detected during the 1st year post-transplant. Percentages are reflective of percentage of biopsies (not patients) falling within each category.

	Total (N = 736)	DSA- (N = 605)	DSA+ (N = 131)	*p*-value
% Patients undergoing for-cause biopsy beyond 1 year	25 (*n* = 181)	25 (*n* = 151)	23 (*n* = 30)	0.62
Number of for-cause biopsies performed beyond 1 year	225 (100%)	189 (84%)	36 (16%)	
Distribution of biopsies				0.35
Negative % (95% CI)	11 (7–15)	12 (7–17)	6 (1–19)	
Clinical inflammation % (95% CI)	30 (24–37)	32 (25–39)	22 (10–39)	
Clinical rejection % (95% CI)	44 (38–51)	42 (35–50)	56 (38–72)	
Non alloimmune events % (95% CI)	15 (10–20)	14 (10–20)	16 (6–33)	
Clinical inflammation % (95% CI)	30 (24–37)	32 (25–39)	22 (10–39)	0.25
Minimal inflammation % (95% CI)	6 (4–11)	8 (4–12)	3 (0.1–15)	0.49
Banff borderline changes % (95% CI)	24 (18–30)	24 (18–31)	19 (8–36)	0.49
Clinical rejection % (95% CI)	44 (38–51)	42 (35–50)	56 (38–72)	0.14
TCMR % (95% CI)	35 (28–41)	36 (29–43)	28 (14–45)	< **0.001**
1A % (95% CI)	24 (18–30)	25 (19–32)	17 (6–33)	0.56
1B % (95% CI)	11 (7–15)	11 (7–16)	11 (3–26)	0.50
≥2A %	0.4 (0–3)	0.5 (0–3)	0 (0–10)	0.70
Mixed (associated TCMR grade) % (95% CI)	9 (6–14)	6 (3–10)	28 (14–45)	0.07
1A % (95% CI)	4 (2–7)	2 (0.6–5)	11 (3–26)	0.87
1B % (95% CI)	4 (2–8)	2 (0.6–5)	14 (5–30)	0.53
≥2A % (95% CI)	1 (0.6–5)	2 (0.3–5)	3 (0.1–15)	0.31
ABMR alone %	0.4 (0–3)	0.5 (0–3)	0 (0–10)	0.66
Non alloimmune events % (95% CI)	15 (10–20)	14 (10–20)	16 (6–33)	0.71
UTI % (95% CI)	2 (0.5–5)	0.5 (0–3)	8 (2–22)	**0.002**
BK virus nephropathy % (95% CI)	7 (4–11)	7 (4–11)	6 (0.7–19)	0.51
Acute tubular injury % (95% CI)	3 (2–7)	4 (2–7)	2 (0.1–15)	0.63
Glomerulonephritis % (95% CI)	2 (0.5–5)	2 (0.5–5)	0 (0–10)	0.31
Oxalate nephropathy % (95% CI)	1 (0.1–3)	1 (0.1–4)	0 (0–10)	0.49

Bold values considered statistically significant with p*-value < 0.05.*

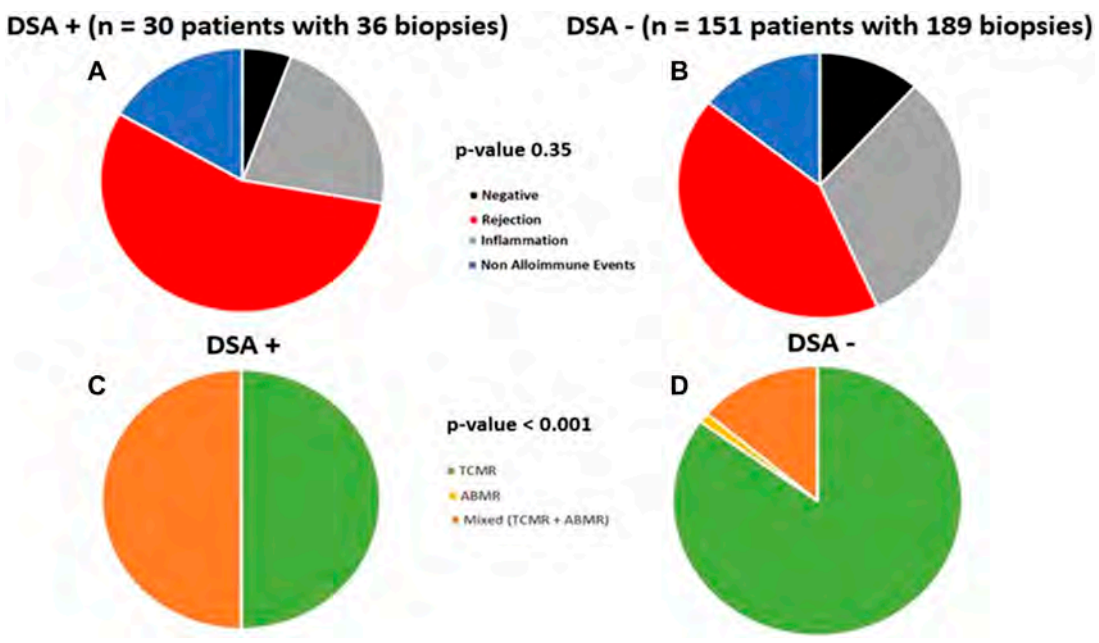


FIGURE 2 | The distribution of for-cause biopsies performed beyond 1 year (*n* = 225, 36 in DSA+ vs. 189 in DSA-) are shown below. A total of 181 patients (25% total) received a for-cause biopsy beyond 1 year and this was similar among the groups (*n* = 30 in DSA+ [23%] vs. *n* = 151 in DSA- [25%], *p* = 0.62). The frequency of for-cause biopsies beyond 1 year demonstrating clinical rejection, clinical inflammation, and non-alloimmune events was similar among DSA+ and DSA-patients (*p* = 0.35, **(A,B)**). However, the type of rejection seen on late for-cause biopsies was different among the groups **(C,D)** as the frequency of biopsies in DSA+ patients trended towards more mixed rejection (50% [95% CI 27–73%] vs. 14% [95% CI 7–23%], *p* = 0.07) and less TCMR (50% [95% CI 27–73%] vs. 85% [95% CI 75–92%], *p* < 0.001) compared to DSA- patients.

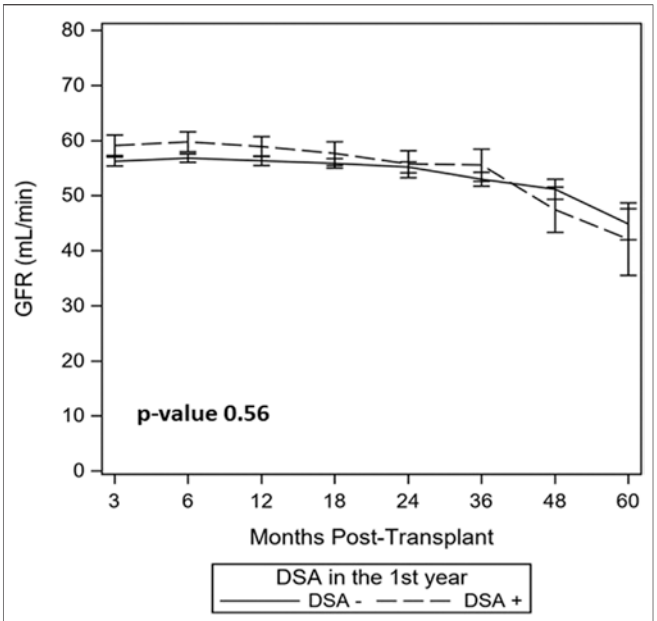


FIGURE 3 | Similar kidney function was seen over study follow up in DSA+ and DSA-kidney transplant recipients with a stable 1st year post-transplant course. A linear mixed model with estimated glomerular filtration rates (GFR) over the study follow up period demonstrating similar GFR between DSA+ and DSA- patients over time (54.8 vs. 53.8 ml/min/1.73 m^2, $p = 0.56$) is shown.

DSA Characteristics

DSA were primarily detected ~1–4 months post-transplant (class I at 40 days [30–108] and class II at 38 days [31–135]) with 77% of first DSA detected within 100 days (**Table 2**). Additionally, 60% of DSA+ patients had at least one multiple positive DSA for the same class and 18% of DSA+ patients had both class I and II DSA detected within 1st year.

Protocol Biopsy Findings Within 1st Year

Protocol biopsy results for those with at least one protocol biopsy ($n = 515$, 70%) are shown in **Table 3**. DSA+ patients had similar protocol biopsy rates vs. DSA- patients (73% [96 patients, 159 biopsies] vs. 69% [419 patients, 696 biopsies], $p = 0.36$). Mean cumulative acute and IFTA scores were similar at 3 and 12 months. Frequency of protocol biopsies with NI, SCI, and SC-TCMR were comparable among groups based on 3-months, 12-months, and maximum 1st year grade (**Table 3**). There was an increased incidence of SC-ABMR during the 1st year in DSA+ vs. DSA- patients (4% vs. 0%, $p < 0.001$), though overall occurrence was rare (0.8%) (**Table 3**). There were seven cases of SC-ABMR in six recipients (1 SC-ABMR alone, 3 with concurrent SCI, 3 with concurrent SC-TCMR).

For-Cause Biopsy Findings Beyond 1st Year

DSA+ patients had similar proportion of for-cause biopsies beyond 1st year vs. DSA- patients (23% [30 patients, 36 biopsies] vs. 25% [151 patients, 189 biopsies], $p = 0.62$, **Table 4**). The distribution of biopsy findings was similar between DSA+ and DSA- cohorts ($p = 0.35$, **Figures 2A,B**), including rates of overall clinical rejection (56% vs. 42%, $p = 0.14$). Clinical TCMR was lower among DSA+ patients (28% vs. 36%, $p < 0.001$), though severity of TCMR was similar (**Table 4**). The distribution of type rejection was different ($p < 0.001$) and favored more mixed rejection (ABMR + TCMR) in DSA+ patients (**Figures 2C,D**). Lastly, distribution of non-alloimmune events on for-cause biopsies was similar (16% vs. 14%, $p = 0.71$, **Table 4**).

Kidney Function

Using a linear mixed model, DSA+ and DSA- groups had similar eGFR over study period (54.8 vs. 53.8 ml/min/1.73 m^2, $p = 0.56$, **Figure 3**). Subgroup exploratory analysis in those patients with protocol biopsy revealed eGFR was similar among DSA+ vs. DSA- patients when stratified by 1st year subclinical events (NI, SCI, SC-TCMR), albeit there was slightly increased eGFR for DSA+ with SCI vs. DSA-with SCI patients ($p = 0.02$, 61 ml/min vs. 54 ml/min, **Supplementary Figure S1**).

Patient and Graft Survival

Overall, DSA+ patients had similar patient survival (83% vs. 90%, $p = 0.15$, **Figure 4A**), combined patient and graft survival (78% vs. 85%, $p = 0.09$, **Figure 4B**), DCGS (91% vs. 94%, $p = 0.30$, **Figure 4C**), and graft failure free survival (76% vs. 82%, $p = 0.11$, **Figure 4D**) vs. DSA- patients. Among DSA+ patients, survival was similar whether based on timing of detection (**Figure 5**) or DSA class (**Figure 6**). We also assessed survival stratified by protocol biopsy status. First, those with protocol biopsy had better patient survival ($p = 0.004$), combined patient and graft survival ($p = 0.02$), and graft failure free survival compared to those without protocol biopsy ($p = 0.045$), but not DCGS ($p = 0.68$) (**Supplementary Figure S2**). Among those without protocol biopsy, DSA+ patients had similar survival vs. DSA- patients (**Supplementary Figure S3**). Conversely, among those with at least one protocol biopsy, DSA+ patients had decreased patient ($p = 0.04$), patient and graft ($p = 0.02$), graft failure free survival ($p = 0.05$), but not DCGS ($p = 0.13$) compared to DSA-patients (**Supplementary Figure S4**).

Multivariate Analysis for Graft Failure

Given a trend towards worse graft failure free survival in DSA+ patients, specifically those who had a protocol biopsy, we performed a backwards cox regression multivariate analysis that found recipient age (1.03, 95% CI 1.01–1.05, $p = 0.01$) and DSA detection within 1st year (1.91, 95% CI 1.03–3.55, $p = 0.04$) as independent predictors for graft failure among those who had a protocol biopsy (**Table 5**).

Unstable Patients

Though excluded from our primary cohort analysis, we did note increased DSA within 1st year in unstable vs. DSA+ stable patients (30% vs. 18%, $p < 0.001$). We explored demographic differences among stable and unstable cohorts based on DSA status (**Supplementary Tables S2, S3**) and DSA characteristics among DSA+ patients (unstable vs. stable) (**Supplementary Table S4**). Interestingly, DSA- unstable patients received kidney transplants with higher KDPI and had more DGF than

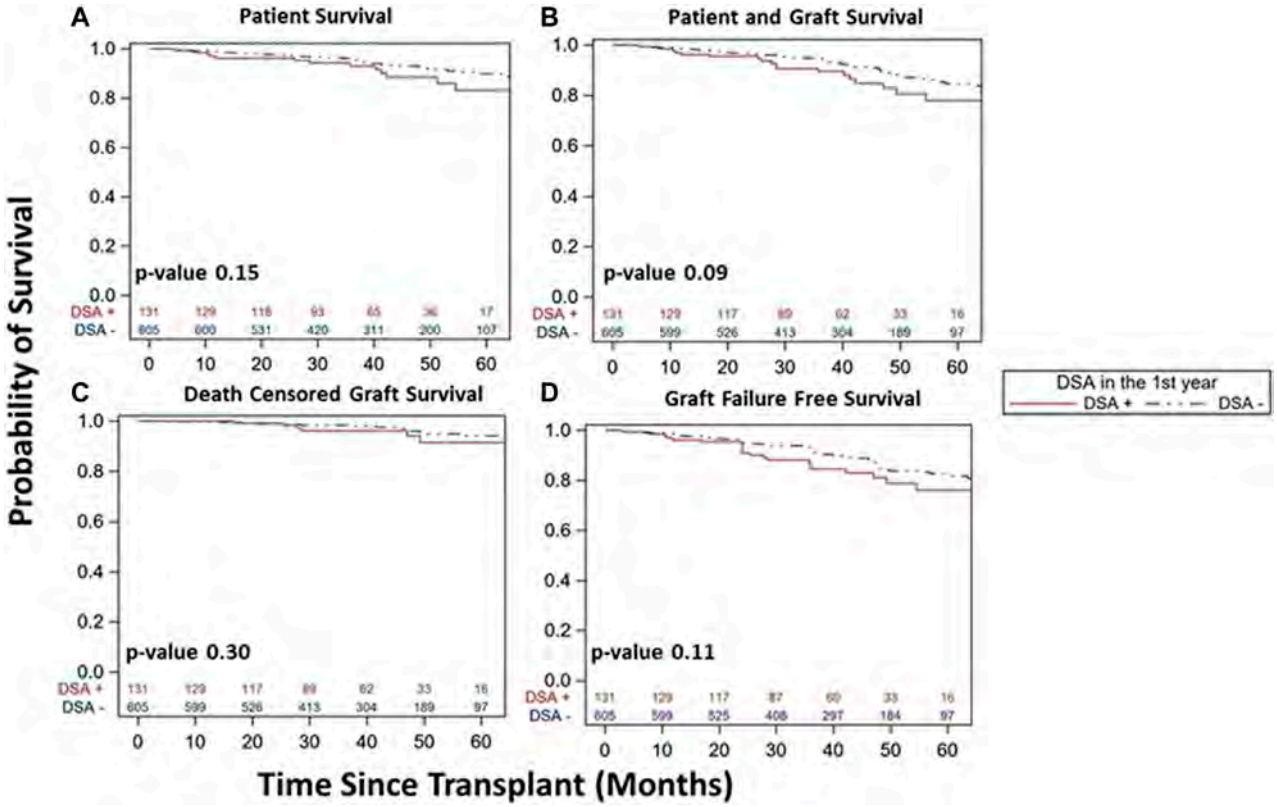


FIGURE 4 | Similar kidney transplant and patient survival was seen over study follow up in DSA+ and DSA- kidney transplant recipients with a stable 1st year post-transplant course. Kaplan-Meier survival curves demonstrated similar patient survival (**(A)**, 83% [95% CI 71–91%]) vs. 90% [95% CI 86–93%], $p = 0.15$), patient and graft survival (**(B)**, 78% [95% CI 65–86%] vs. 85% [95% CI 80–88%], $p = 0.09$), death censored graft survival (**(C)**, 91% [95% CI 80–96%] vs. 94% [95% CI 90–97%], $p = 0.30$), and graft failure free survival (**(D)**, 76% [95% CI 64–85%] vs. 82% [95% CI 77–87%], $p = 0.11$) among DSA+ and DSA-patients over study period follow up.

DSA- stable patients (**Supplementary Table S2**). Further, DSA+ unstable patients were younger (46 vs. 51, $p = 0.02$) with increased overall number of DSA tests, overall number of positive class II tests, and a trend towards more combined Class I and Class II DSA detection vs. DSA+ stable patients, though the timing of 1st positive test (Class I/II) was similar for DSA+ unstable vs. DSA+ stable patients (**Supplementary Tables S3, S4**). Patient survival, patient and graft survival, DCGS, and graft failure free survival were similar among DSA+ vs. DSA- unstable patients (**Supplementary Figure S5**). However, when all four groups were included, there was significant differences in survival among the four groups as the unstable cohort had inferior survival overall, particularly the DSA+ unstable group (**Figure 7**). Interestingly, when including entire population (both stable and unstable), DSA+ stable patients did have inferior patient/graft survival ($p = 0.001$), DCGS ($p = 0.03$), and graft failure free survival ($p = 0.001$) vs. DSA-stable patients (**Figure 7**).

DISCUSSION

While post-transplant DSA detection has been associated with inferior outcomes, not all patients with post-transplant DSA fare poorly. Thus, whether early post-transplant DSA screening should be widely used in stable patients for risk stratification remains unclear. To address the impact of DSA detection as an early post-transplant screening tool, we assessed DSA detection on screening testing in stable patients for associations with key clinical events.

In a cohort of 736 patients with a stable 1st year course, DSA detection was not associated with inferior function or survival. Among those who had a protocol biopsy, DSA was associated with graft failure on multivariate analysis and increased early incidence of SC-ABMR. Specifically, DSA+ patients had increased SC-ABMR, but did not have increased SCI, SC-TCMR, or early chronicity (ie IFTA). Similarly, previous studies displayed increased subclinical rejection (mostly ABMR) with protocol biopsies performed for DSA detection on screening without graft dysfunction, though those often were later (beyond 1 year) and again not all patients had rejection (8,26–30). Still, Loupy et al. noted early SC-ABMR may impact long-term outcomes (31). While data on treatment of early SC-ABMR is limited, treatment of late SC-ABMR (~55 months) may be effective, and thus diagnosing early SC-ABMR may be valuable (32). The reported incidence of SC-ABMR has been variable (~26–51%), which is likely related to DSA and biopsy timing (events beyond 1 year), and differing

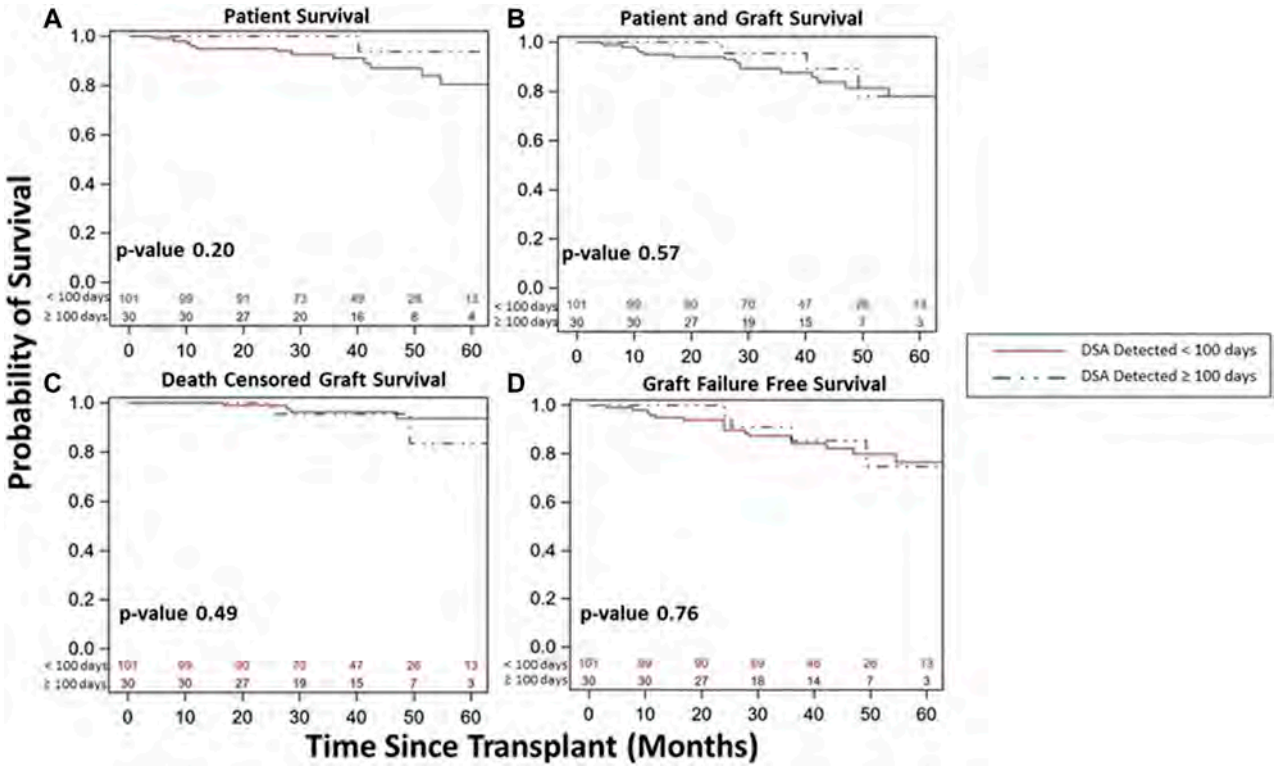


FIGURE 5 | Kidney transplant and patient survival for patients with DSA detected and a stable clinical course during the 1st year post kidney transplant based on timing of DSA detection status within the 1st year post-transplant (<100 days vs. ≥100 days). Kaplan-Meier survival curves demonstrating patient survival (**(A)**, 80% vs. 94%, $p = 0.20$), patient and graft survival (**(B)**, 78% vs. 78%, $p = 0.57$), death censored graft survival (**(C)**, 94% vs. 85%, $p = 0.49$), and graft failure free survival (**(D)**, 76% vs. 75%, $p = 0.76$) over study period follow up among patients with DSA detected and a stable clinical course during the 1st year post-transplant based on timing of DSA detection.

study cohorts and designs (biopsy for DSA detection without graft dysfunction) (8, 27–30). SC-ABMR was a rare overall event in our low risk cohort likely due to DSA detection timing (within 1 year) and biopsy approach (no protocol biopsies for isolated DSA detection), though similar with the rate of ABMR (3.7%), albeit clinical, within 1st year reported by Adebiyi et al in their cohort with pre-transplant DSA that had a negative FC (33).

Beyond the 1st year, DSA+ patients had similar kidney function and rates of clinical rejection, though distribution was towards more mixed rejection (ABMR + TCMR). Comparably, Bartel et al. demonstrated post-transplant anti-HLA antibody detection with a stable 1st year course was not associated with worse eGFR at 5 years (34). Later, Cooper et al. showed patients with DSA had more clinical rejection (TCMR and/or ABMR), though intermediate (up to 24 months) outcomes (eGFR, graft survival) were similar for those with DSA without clinical rejection and DSA detected on screening compared to those without DSA detection (9). Likewise, Devos et al. reported DSA detection was associated with increased clinical rejection and worse DCGS at intermediate follow up (~31 months), but there was no difference in graft survival or function for those with DSA without clinical rejection (35). While we recognize our DSA+ cohort as having DSA that was newly detected post-transplant, pre-existing DSA prior to transplant is possible given early DSA detection, which may explain similar survival outcomes as Aubert et al noted ABMR due to preexisting DSA occurs earlier than ABMR due to *de novo* DSA with better graft survival (36). Further, Adebiyi et al demonstrated a trend towards diminished DCGS in those with pre-transplant DSA, FC negative with post-transplant DSA versus those with no pre-transplant DSA or those with pre-transplant DSA but no post-transplant DSA (33). Likewise, in our study, DSA+ patients had similar survival (patient, DCGS) and function, though 1st year screening DSA detection was an independent predictor of graft failure on multivariate analysis only among those with protocol biopsy.

Previous studies were limited by smaller sample size and mixed testing indication (DSA and biopsy). For-cause DSA testing at time of dysfunction or for-cause biopsy biases towards adverse outcomes and is a different context than DSA screening testing in stable patients. Pediatric literature has demonstrated the reasoning (screening vs. for-cause) for DSA and biopsy testing matters in understanding DSA as a decision tool (37). Now, in a large adult cohort with clear testing indication, we demonstrate DSA detection on screening testing during the 1st year in stable kidney transplant patients was associated with increased SC-ABMR and was an independent predictor for graft failure among those who had a protocol biopsy, but not associated overall with inferior function or survival.

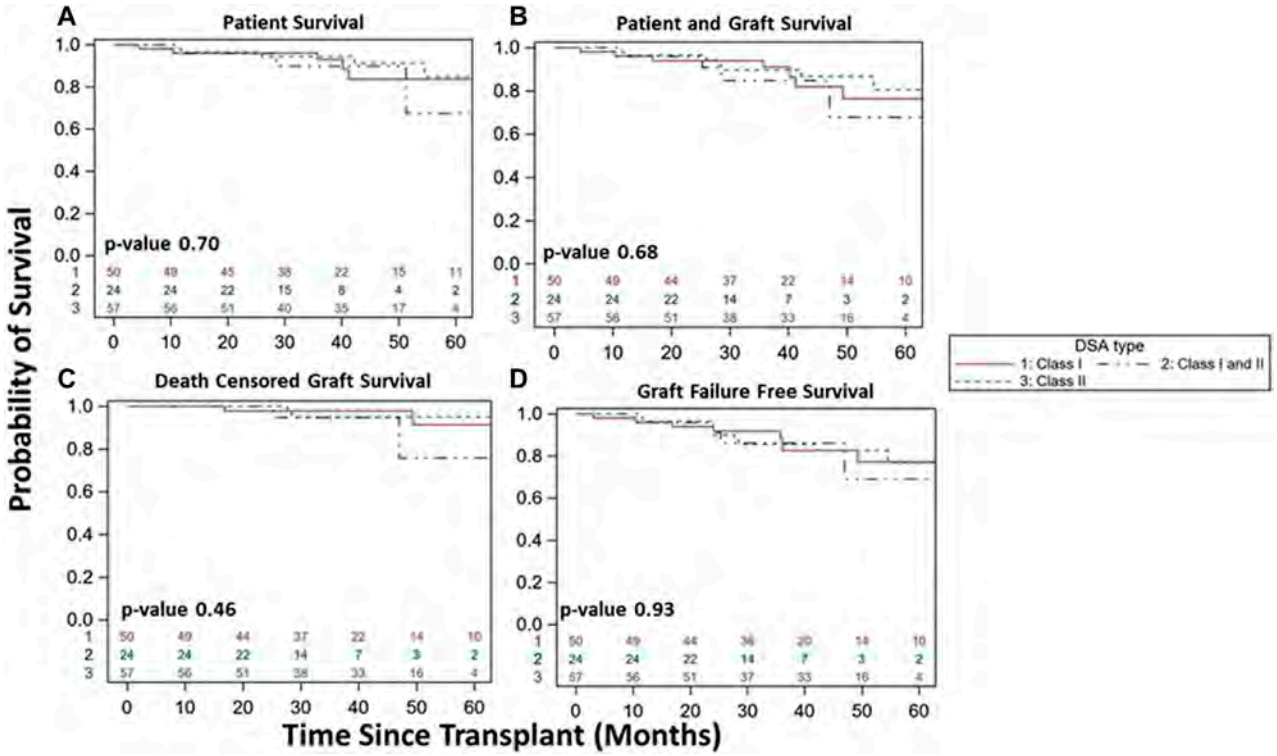


FIGURE 6 | Kidney transplant and patient survival for patients with DSA detected and stable clinical course during the 1st year post kidney transplant based on DSA class detected (Class I vs. Class I/II vs. Class II). Kaplan-Meier survival curves demonstrating patient survival (**(A)**, 84% vs. 67% vs. 85%, $p = 0.70$), patient and graft survival (**(B)**, 76% vs. 68% vs. 81%, $p = 0.68$), death censored graft survival (**(C)**, 91% vs. 76% vs. 95%, $p = 0.46$), and graft failure free survival (**(D)**, 77% vs. 69% vs. 77%, $p = 0.93$) over study period follow up among DSA+ patients with a stable clinical course during the 1st year post-transplant.

TABLE 5 | Adjusted multivariate analysis showing risk factors for developing graft failure among those with at least one protocol biopsy during the 1st year ($n = 515$).

	Hazard ratio (with 95% CI)	*p*-value
DSA during 1st year	1.91 (1.03–3.55)	**0.04**
Recipient Age	1.03 (1.01–1.05)	**0.01**
Subclinical TCMR during 1st year	1.14 (0.48–2.70)	0.76
Subclinical Inflammation during 1st year	0.90 (0.41–1.98)	0.80

Bold values considered statistically significant with p-value < 0.05.

Adjusted multivariate model was adjusted for recipient age, donor type (Living donor vs. Deceased donor), PRA I/II ≥ 90%, cPRA ≥ 90%, DGF, and SC-I/SC-TCMR, using a backward selection Cox Regression Model.

The strengths of our study include our large cohort with detailed histological (including protocol and for-cause biopsies) and clinical information. Further, our design to differentiate the reason for DSA and biopsy testing allowed better analysis of DSA as a screening tool in stable patients. Additionally, our study provided important information regarding the timing, type, and persistence of screening DSA in stable patients as well how these DSA and demographic characteristics for stable patients differed from those who had an unstable 1st year clinical course.

We acknowledge our study has limitations. First, our study is a single center study without an external validation cohort, which may limit broad applicability. Second, while our study was primarily focused on graft outcomes beyond the 1st year, we included the entire study period from the time of transplant for both our linear mixed eGFR model analysis and our Kaplan Meier survival analysis. Thus, we acknowledge results within the 1st year, while appearing similar, should be interpreted with caution as our study groups were defined by DSA detection at the end of the 1st year post-transplantation. Additionally, despite a large sample size, we could not perform adequate subgroup analysis among DSA+ patients for subclinical and clinical events to identify subgroups at higher risk who may benefit from more intense screening. Also, we did not have full information about HLA eplet mismatch load or about all DSA characteristics (MFI, titer, specificity, and complement binding), which both may allow better risk stratification, though limitations with MFI have been previously noted (13–15, 33, 38). Further, follow up period may be insufficient to detect true long-term differences in graft survival, though knowing this limitation, we did assess surrogate markers such as histology and eGFR. We also recognize the temporal relationship of DSA with both subclinical and clinical events, which we did not examine, though timing of first DSA detection was similar for stable vs. unstable groups. Also, previous studies suggest that not all DSA detection may precede rejection and this distinction may not impact associations with later events (5). As previously acknowledged, prior sera (>30 days

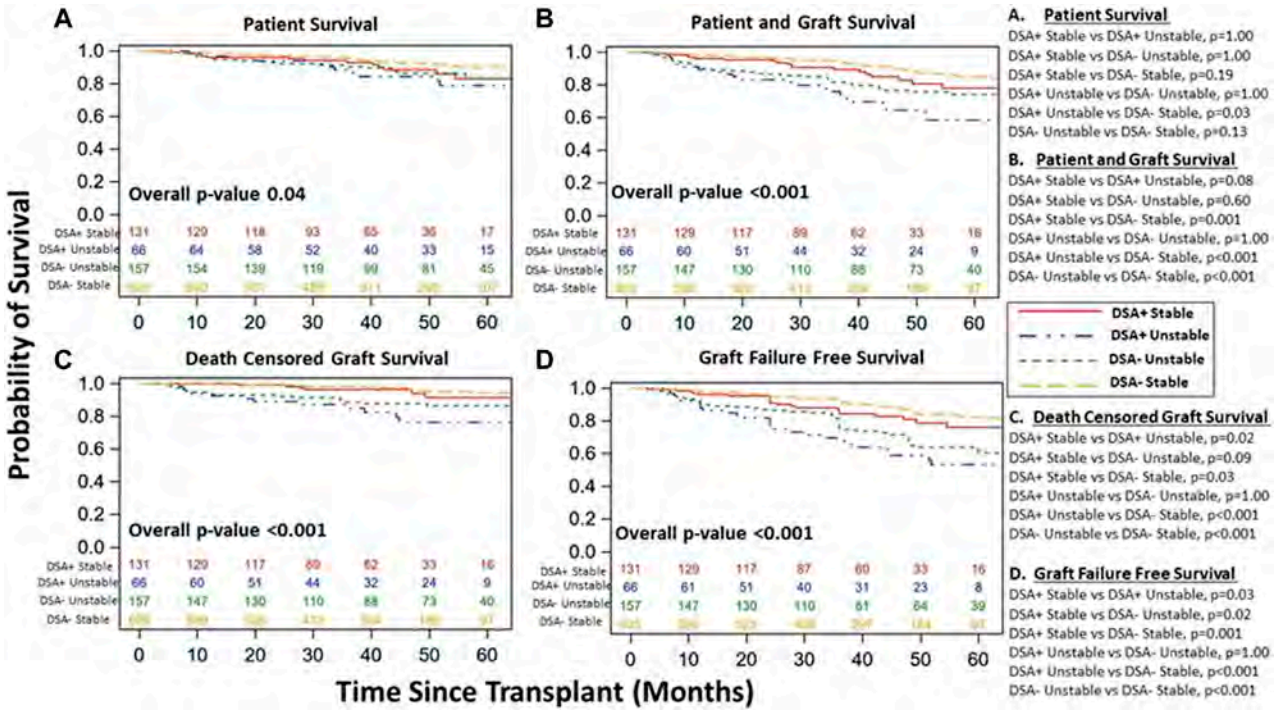


FIGURE 7 | Kidney transplant and patient survival for all patients (included and excluded patients) grouped based on DSA detection status (DSA+ versus DSA-) and clinical course (stable versus unstable) during the 1st year post kidney transplant. Kaplan-Meier survival curves demonstrating patient survival (**(A)**, DSA+ Stable 83% [95% CI 71–91%]) vs. DSA- Stable 90% [95% CI 86–93%] vs. DSA+ Unstable 76% [95% CI 61–86%] vs. DSA- Unstable 86% [95% CI 79–91%], $p = 0.04$), patient and graft survival (**(B)**, DSA+ Stable 78% [95% CI 65–86%] vs. DSA- Stable 85% [95% CI 80–88%] vs. DSA+ Unstable 58% [95% CI 43–71%] vs. DSA- Unstable 74% [95% CI 65–81%], $p < 0.001$), death censored graft survival (**(C)**, DSA+ Stable 91% [95% CI 80–96%] vs. DSA- Unstable 94% [95% CI 90–97%] vs. DSA+ Unstable 76% [95% CI 61–86%] vs. DSA- Unstable 86% [95% CI 79–91%], $p < 0.001$), and Graft Failure free survival (**(D)**, DSA+ Stable 76% [95% CI 64–85%] vs. DSA- Stable 82% [95% CI 77–87%] vs. DSA+ Unstable 53% [95% CI 38–66%] vs. DSA- Unstable 61% [95% CI 51–69%], $p < 0.001$) over study period follow up among all patients. The individualized comparisons for **(A–D)** are provided.

prior to transplant) was not analyzed for historical DSA and we recognize our MFI cut off value of 1,000 may have missed weak DSA (MFI <1,000) at the time of transplant. Thus, early DSA detected in our study may have been pre-formed, which may be different from *de novo* DSA, though we did assess survival outcomes based on timing of DSA detection, which was not different. More, we did not assess medication non-adherence, which has been linked with DSA detection and poor outcomes, though this has been previously explored (5, 39, 40). We also recognize that DSA detection on screening may have influenced optimization of immunosuppression, which we could not account for, and this itself may have affected outcomes. Lastly, we recognize that our study cohort was heterogenous as ~30% were without a protocol biopsy and this limited evaluation of subclinical events for all patients. However, we performed additional analysis assessing differences (demographics, survival) between those who did and did not receive protocol biopsies to give a more complete picture of our study cohort. Again, while heterogenous, our study represented an actual clinical practice where DSA screening would be used.

Nonetheless, we report key findings regarding early DSA screening among stable kidney transplant patients. Overall, DSA+ patients had similar function and survival vs. DSA- patients. In those with a protocol biopsy, DSA+ patients had increased incidence of SC-ABMR, with rare events overall, and similar incidence of SCI/SC-TCMR. Still, DSA detection was independently associated with graft failure among those who had a protocol biopsy. Lastly, DSA+ patients had similar incidence of clinical rejection on for-cause biopsies after 1 year vs. DSA- patients, though rejection was more mixed (ABMR + TCMR) in DSA+ patients. Additional studies involving multiple centers with an increased study population (especially given differences seen when including both stable and unstable cohorts, including between DSA+ vs. DSA- stable patients) and longer follow up may allow for more definitive evidence regarding the utility of DSA as an early post-transplant screening tool. More importantly, these types of studies may help definitively identify those patients who will benefit the most from intense early screening. Still, with our findings, a more targeted screening approach may increase the impact of DSA screening in stable patients and allow for more tailored medicine. Specifically, potential targeted approaches may include more intense screening in highly sensitized patients and/or those with increased DR mismatches, less intense screening in those with competing risk factors for graft loss (possibly similar to the non-protocol biopsy group), and/or using early intensive DSA screening within 6 months in all stable patients (majority of DSA+ tests were noted by this time, both for the stable and unstable cohorts) to guide further testing. Additionally, given

association with subsequent DSA development, the consideration of eplet mismatches to guide early post-transplant screening may also increase impact (41–43). Regardless, with focus on high value care, cost effectiveness for DSA as a screening tool must be assessed as well given a previous study estimated the cost of annual DSA screening at ~$480/year (range $300–1,000) and more recently, a single DSA screening test (combined for both Class I and II) was recently estimated at ~$680 based on recent United States Medicare data, both of which highlight the need for more targeted screening in those low risk patients with stable kidney function (44–45). Lastly, the context and reason for DSA testing matters and should be clearly delineated in further studies as DSA for-cause testing assists in decision making when faced with renal dysfunction or supplements abnormal pathology whereas DSA screening testing in those with stable function may identify those at increased risk but impact may be blunted when widely used.

In conclusion, DSA detected on screening in stable 1st year kidney transplant patients was independently associated with graft failure on multivariate analysis, however this was only true among patients who underwent at least one protocol biopsy.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy considerations.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at the University of Pittsburgh. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AS, DJ, and SH were all involved in the participation of research design, writing of the paper, performance of the research, and in data analysis. RM, PS, CP, CW, AT, MM, and AZ all participated in writing of the paper.

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.2022.10094/full#supplementary-material

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ORIGINAL RESEARCH
published: 14 March 2022
doi: 10.3389/ti.2022.10345

Trends and Outcomes of Hypothermic Machine Perfusion Preservation of Kidney Allografts in Simultaneous Liver and Kidney Transplantation in the United States

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Optimal kidney graft outcomes after simultaneous liver-kidney (SLK) transplant may be threatened by the increased cold ischemia time and hemodynamic perturbations of dual organ transplantation. Hypothermic machine perfusion (MP) of kidney allografts may mitigate these effects. We analyzed U.S. trends and renal outcomes of hypothermic non-oxygenated MP vs. static cold storage (CS) of kidney grafts from 6,689 SLK transplants performed between 2005 and 2020 using the United Network for Organ Sharing database. Outcomes included delayed graft function (DGF), primary non-function (PNF), and kidney graft survival (GS). Overall, 17.2% of kidney allografts were placed on MP. Kidney cold ischemia time was longer in the MP group (median 12.8 vs. 10.0 h; $p < 0.001$). Nationally, MP utilization in SLK increased from <3% in 2005 to >25% by 2019. Center preference was the primary determinant of whether a graft underwent MP vs. CS (intraclass correlation coefficient 65.0%). MP reduced DGF (adjusted OR 0.74; $p = 0.008$), but not PNF ($p = 0.637$). Improved GS with MP was only observed with Kidney Donor Profile Index <20% (HR 0.71; $p = 0.030$). Kidney MP has increased significantly in SLK in the U.S. in a heterogeneous manner and with variable short-term benefits. Additional studies are needed to determine the ideal utilization for MP in SLK.

Keywords: graft survival, delayed graft function, simultaneous liver-kidney transplantation, allograft preservation, allograft outcomes, primary non-function, center variability

OPEN ACCESS

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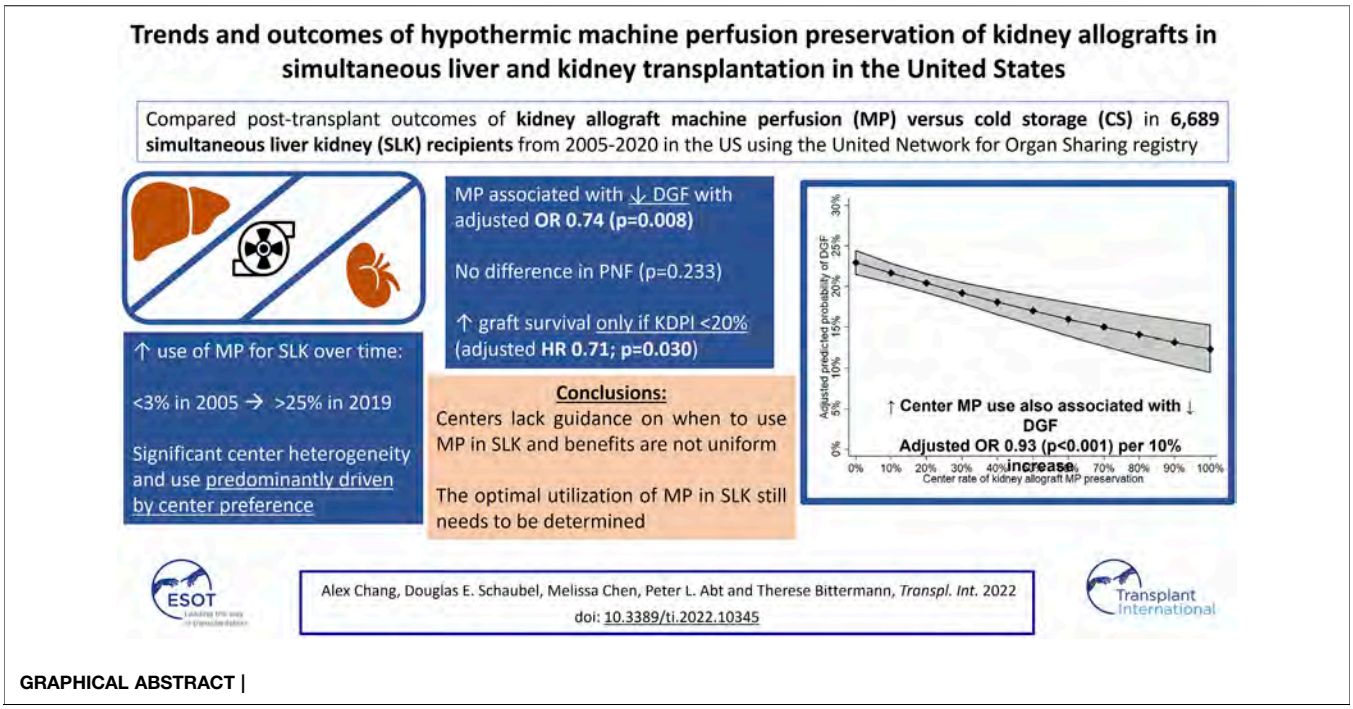
Received: *07 January 2022*
Accepted: *18 January 2022*
Published: *14 March 2022*

Citation:
Chang A, Schaubel DE, Chen M, Abt PL and Bittermann T (2022) Trends and Outcomes of Hypothermic Machine Perfusion Preservation of Kidney Allografts in Simultaneous Liver and Kidney Transplantation in the United States. Transpl Int 35:10345. doi: 10.3389/ti.2022.10345

INTRODUCTION

Outcomes after orthotopic liver transplantation (LT) are strongly associated with pre- and post-operative renal failure, and patient survival is significantly lower in recipients requiring long-term dialysis post-transplant (1). Thus, it is widely accepted that selected patients with pre-LT renal dysfunction be considered for simultaneous liver-kidney transplant (SLK) to improve their outcomes after LT (2). In the years following the introduction of the Model for End-stage Liver

Abbreviations: BMI, body mass index; CIT, cold ischemia time; COD, cause of death; CS, cold storage; DCD, donation after circulatory death; HCV, hepatitis C virus; HLA, human leukocyte antigen; KDPI, kidney donor profile index; LT, liver transplant; MELD, Model for End Stage Liver Disease; MP, machine perfusion; OPO, organ procurement organization; OPTN, Organ Procurement and Transplantation Network; PNF, primary non-function; SLK, simultaneous liver and kidney; UNOS, United Network for Organ Sharing.



GRAPHICAL ABSTRACT |

Disease (MELD) allocation system in 2002, the rate of SLKs increased dramatically (3). This partly resulted from an organ allocation system that prioritized candidates with worse renal function and from the implementation of policies that facilitated access to SLK. According to the most recently published national data, 7.1% of candidates on the LT waitlist were awaiting SLK and 8.6% of completed LTs were performed with a concurrent kidney transplant (KT) in 2018 (4). However, despite being of higher quality, kidney graft survival after SLK has been shown to be worse than after KT alone, particularly in the early post-LT period, which has been primarily attributed to the greater severity of illness of SLK recipients (5, 6).

Machine perfusion (MP) of deceased donor kidney grafts has been used as an alternative to static cold storage (CS) as a means to improve post-transplant kidney function, particularly for allografts of reduced quality (7). After the allograft is flushed free of blood, MP pumps hypo- or normothermic preservation solution through the renal vasculature in a manner that simulates natural organ perfusion, leading to clearance of toxic metabolites and reduced renovascular resistance (8). While MP has primarily been used in the setting of marginal kidney allografts for KT alone, a recent observational study by Lunsford et al. conducted at two U.S. transplant centers has suggested that MP may also improve kidney graft outcomes among SLK recipients (9).

Given these recent findings, we sought to evaluate 1) temporal and geographic changes in the use of kidney graft MP preservation in SLK and 2) evaluate the potential benefit of MP on patient and kidney graft outcomes in a national cohort.

METHODS

This was a retrospective cohort study using the United Network for Organ Sharing (UNOS) database. All adult (≥18 years), deceased-donor simultaneous liver-kidney (SLK) transplant recipients between January 1, 2005 and December 6, 2020 were identified. Recipients of prior solid organ transplant of any kind were excluded. Status 1 (i.e., emergent LT) recipients were additionally excluded.

The primary exposure of interest was receipt of a kidney allograft preserved using MP versus CS. Given the focus of the study, all analyses were restricted to SLK recipients for whom kidney allograft preservation data was available (98.8% of the initial cohort). While detailed information regarding MP protocols used was not available (e.g., duration, flow, resistance), it should be noted that all currently approved devices by the US Food and Drug Administration are hypothermic non-oxygenated systems. Recipient characteristics obtained at the time of SLK included: age, sex, race/ethnicity, kidney disease etiology, history of diabetes, native Model for End-stage Liver Disease (MELD), cirrhosis decompensations (ascites, hepatic encephalopathy), patient location prior to SLK (home, inpatient ward, intensive care unit), severity of renal disease at SLK (on dialysis, eGFR <30 ml/min/1.73 m^2 not on dialysis and eGFR ≥30 ml/min/1.73 m^2 not on dialysis), and duration of dialysis (among those on dialysis at SLK). Donor characteristics included: age, sex, race/ethnicity, hypertension, diabetes, body mass index (BMI), terminal creatinine, hepatitis C virus (HCV) antibody status, distance from recipient hospital and cause of death (COD). Additional allograft characteristics included donation after circulatory determination of death status (DCD), cold ischemic time (CIT), whether liver allograft

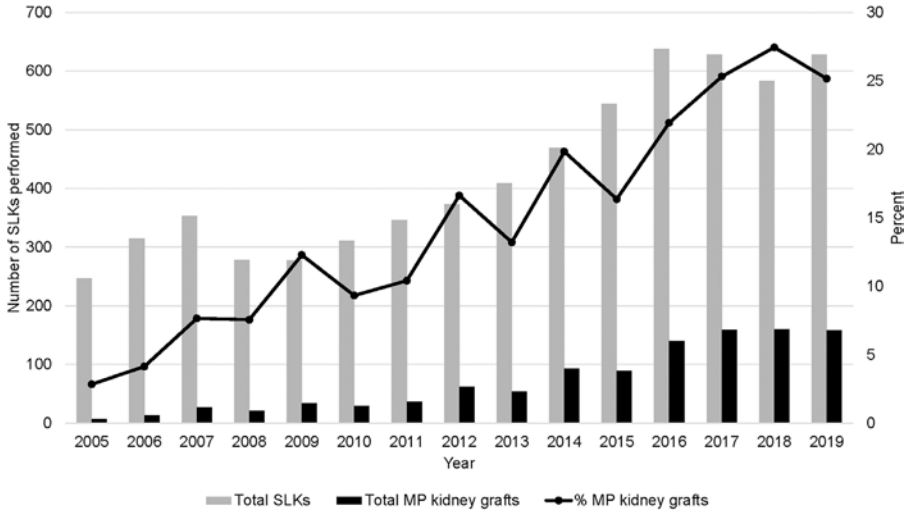


FIGURE 1 | Nationwide trends in MP use in SLK transplants from 2005–2019.

was split, Kidney Donor Profile Index (KDPI; categorized as <20%, 30–34%, 35–85%, and >85% (10, 11)) and share type (local, regional, national). Lastly, we also evaluated whether kidney implantation occurred on the same versus ≥1 day after the date of LT.

Recipient, donor and allograft characteristics were compared descriptively according to preservation using MP versus CS. Chi-squared tests and Kruskal-Wallis tests were used for categorical and continuous variables, respectively. Temporal, regional and center trends in MP use were also described. The geographic distribution of the 11 UNOS regions can be visualized for reference here: https://unos.org/community/regions/. In the first analysis, mixed-effects multivariable logistic regression was employed to evaluate the predictors of MP kidney allograft preservation. This model was adjusted for the aforementioned exposures as fixed effects (with the exception of KDPI to avoid collinearity, as the individual index components were already included) and transplant center as a random effect. From this model, the intraclass correlation coefficient (ICC) was obtained, which indicates the percent variability in MP perfusion across recipients that is explained by transplant center alone.

All subsequent statistical analyses evaluated receipt of MP as a predictor of recipient outcomes. Mixed-effects multivariable logistic regression was used to investigate kidney delayed graft function (DGF) and 2) kidney allograft primary non-function (PNF). Adjustment covariates included each of the aforementioned recipient and donor/allograft characteristics (except KDPI), as well as transplant era (2005–2009, 2010–2014, 2015–2020). All covariates were represented by fixed effect, with the exception of transplant center which was specified as a random effect in order to efficiently account for correlation among patients within-center. DGF was defined as receipt of dialysis within the first week after SLK (12, 13). PNF was defined as kidney graft failure ≤90 days from the date of SLK (14). Kidney graft survival was the time between transplantation and the earliest of retransplantation or death.

DGF was modeled using mixed logistic regression using all of the above-listed adjustment covariates and a random center effect. In the multivariable model investigating PNF, a parsimonious model was developed given the low number of events (a total of 124 patients experienced PNF). Stepwise forwards selection with p-value thresholds of <0.05 and ≥0.1 for entry and removal, respectively, was used to select covariates for the final model. Cox regression was used to model graft survival. Analogous to DGF, all of the adjustment covariates were included, with center again represented through a random effect.

After fitting each of the above-described models, we evaluated interactions with kidney allograft perfusion strategy. To evaluate the interactions with MP, we adopted the same general strategy for each of the tree outcomes. In particular, all main effects remained in the model. First, we evaluated each interaction separately one at a time. Second, any significant interactions would then be evaluated simultaneously to avoid confounding. In order to ensure clinical interpretability of our findings, we restricted attention to a pre-specified set of covariates for which interaction with MP was felt by the investigators to have biological plausibility. This set included each of the KDPI components (i.e., donor age, race/ethnicity, BMI, history of hypertension, history of diabetes, cause of death, terminal creatinine, HCV antibody status, and DCD status (15)), KDPI (categorized as <20%, 30–34%, 35–85%, and >85% (10, 11)), renal allograft CIT (continuous) and recipient renal disease severity (on dialysis, eGFR <30 ml/min/1.73 m^2 not on dialysis and eGFR ≥30 ml/min/1.73 m^2 not on dialysis). In models evaluating the interaction of MP and KDPI, the individual components of the KDPI were not included given concern for collinearity and lack of interpretability. Note that, for PNF, we excluded covariates not chosen earlier (for the main effects model) from the above list of potential interaction variables.

Next, we carried out secondary analyses. First, we evaluated unadjusted rates of each outcome according to whether kidney implantation was delayed or not among those undergoing MP preservation using descriptive statistics. Second, we replaced the

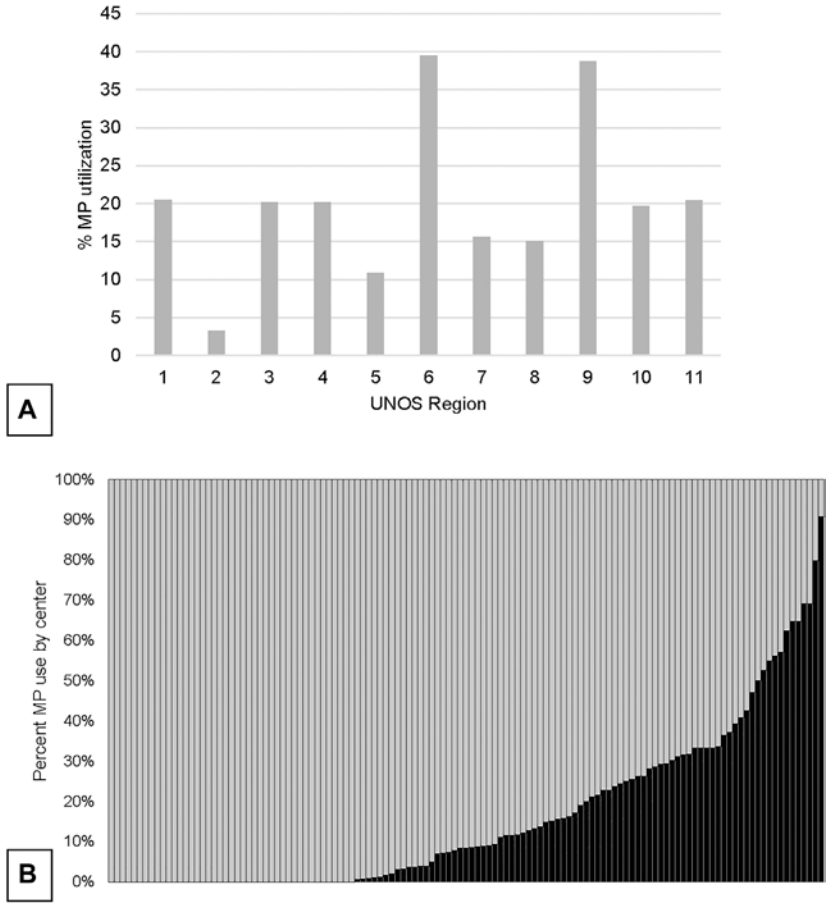


FIGURE 2 | Variation in overall utilization of MP in SLK by UNOS region **(A)** and by center **(B)**.

(patient-level) MP indicator with center-level percentage of patients transplanted with a perfused kidney. This equates to changing the question posed from "what is the effect of MP on patients" outcome in the primary analyses to "what is the effect of a center using more kidney MP on patients" outcome (i.e., irrespective of type of kidney perfusion strategy received). Center MP rate was evaluated as a predictor of each of the three outcomes (DGF, PNF, kidney graft survival) without adjusting for center (since doing so is inappropriate in the presence of center-level covariates). For the models evaluating DGF and kidney graft survival, the final multivariable model adjusted for all covariates. For the model evaluating PNF, the same covariate selection method described previously was used, which selected the same covariates as in the primary multivariable model.

All analyses were performed using STATA v16 (College Station, TX, United States). This study was approved by the Institutional Review Board of the University of Pennsylvania.

RESULTS

There were 6,689 recipients of SLK between January 1, 2005 and December 6, 2020. Allograft storage type (i.e., MP vs. CS) was available in 6,610 (98.8%) recipients. Of these, 5,474 (82.8%) kidney allografts for SLK underwent static CS, while 1,136 (17.2%) received MP preservation.

Concurrent to the increase in SLK volume between 2005 and 2018, the utilization of kidney allograft MP also increased from 2.8% in 2005 to 25.2% in 2019 (**Figure 1**). There was significant geographic variability in the utilization of MP for SLK between UNOS regions and individual transplant centers (**Figure 2**). UNOS region two had the lowest utilization, with 3.3% of 668 SLKs between 2005 and 2020, while region six had the highest with 39.5% of 119 SLKs. Of the 125 centers included in the analysis, 34.4% (N = 43 centers) exclusively used CS in SLK. MP use at the remaining 82 centers ranged from 0.6% to 90.9%. There was no correlation between center MP use and center SLK volume (p = 0.131), median KDPI (p = 0.743) or median SLK waiting time (p = 0.455).

Donor and Recipient Characteristics According to Kidney Allograft Preservation Technique

Donors whose kidneys underwent MP were older than those undergoing CS: median 36 (IQR: 24–47) versus 34 (IQR: 26–49)

TABLE 1 | Donor characteristics according to kidney allograft preservation technique (N = 6,610).

	Cold preservation	Machine perfusion	*p*-value
	N = 5,474	N = 1,136	
Sex, N (%)			0.660
Male	3,388 (61.9)	711 (62.6)	
Female	2,086 (38.1)	425 (37.4)	
Age (years), median (IQR)	34 (24–47)	36 (26–49)	<0.001
Race/ethnicity, N (%)			0.341
White	3,569 (62.5)	750 (66.0)	
Black	849 (15.5)	169 (14.9)	
Hispanic	836 (15.3)	183 (16.1)	
Asian/other	220 (4.0)	34 (3.0)	
Hypertension, N (%)	1,153 (21.2)	264 (23.5)	0.091
Diabetes, N (%)	228 (4.2)	71 (6.3)	0.002
KDPI category, N (%)			0.003
<20%	1,933 (36.5)	356 (31.4)	
20–34%	1,022 (18.7)	237 (20.9)	
35–85%	2,235 (40.9)	482 (42.5)	
>85%	210 (3.9)	59 (5.2)	
DCD donor, N (%)	224 (4.5)	90 (7.9)	<0.001
Kidney CIT (hours), median (IQR)	10.0 (7.7–12.8)	12.8 (9.4–21.7)	<0.001
Liver CIT (hours), median (IQR)	6.1 (5.0–7.7)	6.0 (4.7–7.6)	0.074
Split liver, N (%)	81 (1.5)	12 (1.1)	0.270
Distance to donor (miles), median (IQR)	59 (8–158)	52 (8–166)	0.492
Share type, N (%)			0.018
Local	4,099 (74.9)	858 (75.5)	
Regional	1,241 (22.7)	235 (20.7)	
National	134 (2.5)	43 (3.8)	
Cause of death, N (%)			0.002
Anoxia	1,625 (29.7)	407 (35.8)	
Stroke	1,494 (27.3)	277 (24.4)	
Head trauma	2,188 (40.0)	420 (37.0)	
CNS tumor	35 (0.64)	5 (0.4)	
Other	132 (2.4)	27 (2.4)	
BMI (kg/m^2), median (IQR)	25.8 (22.8–29.8)	26.4 (23.3–30.1)	0.001
Terminal creatinine (mg/dl), median (IQR)	0.9 (0.7–1.2)	0.9 (0.7–1.2)	0.267
HCV antibody positive, N (%)	399 (7.3)	88 (7.8)	0.599

years ($p < 0.001$, **Table 1**), though this difference was small. They were also more likely to have diabetes: 6.3% versus 4.2% ($p = 0.002$). There was no statistically significant difference in donor sex, race/ethnicity, terminal creatinine, or HCV antibody status between allografts preserved using MP versus CS. Kidney allografts undergoing MP were more often DCD organs (7.9% vs. 4.5%, $p < 0.001$) and had longer CIT (median 12.8 vs. 10.0 h, $p < 0.001$). Of note, there was no statistical difference in liver allograft CIT between groups ($p = 0.074$). A trend towards higher KDPI among recipients of MP preserved kidney allografts was noted ($p = 0.003$; **Table 1**).

Few recipient characteristics were associated with kidney allograft MP versus CS preservation (**Table 2**). For example, no statistically significant differences were observed with regards to age, sex, or native MELD score. While statistically significant, differences in cirrhosis decompensations such as ascites severity or hepatic encephalopathy grade were clinically less relevant, as they were very small ($p < 0.001$ and $p = 0.018$, respectively). There was no statistically significant difference in pre-LT renal disease severity between groups ($p = 0.458$). However, among recipients on dialysis pre-SLK (N = 4,590), pre-transplant dialysis duration was longer for patients receiving allografts preserved using MP: median 6.1 months versus 3.7 months ($p < 0.001$). Etiology of kidney disease was also different ($p < 0.001$) with those having hepatorenal syndrome receiving MP kidney grafts more frequently than those with cold storage (40.9% vs. 30.2%). Kidney implantation occurred ≥1 day after LT for 34.9% of patients in the MP group versus 13.9% in the CS group ($p < 0.001$).

Predictors of Kidney Allograft MP Preservation in SLK

In adjusted analyses, several predictors of kidney allograft MP preservation were identified (**Supplementary Table S1**). These included: increasing donor age (OR 1.02 per 1 year increase, 95% CI: 1.01–1.03, $p < 0.001$), DCD status (OR 2.81, 95% CI: 1.88–4.20; $p < 0.001$), kidney allograft CIT (1.10 per 1 h increase, 95% CI: 1.08–1.11; $p < 0.001$), donor terminal creatinine (OR 1.22 per 1 mg/dl increase, 95% CI: 1.08–1.39; $p = 0.001$), and donor BMI (OR 1.02 per 1 kg/m^2 increase; 95% CI 1.00–1.04; $p = 0.020$). Regionally shared kidney allografts were associated with less use of MP preservation (OR 0.47 vs. local, 95% CI: 0.36–0.61; $p < 0.001$). Transplant era was strongly associated with MP use: OR 2.42 (95% CI: 1.72–3.39) for

TABLE 2 | Recipient characteristics at LT according to donor kidney allograft preservation technique (N = 6,610).

	Cold storage	Machine perfusion	*p*-value
	N = 5,474	N = 1,136	
Sex, N (%)			0.324
Male	3,482 (63.6)	705 (62.1)	
Female	1,992 (36.4)	431 (37.9)	
Age (years), median (IQR)	58 (51–63)	58 (52–64)	0.222
Race/ethnicity			0.072
White	3,386 (61.9)	724 (63.7)	
Black	807 (14.7)	158 (13.9)	
Hispanic	992 (18.1)	196 (17.3)	
Asian	211 (3.9)	32 (2.8)	
Other	78 (1.4)	26 (2.3)	
Native MELD at SLK, median (IQR)	28 (23–35)	28 (23–35)	0.457
Ascites, N (%)			<0.001
None	885 (16.2)	230 (20.3)	
Mild	2,182 (40.1)	385 (34.0)	
Moderate-severe	2,381 (43.7)	519 (45.8)	
Encephalopathy, N (%)			0.018
None	1,697 (31.2)	401 (35.4)	
Grade 1–2	2,991 (54.9)	577 (50.9)	
Grade 3–4	760 (14.0)	156 (13.8)	
Preop location, N (%)			0.514
Home	3,142 (57.5)	674 (59.4)	
Inpatient ward	1,311 (24.0)	262 (23.1)	
ICU	1,008 (18.5)	199 (17.5)	
Diabetes, N (%)	2,356 (43.3)	488 (43.2)	0.905
Kidney disease severity, N (%)			
eGFR ≥30 ml/min/1.73 m^2[a]	677 (12.7)	157 (14.1)	0.458
eGFR <30 ml/min/1.73 m^2[a]	1,445 (27.1)	295 (26.4)	
On dialysis	3,213 (60.2)	665 (59.5)	
Dialysis time[b] (months), median (IQR)	3.7 (0.9–14.9)	6.1 (1.5–21.5)	<0.001
Etiology of kidney disease, N (%)			<0.001
Hepatorenal syndrome	1,655 (30.2)	465 (40.9)	
Diabetes	1,134 (20.7)	225 (19.8)	
Glomerular disease	426 (7.8)	78 (6.9)	
Polycystic kidney disease	278 (5.1)	88 (7.8)	
Hypertension	476 (8.7)	74 (6.5)	
Other	1,505 (27.5)	206 (18.1)	
KT implantation ≥1 day after LT, N (%)	760 (13.9)	396 (34.9)	<0.001

[a]*Not on dialysis pre-LT.*
[b]*Among patients receiving dialysis prior to SLK (N = 4,590).*

TABLE 3 | Summary of findings obtained from multivariable models evaluating kidney allograft preservation type as a predictor of kidney graft outcomes after SLK.

	Point estimate (95%CI) for kidney allograft MP compared to CS	*p*-value
Kidney delayed graft function	OR 0.74 (0.60–0.92)	0.008
Kidney primary non-function	OR 0.88 (0.52–1.49)	0.637
Kidney graft survival	HR 0.91 (0.78–1.06)	0.230

2010–2014 and OR 6.03 (4.30–8.44) for 2015–2020 versus 2005–2009 ($p < 0.001$). The ICC for transplant center in this model was 65.0%. This indicates that nearly two-thirds of the variability in MP use across SLK recipients was explained by the transplanting center alone, while donor and recipient factors explained only a minority.

Kidney Allograft Preservation Technique and Delayed Graft Function

DGF occurred in 256 recipients after MP and 1,311 recipients after CS (22.5% vs. 24.0%, $p = 0.293$). There was no statistical difference in DGF rates among MP allografts implanted on the same versus on a subsequent date from LT (22.0% vs. 23.6%; $p = 0.554$). Accounting for recipient and donor covariates, transplant era and transplant center, MP was significantly associated with DGF in the final multivariable model with a covariate-adjusted OR of 0.74 (95% CI: 0.60–0.92; $p = 0.008$; **Table 3**). The results of the full multivariable model are shown in **Supplementary Table S2**. There were no statistically significant interactions found between kidney allograft preservation type and any of the covariates evaluated.

As a secondary analysis, center kidney allograft MP use was evaluated as an independent predictor of recipient DGF. Center practice was found to be associated with a reduction

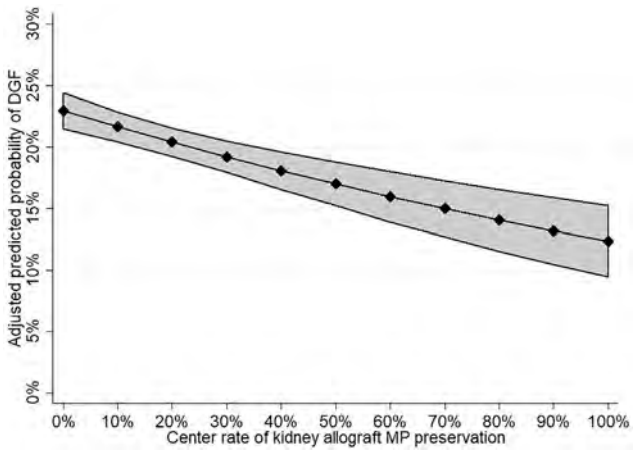


FIGURE 3 | Adjusted predicted probability of DGF according to increasing center MP use.

in the odds of DGF in both univariable (OR 0.94 per 10% increase in center MP use, 95% CI: 0.92–0.97; $p < 0.001$) and multivariable analyses (OR 0.93 per 10% increase in MP use, 95% CI: 0.90–0.96; $p < 0.001$; **Supplementary Table S3**). The predictive margins of DGF by increasing center kidney allograft MP are shown in **Figure 3**.

Kidney Allograft Preservation Technique and Primary Non-function

Kidney allograft PNF occurred in 19 patients after MP and 105 patients after CS (1.9% vs. 2.1%, $p = 0.666$). There was no difference in PNF rate for MP kidneys with delayed implantation (2.0% vs. 1.9%; $p = 0.849$). MP was not associated with PNF in the final multivariable model: covariate-adjusted OR 0.88 (95% CI: 0.52–1.49; $p = 0.637$; **Supplementary Table S4**). No statistically significant interaction was found between MP use and any of the covariates studied, which included recipient renal disease severity, kidney donor KDPI, donor age, donor BMI, donor hypertension, donor cause of death or kidney allograft CIT. In secondary analyses, center MP use was not associated with kidney allograft PNF on either univariable (OR 0.94 per 10% increase in MP use, 95% CI: 0.86–1.03; $p = 0.180$) or multivariable analyses (OR 0.94, 95% CI: 0.85–1.04; $p = 0.233$; **Supplementary Table S5**).

Kidney Allograft Preservation Technique and Kidney Allograft Survival

Kidney allograft MP was not associated with unadjusted or adjusted kidney graft survival, defined as a combined end-point of kidney graft failure or patient death: HR 0.95 (95% CI: 0.82–1.10, $p = 0.481$) and HR 0.91 (95% CI: 0.76–1.03; $p = 0.230$; **Supplementary Table S6**), respectively. Of the covariates evaluated for interaction with kidney allograft perfusion type, the following were statistically significant: donor KDPI category ($p = 0.029$) and donor cause of death ($p = 0.039$). The results of the multivariable model including the interaction of perfusion type and KDPI category are shown in **Supplementary Table S7**. In stratified models by KDPI category, MP was associated with improved graft survival in the setting of KDPI <20% (adjusted HR 0.71, 95% CI: 0.53–0.97; $p = 0.030$, but not with higher KDPI ($p = 0.677$ for KDPI 20–34%, $p = 0.339$ for 35–85% and $p = 0.071$ for >85%; **Figure 4**). Unfortunately, the interaction between perfusion type and donor cause of death was entirely driven by the "other" category, which is clinically uninterpretable and, thus, not included in the final model. Center MP use was not associated with kidney graft survival in multivariable analyses: covariate-adjusted HR 1.00 (95% CI: 0.98–1.03; $p = 0.851$; **Supplementary Table S8**). In unadjusted analyses, there was no improvement in kidney graft survival among MP allografts with delayed implantation (log-rank $p = 0.741$).

DISCUSSION

In this analysis of national data over a 15-year period, we show that the use of MP preservation in SLK has markedly increased in the U.S. over time, accounting for 1 in 4 kidney allografts since 2017. Several studies have shown benefits of MP preservation compared to static CS in the setting of KT alone (16–18). However, its potential benefits have not been rigorously studied in SLK transplantation until now, a scenario in which 1) increased kidney allograft quality and 2) the added complexity of dual-organ transplantation may reduce the advantages of MP preservation. In this study, we find a significant reduction in kidney DGF with MP preservation and increased center MP utilization also predicted lower DGF. In contrast, we found no association between kidney allograft MP and PNF, and only benefits with respect to kidney graft survival among the highest quality kidney allografts. The present study additionally demonstrates large practice variability between transplant centers in the choice of kidney allograft preservation modality in SLK. In fact, where one undergoes SLK explained the majority of the variability in kidney allograft MP use, while the 25 other donor and recipient factors were lesser determinants. It is likely that anecdotal experience and the existing evidence-base in the KT alone population has driven the rapid expansion of MP preservation for SLKs at these centers. However, further studies are needed to more clearly delineate which SLK recipients stand most to benefit from the added resources and associated costs of kidney MP preservation.

It is well-established that SLK recipients have access to the highest quality kidney allografts (19). In several meta-analyses of clinical trials, MP preservation in KT alone has benefits with regards to short- and long-term graft outcomes. This has not only been demonstrated in marginal donor kidneys but also in standard quality organs (7, 20). This technique has also been shown to be more cost-effective over CS, irrespective of kidney graft quality (21). Yet, perhaps surprisingly, in the SLK population, we only found evidence of reduced DGF and limited improvements in long-term graft outcomes, despite accounting for other measures of inferior allograft quality in our analyses. Nevertheless, the significant reduction in DGF in this population should not be overlooked, particularly given

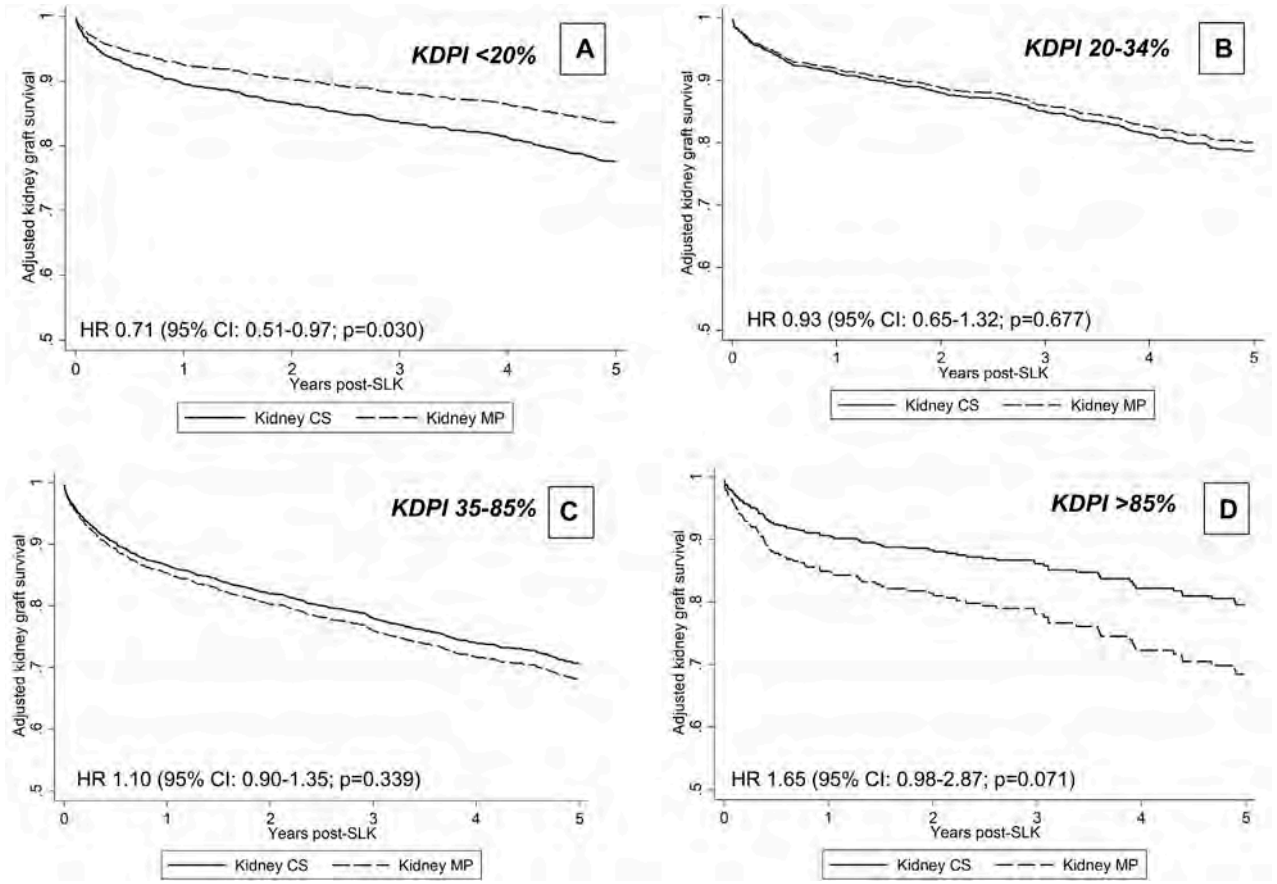


FIGURE 4 | Adjusted graft survival associated with MP preservation according to KDPI group.

the high quality of kidney allografts allocated to SLK recipients.

Recipient factors play important and unique roles in the development of poor kidney graft outcomes in the SLK population. These include, among others, increased liver disease severity, intra-operative challenges (e.g., volume shifts, transfusion requirements, electrolyte disturbances) and prolonged post-transplant recovery than KT alone recipients. The significant contribution of recipient factors on graft outcomes may explain why the benefits of MP were only observed in those receiving allografts with KDPI <20%, which represented 35.5% of the cohort. Delayed kidney implantation (as evidenced by the longer kidney CIT and difference in transplant dates recorded) was more frequent in the MP group. However, we did not observe any differences in unadjusted graft outcomes according to timing of kidney implantation in the MP group. Thus, the proposed benefits of MP preservation to allow for delayed KT in a more optimal recipient milieu after LT remain uncertain.

Our results using national data differ from those published by others reporting their own center-specific experiences, in which MP preservation with delayed KT implantation offered clear superior results, including resultant effects on patient survival (9, 22). These differences in findings are likely partly explained by the association between increasing center preference for MP and the associated reduction in DGF identified in this study, as centers with established MP protocols are more likely to publish on their experiences compared to those that seldom use MP. In addition, while we were able to determine type of preservation modality and duration of CIT, whether centers and organ procurement organizations (OPOs) differed with respect to the proportion of time spent on pump, time from procurement to placement on pump, other aspects of MP-related management and decision-making regarding potential delayed timing of implantation were not known. This may also explain why smaller gains were observed with kidney allograft MP preservation when this practice was evaluated nationally, and which would highlight the need for more clearly defined "best practices" regarding when and how to employ MP preservation to maximize its impact on kidney graft outcomes in SLK. Further research using more comprehensive donor data and allograft quality indicators, such as that collected from OPOs, may provide greater insights into the ideal setting to use MP preservation in SLK.

While the use of a national cohort offers advantages, there are also inherent study limitations. All commercially available MP devices for kidney allografts in the US are hypothermic non-oxygenated systems. However, more granular data regarding the duration of pumping and other MP parameters (e.g., flow, resistance) were not available and likely varied by center and OPO. This may have biased

certain results towards the null. In addition, while we were able to examine common recipient and allograft predictors of MP use, more comprehensive details on centers' decision-making and protocols are not known. Similarly, there may be differences regarding kidney allograft management that occurred at the OPO-level before the organ arrived at the transplanting center. If heterogeneity in MP protocol is indeed the explanation for the null result obtained in our study, then this speaks to the need for greater evidence-based guidance on its use and further multi-center studies are warranted that could address this evidence gap. The relationship observed between increasing center MP use and declining DGF rates may support the notion that centers with more MP experience use this technology more effectively and thus a "learning curve" for MP exists, which may further contribute to the outcomes seen.

Other limitations of registry data include diminished donor and recipient clinical detail. This could have led to unmeasured confounding and subsequent bias in our results. There were also no recipient peri- or post-operative clinical details between transplant surgeries to confirm that the longer kidney CIT and differences in KT versus LT transplant dates recorded for the MP group indeed reflected the intention to delay kidney implantation to allow for a more favorable recipient clinical status. Supporting this is the fact that indicators of kidney allograft quality and recipient factors explained only a minority of the variability in MP use across centers, and thus this decision-making infrequently takes into account key variables known to be associated with inferior kidney graft outcomes (23–25). Given the available variables, geographic trends analysis was limited to UNOS regions. This issue should be re-evaluated in the future, particularly in the context of the new liver allocation system in the U.S, which has led to greater transportation of allografts (26). Lastly, the imbalance between the MP and CS sample sizes may have led to imprecision in the point estimates and the adjustment of measured confounders in the multivariable models. In particular, given the low number of PNF events particularly among MP patients, it is likely that power was inadequate to detect a significant difference. Moreover, given the low frequency of high KDPI kidneys in this SLK cohort, a potential difference in graft survival with MP may have been missed.

A rapidly increasing use of MP for storage of kidney allografts prior to SLK transplantation has occurred in the U.S. that is predominantly driven by transplant center preference. While MP kidney allograft preservation affords a reduction in DGF, its impact on longer-term outcomes for the majority of recipients remain uncertain. There is a need to understand the cost-effectiveness and logistical implications of this increasing MP use (with or without kidney implantation delay), and more comprehensive guidance is also warranted with respect to when and how to best use this potentially valuable technology in the SLK population.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://unos.org/data/.

ETHICS STATEMENT

This study was approved by the Institutional Review Board of the University of Pennsylvania.

AUTHOR CONTRIBUTIONS

AC: research design, data interpretation, and writing of the manuscript. DS: data interpretation, editing of the manuscript MC: research design and writing of the manuscript. PA: research design and editing of the manuscript. TB: research design, performance of the statistical analyses, data interpretation, and writing of the manuscript.

FUNDING

TB is supported by a Career Development Award from the National Institute of Diabetes and Digestive and Kidney Diseases (K08-DK117013). DS is supported by R01-DK070869 from the National Institute of Diabetes and Digestive and Kidney Diseases.

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.2022.10345/full#supplementary-material

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ORIGINAL RESEARCH
published: 17 March 2022
doi: 10.3389/ti.2022.10078

The Utility of Pre- and Post-Transplant Oral Glucose Tolerance Tests: Identifying Kidney Transplant Recipients With or at Risk of New Onset Diabetes After Transplant

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Background: New onset diabetes after transplant (NODAT) is common in kidney transplant recipients (KTRs). Identifying patients at risk prior to transplant may enable strategies to mitigate NODAT, with a pre-transplant oral glucose tolerance test (OGTT) suggested by the KDIGO 2020 Guidelines for this purpose.

Methods: We investigated the utility of pre- and post-transplant OGTTs to stratify risk and diagnose NODAT in a retrospective, single-centre cohort study of all non-diabetic KTRs transplanted between 2003 and 2018.

Results: We identified 597 KTRs who performed a pre-transplant OGTT, of which 441 had their post-transplant glycaemic status determined by a clinical diagnosis of NODAT or OGTT. Pre-transplant dysglycaemia was identified in 28% of KTRs and was associated with increasing age ($p < 0.001$), BMI ($p = 0.03$), and peritoneal dialysis ($p < 0.001$). Post-transplant dysglycaemia was common with NODAT and impaired glucose tolerance (IGT) occurring in 143 (32%) and 121 (27%) patients, respectively. Pre-transplant IGT was strongly associated with NODAT development (OR 3.8, $p < 0.001$).

Conclusion: A pre-transplant OGTT identified candidates at increased risk of post-transplant dysglycaemia and NODAT, as diagnosed by an OGTT. Robust prospective trials are needed to determine whether various interventions can reduce post-transplant risk for candidates with an abnormal pre-transplant OGTT.

Keywords: new onset diabetes after transplant, impaired glucose tolerance, oral glucose tolerance test, kidney transplant, cohort study, NODAT, OGTT, transplant recipients

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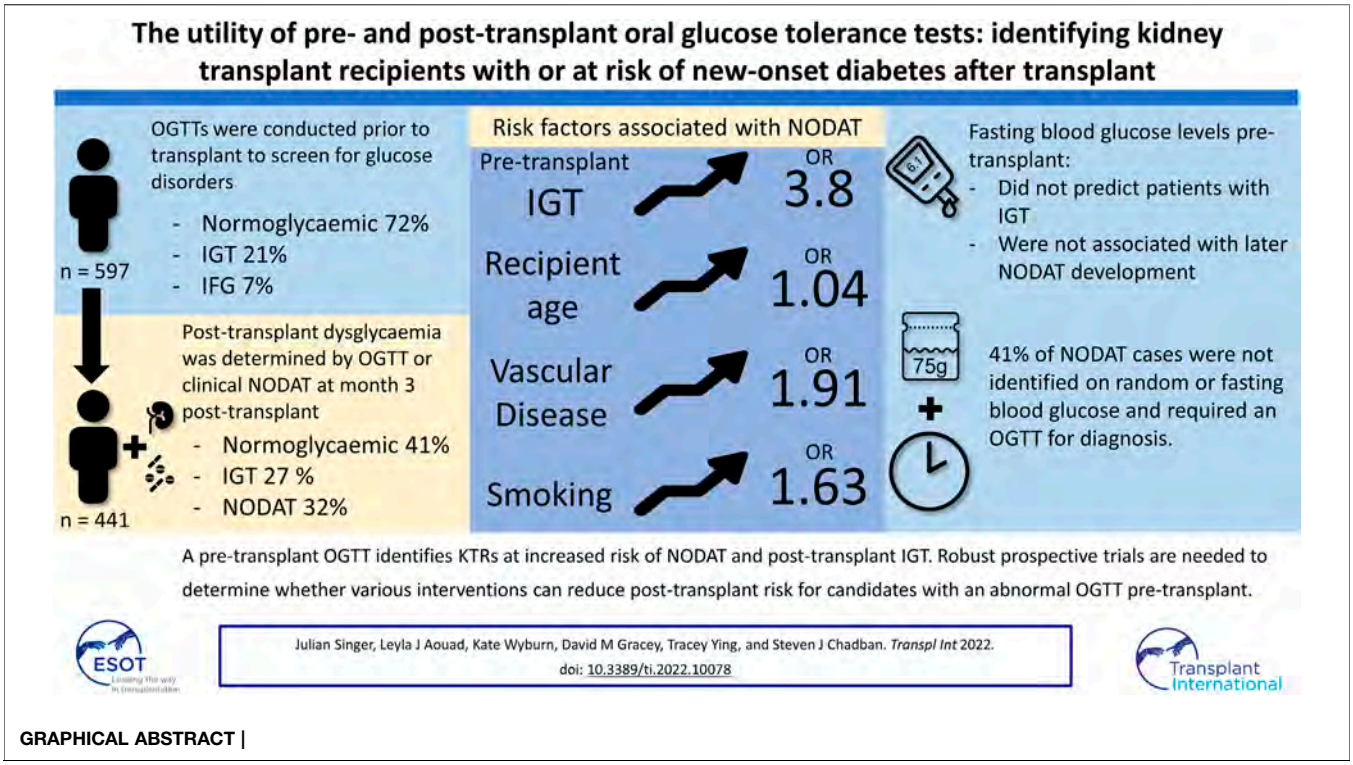
Received: *30 September 2021*
Accepted: *24 February 2022*
Published: *17 March 2022*

Citation:
Singer J, Aouad LJ, Wyburn K, Gracey DM, Ying T and Chadban SJ (2022) The Utility of Pre- and Post-Transplant Oral Glucose Tolerance Tests: Identifying Kidney Transplant Recipients With or at Risk of New Onset Diabetes After Transplant. Transpl Int 35:10078. doi: 10.3389/ti.2022.10078

Abbreviations: 2hPG, 2-h plasma glucose; ANZDATA, Australia and New Zealand Dialysis and Transplant Registry; BMI, body mass index; CNI, calcineurin inhibitor; eGFR, estimated glomerular filtration rate; ESKD, end-stage kidney disease; FPG, fasting plasma glucose; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; KTRs, kidney transplant recipients; mTORi, mammalian target of rapamycin inhibitor; NODAT, new onset diabetes after transplant; OGTT, oral glucose tolerance test.



GRAPHICAL ABSTRACT |

INTRODUCTION

New onset diabetes after transplant (NODAT) occurs commonly following kidney transplantation and is associated with an increase in recipient morbidity and mortality, primarily through the development of cardiovascular disease (1–5). As older age and obesity are becoming more prevalent among kidney transplant candidates and recipient populations over time (6–9), the frequency of NODAT is likely to increase. Identifying patients at risk for NODAT prior to transplantation is therefore of importance to both clinicians and kidney transplant recipients (KTRs). Early recognition of patients at risk for NODAT prior to kidney transplantation may allow for informed risk counselling, a tailored approach towards immunosuppression, and the implementation of targeted interventions to address modifiable risk-factors before and after transplantation.

Abnormalities of glucose metabolism prior to transplant have been shown to predispose recipients to the development of NODAT, although consensus is lacking over which glycaemic parameters are best measured to assess this risk. In general populations, patterns of oral glucose tolerance test (OGTT) results are predictive of future progression to diabetes (10, 11). In kidney transplant candidates, small studies have suggested that random or fasting blood glucose levels may identify patients at risk (12), although larger studies have not borne this out. Stronger evidence supports the role of a pre-transplant OGTT in identifying patients at risk for NODAT, with patients exhibiting impaired glucose tolerance (IGT) following a glucose challenge incurring greater risk (13–16). However, studies to date have been limited by small sample sizes, cyclosporine-based immunosuppression, restriction to recipients from living donors, and variable diagnostic criteria for NODAT (15, 16).

Similarly, current guidelines suggest a number of glycaemic parameters including fasting plasma glucose (FPG), HbA1c, and OGTT to be suitable tests for the detection of diabetes post-transplant (17). Whilst an OGTT remains the gold-standard, practical and economic limitations may constrain its use, leading many centres to rely on FPG alone to screen at risk recipients. However, the performance of FPG as a tool to screen for diabetes post-transplant remains questionable (18).

In this single centre study from a metropolitan transplant referral hospital, we used routine OGTTs to prospectively determine the glycaemic status of kidney transplant recipients prior to and following transplantation between 2003 and 2018. Records were linked to the ANZDATA registry to obtain recipient factors and transplant outcomes. We hypothesised that OGTTs performed prior to and following kidney transplant would outperform FPG in identifying at-risk transplant candidates and KTRs with NODAT, respectively.

MATERIALS AND METHODS

Study Population and Setting

This single centre retrospective cohort study included all non-diabetic adult kidney transplant recipients transplanted at Royal Prince Alfred Hospital, Sydney, Australia, between 1st January 2003 and 31st March 2018. Patients with a diagnosis of diabetes

prior to transplant, recipients of combined organ transplants (kidney and liver), patients with a functioning renal allograft *in situ*, and permanent residents of overseas territories were excluded (19).

Results of pre- and post-transplant 2-h 75-g OGTT were obtained from the hospital Electronic Medical Record, the Departmental Database, and patient files.

The deidentified dataset was linked to the ANZDATA registry using deterministic record linkage (transplant centre, date of birth, date of transplant, and sex) to obtain recipient factors including ethnicity, primary kidney disease, history of prior kidney transplants, smoking history, weight, and comorbidities present at time of transplantation (coronary artery disease, peripheral vascular disease, diabetes mellitus, cerebrovascular disease, and chronic lung disease); and transplant characteristics including donor type, donor age, ischaemia time, HLA mismatch, delayed graft function, induction therapy, and transplant outcomes.

ANZDATA is a bi-national registry that collects demographic and kidney-related treatment and outcomes data for all dialysis and transplant patients within Australia and New Zealand. Data is provided on a yearly and voluntary basis by nephrology units with an opt-out system of consent. ANZDATA collection methods and validity have been previously described (20).

The study was conducted following approval by the institutional ethics committee under protocol 2019/ETH06370.

Oral Glucose Tolerance Testing, Dysglycaemia, and New Onset Diabetes After Transplant

A 75-g OGTT was performed pre- and post-transplant for each participant, conducted according to American Diabetes Association (ADA) guidelines. On the basis of fasting plasma glucose (FPG) and 2-h plasma glucose (2hPG) levels, patients were categorised as having pre-transplant normoglycaemia (FPG <5.6 mmol/L and 2hPG <7.8 mmol/L), impaired fasting glucose (IFG, FPG ≥5.6 mmol/L to 6.9 mmol/L), or impaired glucose tolerance (IGT, 2hPG ≥7.8 mmol/L to 11.0 mmol/L). Patients with a new diagnosis of diabetes (FPG ≥7, or 2hPG ≥11.1) based on their pre-transplant OGTT were excluded from the primary analysis.

The glycaemic status of KTRs was censored at week 12 post-transplant. NODAT was determined by either a positive OGTT result (FPG ≥7, or 2hPG ≥11.1) performed at weeks 10–12 post-transplant, or by a clinical diagnosis defined as repeated elevations in fasting (≥7.0 mmol/L) or random/post-prandial (≥11.0 mmol/L) blood glucose levels throughout the post-transplant period that required ongoing treatment with antidiabetic medication at week 12 post-transplant. Patients not requiring antidiabetic medication and for whom the results of a 75g OGTT were not attainable were classified as having an unknown glycaemic state due to insufficient evaluation.

Statistical Analysis

Data in the manuscript are expressed as means ± standard deviation for normally distributed data or median ± interquartile range for non-normally distributed data, and as frequencies for categorical variables.

Differences in continuous variables between groups were examined by analysis of variance (ANOVA) for normally distributed data, or by the non-parametric Kruskal-Wallis log rank test for non-normally distributed data. Categorical variables were compared using the Chi squared test. Cohen's kappa was used to determine the agreement between the fasting and 2-h plasma glucose criteria for NODAT, and the correlation between fasting and subsequent 2-h glucose levels by the Pearson correlation coefficient. Receiver operating characteristic (ROC) curve analysis was conducted to identify the diagnostic utility of FPG value at time of OGTT in identifying pre and post-transplant dysglycaemia.

To ascertain the associations between patient factors and the development of NODAT we performed multivariate analysis using a generalised linear model with a logit link function. Variables were included if they were statistically associated with the outcome by univariate analysis ($p < 0.1$) or selected a priori on the basis of published associations. The results of the model are expressed as crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs).

Patient and graft survival were analysed by the Kaplan-Meier method and compared using the log-rank test for unadjusted survival, with Cox proportional hazard regression used for multivariate analyses.

For all analyses, a two-sided $p < 0.05$ was considered statistically significant. All statistical analysis was performed using R Statistical Software (2019; R Foundation for Statistical Computing, Vienna, Austria).

Sensitivity Analyses

As not all kidney transplant recipients at our centre underwent pre-transplant assessment with an OGTT, we conducted a sensitivity analysis to determine whether the association of post-transplant dysglycaemia and transplant outcomes remained consistent when the entire transplant cohort with known glycaemic status post-transplant were examined. This cohort consisted of an additional 114 KTRs who did not undergo pre-transplant assessment with an OGTT but had their post-transplant glycaemic status accurately determined by either a clinical diagnosis of NODAT or the results of an OGTT. A further 197 KTRs who had a pre-transplant diagnosis of diabetes were included as a third comparator.

RESULTS

Patient Characteristics

A total of 1212 kidney only transplants were performed in our centre between January 2003 and the end of April 2018. We excluded 56 transplants performed with recipients whose usual residence was outside of Australia (19), 2 recipients with a prior functioning renal allograft at the time of transplant, and a further 185 patients with a pre-existing diagnosis of diabetes. For the remaining cohort, results of a pre-transplant 75-g OGTT were obtained for 609 recipients, with an additional 12 cases of

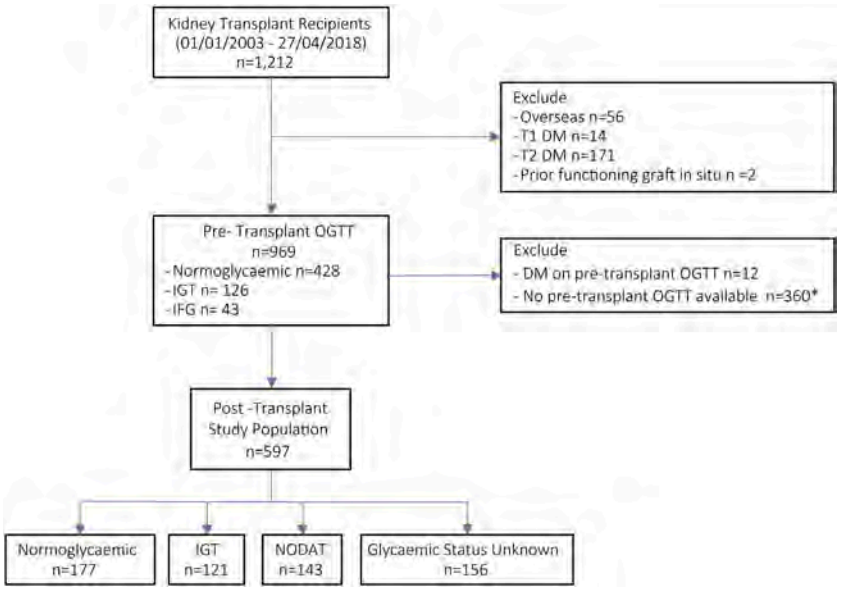


FIGURE 1 | Flow diagram for enrolment and stratification of recipients according to pre- and post-transplant glycaemic status. (*114 transplant recipients who did not perform an OGTT pre-transplant had a known post-transplant glycaemic status and were included in the sensitivity analyses, in addition to 197 recipients with pre-transplant DM.) DM, diabetes mellitus; OGTT, oral glucose tolerance test; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; NODAT, new onset diabetes after transplant.

unrecognised diabetes identified and subsequently excluded from the study (**Figure 1**).

Baseline characteristics of the final study population (n = 597) are shown in **Table 1**. The mean age of recipients was 47 ± 14, with 64% being male and 73% of white descent. The mean body mass index (BMI) was 26 ± 5 kg/m^2, and the primary cause of ESKD was glomerulonephritis (49.2%), polycystic kidney disease (15.2%), reflux nephropathy/posterior urethral valves (PUV) (8%), renovascular disease in 8%, and other causes in the remaining 19.4%. 370 (62%) recipients were receiving maintenance haemodialysis prior to transplantation, 136 (22.8%) peritoneal dialysis, and 91 (15.2%) were pre-emptively transplanted before commencing dialysis. Donor organs were received from living (n = 297) or deceased (n = 300) donors.

The majority of patients received induction with intravenous methylprednisolone and basiliximab (83%), with antithymocyte induction (3.7%) and/or intravenous immunoglobulin (10.7%) administered to higher-immunologic risk recipients. Initial immunosuppression was with tacrolimus (89%) or cyclosporine (10%), mycophenolate (98%) and/or sirolimus/everolimus (20%); and all except one recipient received maintenance prednisolone. Tacrolimus trough concentrations of 10–12 ng/ml were targeted during the first 3 months post-transplant, and 5–8 ng/ml from month 3 onward depending on immunological risk.

Pre-transplant OGTTs were performed at a median of 367 (IQR: 166–714) days prior to transplantation. Dysglycaemia determined by OGTT before transplantation was common, affecting 27% of the cohort, with IGT (126, 21%) more prevalent than IFG (43, 7%); the remaining 428 tests (72%) were normal (**Table 2**).

Patients with pre-transplant dysglycaemia (IGT or IFG) were older (52 ± 12 years vs. 45 ± 14 years, $p < 0.001$), had a higher BMI (27 ± 5 m/kg^2 vs. 26 ± 5 m/kg^2, $p = 0.03$), and were more commonly undergoing peritoneal dialysis (35.3% vs. 17.7%, $p < 0.001$). The association with peritoneal dialysis was largely driven by a higher prevalence of IFG, potentially relating to glucose absorption from dialysate (**Supplementary Table S1**).

Pre-Transplant FPG and Prediction of IGT

As elevated FPG levels have been advocated as a screening test to identify patients who would benefit from further investigation with an OGTT pre-transplant, we examined the predictive value of this approach. The mean FPG of patients in the normoglycaemic group was 4.8 ± 0.5 mmol/L compared to 5.2 ± 0.7 mmol/L in those with IGT ($p < 0.001$). However, FPG values only weakly correlated with subsequent 2hPG levels (r = 0.36, $p < 0.001$). The receiver operating characteristic (ROC) curve for FPG predicting an abnormal OGTT is shown in **Figure 2A**. The AUC was 0.66 (95% CI: 0.62–0.71), suggesting that FPG has little value in identifying patients who would be found to have IGT or diabetes by OGTT pre-transplant. **Table 3** displays the test characteristics for FPG cut-off values predictive of IGT pre-transplant, identifying a FPG of 5.05 mmol/L as having the optimal test performance, but with a sensitivity and specificity of 53% and 70% respectively. Thus, if patients were only selected to undergo an OGTT based on an abnormal FPG reading (≥5.6 mmol/L), 78% of KTRs with pre-transplant dysglycaemia would not be identified.

TABLE 1 | Characteristics of kidney transplant recipients stratified by post-transplant glycaemic status.

	Normoglycaemic	IGT	NODAT	Unknown	p
	n = 177	*n* = 121	*n* = 143	*n* = 156	
Age (mean (SD))	41.5 ± 13.5	49.7 ± 12.8)	53.9 ± 11.4	47.7 ± 14.2	<0.001
Age ≥ 50 (%)	49 (27.7)	69 (57.0)	94 (65.7)	70 (44.9)	<0.001
Gender					0.967
Male (%)	116 (65.5)	76 (62.8)	91 (63.6)	102 (65.4)	
Female (%)	61 (34.5)	45 (37.2)	52 (36.4)	54 (34.6)	
BMI (mean (SD))	25.4 ± 5.5	26.3 ± 4.6	26.2 ± 4.5	26.3 ± 4.9	0.326
BMI Category (%)					0.593
Underweight (<18.5)	9 (5.1)	4 (3.3)	5 (3.5)	6 (3.8)	
Normal (≥18.5 to <25.0)	84 (47.5)	45 (37.2)	58 (40.6)	57 (36.5)	
Overweight (≥25.0 to <30.0)	52 (29.4)	45 (37.2)	46 (32.2)	52 (33.3)	
Obese (≥30)	27 (15.3)	25 (20.7)	31 (21.7)	34 (21.8)	
Not Available	5 (2.8)	2 (1.7)	3 (2.1)	7 (4.5)	
Racial Background (%)					0.243
Caucasian	129 (72.9)	93 (76.9)	99 (69.2)	116 (74.4)	
Asian	32 (18.1)	21 (17.4)	33 (23.1)	17 (10.9)	
Aboriginal/Torres Strait Islander	1 (0.6)	1 (0.8)	4 (2.8)	5 (3.2)	
Other	15 (8.4)	6 (5.0)	7 (4.9)	18 (11.5)	
Primary Renal Disease (%)					0.068
Glomerulonephritis	93 (52.5)	67 (55.4)	69 (48.3)	65 (41.7)	
Polycystic Kidney Disease	22 (12.4)	23 (19.0)	20 (14.0)	26 (16.7)	
Reflux Nephropathy/PUV	13 (7.3)	8 (6.6)	9 (6.3)	18 (11.5)	
Hypertension	17 (9.6)	4 (3.3)	18 (12.6)	9 (5.8)	
Other	32 (18.1)	19 (15.7)	27 (18.9)	38 (24.4)	
RRT Prior To Transplant (%)					0.327
Haemodialysis	109 (61.6)	68 (56.2)	88 (61.5)	105 (67.3)	
Peritoneal	34 (19.2)	32 (26.4)	38 (26.6)	32 (20.5)	
Pre-emptive transplant	34 (19.2)	21 (17.4)	17 (11.9)	19 (12.2)	
Living Donor (%)	110 (62.1)	65 (53.7)	67 (46.9)	55 (35.3)	<0.001
Prior Kidney Transplant (%)	18 (10.2)	8 (6.6)	11 (7.7)	25 (16.0)	0.055
Smoking History (%)	48 (27.0)	36 (29.8)	62 (43.3)	70 (44.9)	0.001
Prior Vascular Disease[a] (%)	28 (15.8)	21 (17.4)	45 (31.5)	26 (16.7)	0.002
Induction Immunosuppression					
IL-2 Receptor antibody (%)	149 (84.2)	109 (90.1)	124 (86.7)	114 (73.1)	0.001
T cell depleting antibody (%)	7 (4.0.)	5 (4.1)	4 (2.8)	6 (3.8)	0.931
B cell depleting antibody (%)	4 (2.3)	2 (1.7)	3 (2.1)	0 (0.0)	0.335
Intravenous Immunoglobulin (%)	17 (9.6)	13 (10.7)	15 (10.5)	19 (12.2)	0.937
Maintenance Immunosuppression					
Tacrolimus v CSA (%)	152 (88.4)	97 (80.8)	127 (89.4)	148 (95.5)	0.002
CNI Free (%)	5 (2.8)	1 (0.8)	1 (0.7)	1 (0.6)	0.242
mTOR (%)	49 (27.7)	26 (21.5)	34 (23.8)	11 (7.1)	<0.001
Prednisolone (%)	177 (100.0)	121 (100.0)	143 (100.0)	155 (99.4)	0.416
- Dose (mg) at 3 m (mean, SD)	11.1 ± 5.5	10.7 ± 2.9	11.5 ± 8.1	11.1 ± 3.6	0.790
HLA MM (%)					0.068
1–2	63 (35.6)	44 (36.4)	48 (33.6)	46 (29.5)	
3–4	70 (39.5)	41 (33.9)	41 (28.7)	47 (30.1)	
5–6	44 (24.9)	36 (29.8)	54 (37.8)	63 (40.4)	
Rejection episode (any) (%)	38 (21.5)	25 (20.7)	32 (22.4)	37 (23.7)	0.931
Early rejection (≤ 90 days post-transplant) (%)	26 (14.7)	15 (12.4)	29 (20.3)	32 (20.5)	0.179
Delayed graft function (%)	20 (11.3)	17 (14.0)	25 (17.5)	38 (24.4)	0.011
eGFR (CKD-EPI)					
at 3 m (mean, SD)	55.9 ± 18.5	53.1 ± 18.1	51.3 ± 16.5	48.7 ± 17.5	0.004
at 1 year (mean, SD)	60.2 ± 18.8	52.2 ± 15.4	52.6 ± 18.6	51.2 ± 18.5	<0.001

[a]*Coronary artery disease, peripheral vascular disease, or cerebrovascular disease.*

Post-Transplant Glycaemic Status

Of the 597 KTRs assessed, post-transplant glycaemic status could be accurately determined in 441 cases by either a clinical diagnosis of NODAT (*n* = 85), or by the results of an OGTT (*n* = 358) conducted at a median of 74 days post-transplant (IQR: 67–91 days). Disorders of glycaemia were

TABLE 2 | Results of oral glucose tolerance tests performed prior to and following kidney transplantation, stratified by post-transplant glycaemic status.

	Normoglycaemic	IGT	NODAT	Unknown	p
	n = 177	*n* = 121	*n* = 143	*n* = 156	
Pre-Transplant OGTT					
Day pre-transplant (median [IQR])	−282 [−551, −146]	−407 [−746, −211]	−367 [−672, −142]	−440 [−736, −227]	0.002
FPG mmol/L [mean (SD)]	4.77 (0.49)	5.07 (0.59)	5.07 (0.73)	4.81 (0.56)	<0.001
2hPG mmol/L [mean (SD)]	5.58 (1.49)	6.54 (1.60)	7.37 (1.90)	5.98 (1.80)	<0.001
Glycaemic status pre-transplant					<0.001
Normoglycaemic (%)	151 (85.3)	81 (70.0)	74 (51.7)	122 (78.2)	
IFG (%)	9 (5.1)	16 (13.2)	9 (6.3)	9 (5.8)	
IGT (%)	17 (9.6)	24 (19.8)	60 (42.0)	25 (16.0)	
Post-Transplant OGTT					
Day post-transplant (median [IQR])	77 [68, 92]	72 [69, 91]	73 [65, 88]	—	0.312
FPG mmol/L (mean (SD))	4.95 (0.46)	5.24 (0.58)	5.76 (0.89)	—	<0.001
2hPG mmol/L (mean (SD))	6.21 (1.12)	9.14 (0.95)	13.08 (2.20)	—	<0.001

OGTT, oral glucose tolerance test; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; NODAT, new onset diabetes after transplant.

common post-transplant with 143 patients (33%) developing NODAT and a further 121 (28%) displaying IGT. For the remainder, the OGTT was normal (*n* = 159, 37%) or revealed isolated IFG (*n* = 18, 4%).

Comparison of NODAT Evident by FPG or Post 2-h Glucose Load

Of the 143 KTRs with NODAT, 59 (41%) diagnoses were not established on clinical grounds and were detected by protocolised OGTT at 10 weeks post-transplant. Whilst all 59 patients met ADA diagnostic criteria by an elevated 2hPG, only 3 patients met FPG criteria (FPG ≥7 mmol/L). The concordance between the fasting and 2-h glucose criteria for the diagnosis of NODAT was poor (κ = 0.07).

In patients without clinical NODAT, post-transplant FPG levels were a poor indicator of KTRs likely to have dysglycaemia on formal testing (**Figure 2B**, AUC 0.71, 95% CI: 0.66–0.78). The optimum decision threshold for an FPG to proceed to an OGTT was 5.15 mmol/L, with a sensitivity and specificity of 66% and 76%, respectively. If the ADA criteria for an abnormal FPG (≥5.6 mmol/L) was applied to identify KTR without clinical NODAT who should undergo an OGTT post-transplant, 60% of KTR with occult dysglycaemia would be missed (**Table 4**).

Of our cohort, 156 (26%) patients did not develop clinical NODAT and did not undergo post-transplant OGTT. This group were similar in age (47 ± 14 vs. 46 ± 13, $p = 0.63$) and BMI (26.3 ± 4.9 vs. 25.7 ± 5.1, $p = 0.246$) to those for whom an OGTT was recorded, with similar glucose profiles recorded prior to transplant (FPG 4.8 ± 0.6 mmol/L v 4.9 ± 0.6 mmol/L, $p = 0.107$; and 2hPG 6.0 ± 1.8 mmol/L v 6.1 ± 1.7 mmol/L, $p = 0.46$) (**Supplementary Table S2**). We conducted multivariate analysis to determine whether this group differed significantly from the cohort with recorded OGTTs (**Supplementary Table S3**), and found they were more likely to have been referred from and returned to care outside the transplant centre (OR = 2.42, 95% CI: 1.62–3.62, $p < 0.001$), and to have received a kidney from a deceased donor (OR = 2.25, 95% CI: 1.46–3.48, $p < 0.001$).

Risk Factors for the Development of NODAT

Covariates associated with the development of NODAT are shown in **Figure 3**. Patient factors not associated with the development of NODAT by univariate analysis included BMI, gender, primary renal disease, prior kidney transplantation, the type of induction therapy or calcineurin inhibitor used, and the occurrence of rejection within the first 90 days post-transplant. After multivariate analysis, age at transplant remained a significant risk-factor, conferring a 4% increase in risk of NODAT per year of age. Pre-transplant IGT (OR = 3.79, 95% CI: 2.27–6.35, $p < 0.001$), but not IFG, was significantly associated with NODAT (**Figure 3**).

Patient and Graft Outcomes

Kaplan-Meier plots of graft survival, death-censored graft survival, and patient survival are shown in **Figure 4**. In the cohort of patients with a known glycaemic status post-transplant, graft survival at 5-years was 91% (95% CI: 89–94%) and 95% (95% CI: 92–97%) when censored for death. No significant difference in graft survival was observed between the glycaemic cohorts, without or with censoring for death of the patient (**Figures 4A,B**, $p = 0.2$ and $p = 0.76$). Patient survival was inferior for patients with NODAT compared to normoglycaemic recipients. (**Figures 4C**, $p = 0.032$). Whilst patients with NODAT experienced higher rates of mortality compared to normoglycaemic KTRs (HR 2.29, 95% CI: 1.21–4.32, $p = 0.012$), only increasing recipient age (HR 1.03, 95% CI: 1.01–1.06, $p = 0.034$) and a pre-transplant history of vascular disease (HR 2.65, 95% CI: 1.35–5.28, $p = 0.006$) were associated with an increased risk of death in a multivariate analysis (**Table 5**).

Sensitivity Analyses

Five-years graft survival of all KTRs transplanted during the study period with a known post-transplant glycaemic status and 182 KTRs previously excluded because of known pre-transplant DM are shown in **Figure 4D**. The Kaplan-Meier plots reveal a hierarchy of risk for mortality, strongest for pre-transplant DM over NODAT, IGT and normoglycaemia. By multivariate analysis, pre-transplant diabetes (HR 2.77, 95%CI: 1.54–4.98, $p < 0.001$), but not NODAT or IGT, was strongly associated with decreased survival post-transplant.

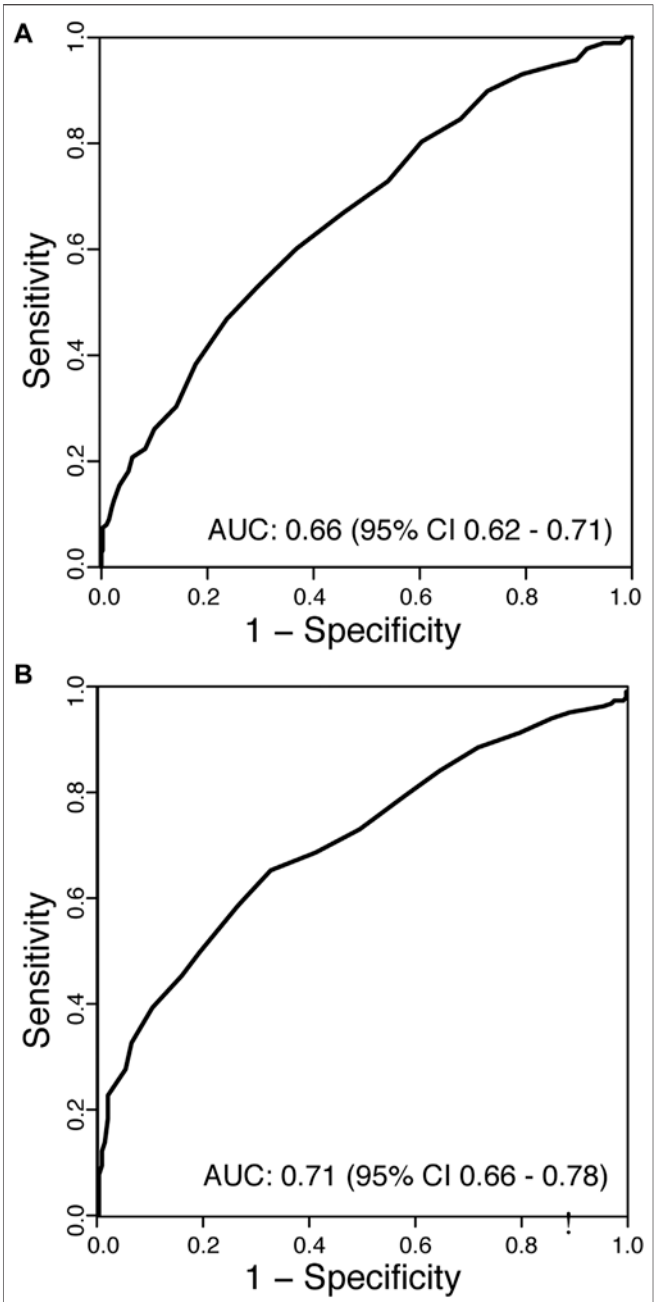


FIGURE 2 | Receiver operating characteristic (ROC) curves for **(A)** fasting plasma glucose (FPG) predicting impaired glucose tolerance (IGT) in patients pre-transplant, and **(B)** FPG predicting dysglycaemia (NODAT or IGT) post-transplant. AUC, area under the curve.

DISCUSSION

In a large cohort of KTRs managed with contemporary immunosuppression, an OGTT conducted as part of pre-transplant candidate evaluation revealed unrecognised diabetes in 2% and IGT in 28%. Following transplantation, those with IGT incurred a greater than 3-fold higher incidence of NODAT as compared to their normoglycaemic peers. Elevated fasting glucose pre-transplant was not predictive of NODAT, nor did it identify a subset of candidates likely to manifest IGT or DM pre-transplant. These findings highlight the utility of routine pre-transplant OGTT to identify risk of NODAT, and thereby provide opportunities to recognise, discuss and potentially mitigate the negative impacts of NODAT on post-transplant survival. This data lends support to the 2020 KDIGO Guidelines on the management of Candidates for Kidney Transplantation where evaluation with a pre-transplant OGTT has been suggested for this purpose (21).

A secondary finding of our study was the utility of a protocolised, post-transplant OGTT to diagnose clinically inapparent NODAT and to identify KTRs with IGT. In addition to the 19% of KTRs with clinically apparent NODAT, OGTT detected NODAT in a further 14% yielding a total incidence of 33% in those who underwent thorough assessment. A further 121 KTRs exhibited IGT, thus use of post-transplant OGTT identified clinically unrecognised dysglycaemia in 42% of our cohort. Given the increase in cardiovascular risk associated with NODAT and IGT following kidney transplantation (2, 22), an OGTT is essential in order to identify at risk KTRs and create an opportunity for the implementation of appropriate risk-reduction strategies.

We recognise that widespread uptake of OGTTs has been limited by practical and economic constraints. For this reason, its use as a screening tool in transplant assessment has often been restricted to those with identified risk factors, such as a prior elevated FPG level (21). Our findings suggest that this approach is of little value. We found that pre-transplant FPG levels, in our study taken at the time of an OGTT, correlated poorly with subsequent 2hPG levels. Furthermore, FPG levels were of no discriminatory value in predicting transplant candidates who had IGT, and unlike IGT were not associated with the development of NODAT post-transplant.

The prevalence of pre-transplant dysglycaemia in our cohort is concordant with previously reported rates of IGT amongst kidney transplant candidates (23). These rates are significantly higher than the general, age-matched Australian population (24), and may reflect the increase in basal insulin resistance amongst patients with ESKD (25). The insensitivity of FPG to detect dysglycaemia, coupled with the high incidence of dysglycaemia amongst candidates for kidney transplantation highlights the need for an OGTT to be performed as part of routine candidate assessment.

NODAT occurs commonly in KTRs although the reported incidence varies according to the diagnostic criteria employed, timing post-transplant, and the type of immunosuppression used. At month three post-transplant the incidence of recorded NODAT in our cohort was 24%, consistent with previous studies where protocolised OGTTs have been performed (13, 26, 27). Lower rates have been reported in cohorts which have relied on clinical records or non-dynamic glucose testing (28–30), and higher rates in studies which included dysglycaemia recorded during the early post-transplant period (31).

TABLE 3 | Fasting plasma glucose cut-off values for the detection of impaired glucose tolerance pre-transplant.

FPG (mmol/L)	Sensitivity (%)	Specificity (%)	FPR	FNR	PPV	NPV	Youden index
4.60	85	32	0.68	0.15	0.36	0.82	1.17
4.80	73	46	0.54	0.27	0.38	0.79	1.19
5.00	60	63	0.37	0.40	0.43	0.78	1.23
5.05	53	70	0.30	0.47	0.45	0.77	1.24
5.20	47	76	0.24	0.53	0.48	0.76	1.23
5.40	30	86	0.14	0.70	0.50	0.73	1.16
5.60	22	92	0.08	0.78	0.55	0.72	1.14
5.80	18	95	0.05	0.82	0.62	0.72	1.13
6.00	13	98	0.02	0.87	0.71	0.71	1.10

FPR, false positive ratio; FNR, false negative ratio; PPV, positive predictive value; NPV, negative predictive value.

TABLE 4 | Fasting plasma glucose cut-off values for the detection of dysglycaemia (IGT or NODAT) post-transplant.

FPG (mmol/L)	Sensitivity (%)	Specificity (%)	FPR	FNR	PPV	NPV	Youden index
4.60	92	20	0.80	0.08	0.54	0.72	1.12
4.80	85	35	0.65	0.15	0.57	0.70	1.20
5.00	74	51	0.49	0.26	0.60	0.66	1.24
5.15	66	67	0.33	0.34	0.67	0.66	1.33
5.20	66	67	0.33	0.34	0.67	0.66	1.33
5.40	50	81	0.19	0.50	0.73	0.62	1.31
5.60	40	90	0.10	0.60	0.80	0.60	1.30
5.80	28	95	0.05	0.72	0.85	0.57	1.23
6.00	18	98	0.02	0.82	0.92	0.55	1.17

FPR, false positive ratio; FNR, false negative ratio; PPV, positive predictive value; NPV, negative predictive value.

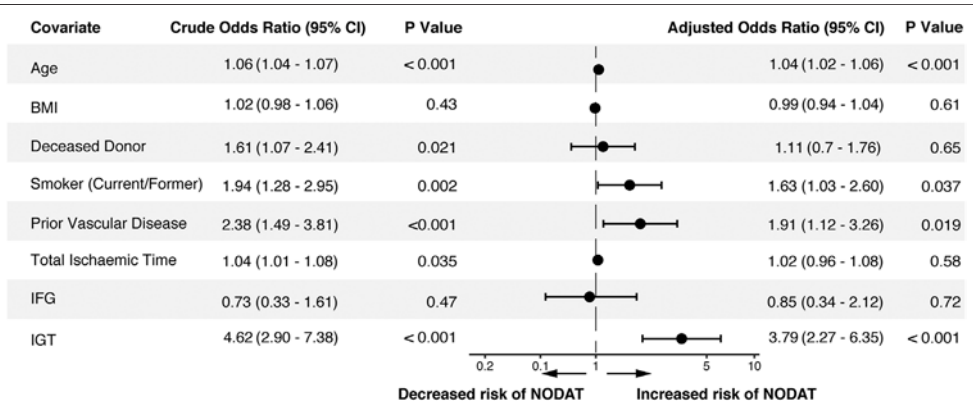


FIGURE 3 | Risk factors for the development of NODAT following univariate and multivariate analysis. BMI, body mass index; IGT, impaired glucose tolerance; IFG, impaired fasting glucose; NODAT, new onset diabetes after transplant.

In this study, 41% of NODAT cases were not identified by routine surveillance of blood glucose levels and were only diagnosed by the use of a screening OGTT. We found FPG to not only lack sufficient sensitivity to identify patients with NODAT, but to poorly predict KTRs who would return an abnormal OGTT. Importantly, as the diagnosis of IGT in KTRs is clinically significant (32, 33) and can only be achieved with an OGTT, our findings suggest that all kidney transplant recipients without clinically evident NODAT, should undergo an OGTT to screen for the presence of occult NODAT or IGT (34).

We confirmed well-known risk factors for NODAT such as increasing age and bring attention to the impact of smoking (35). Interestingly, in our cohort BMI was not associated with the development of dysglycaemia post-transplant. Our study is not alone in presenting this finding (28, 36, 37), which may be due to different demographic populations, the short follow-up time and differences in the diagnostic criteria for NODAT. Populations with a strong association between BMI and NODAT, such as African Americans were not represented in our cohort (3), whilst Asian populations, which contributed to 17% of our cohort are at an increased risk for NODAT despite lower BMIs (38, 39). Other

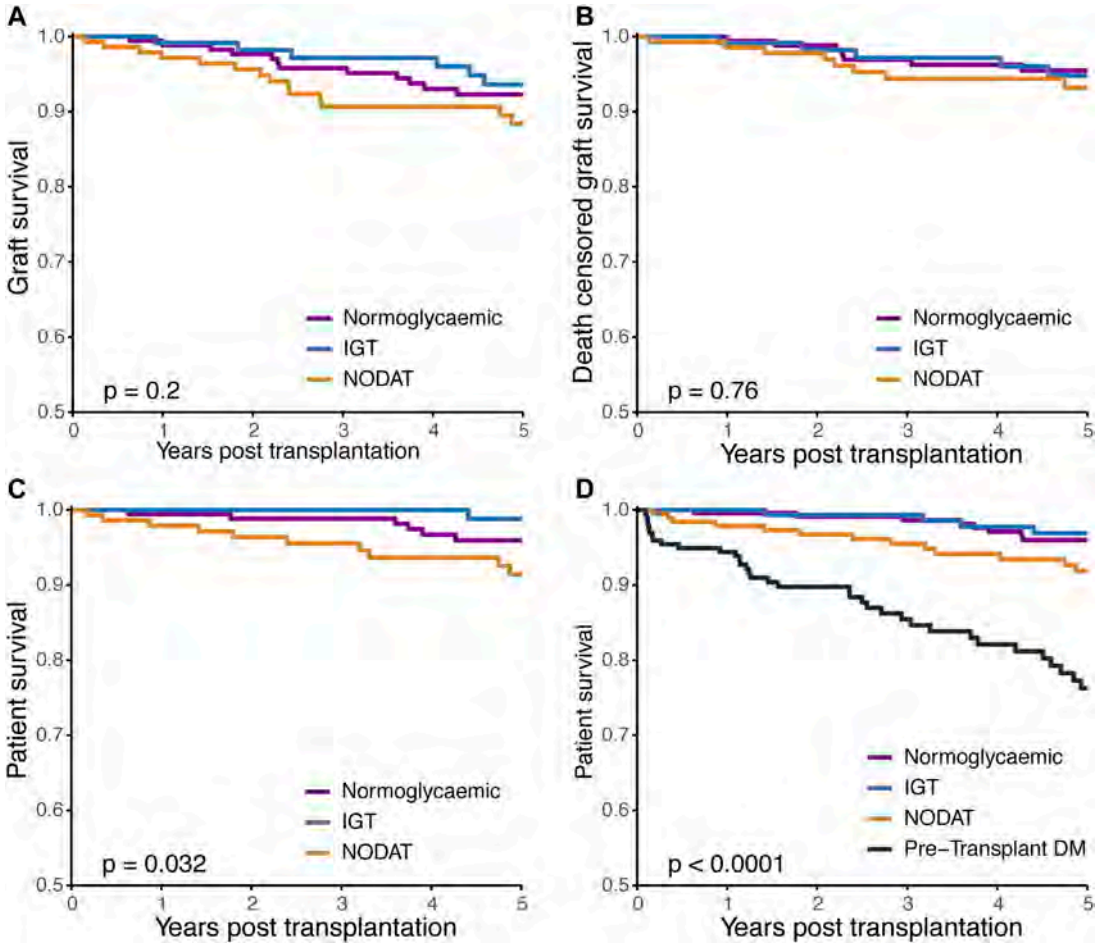


FIGURE 4 | Kaplan-Meier plots of **(A)**, graft survival **(B)**, death censored graft survival, and **(C)**, patient survival according to post-transplant glycaemic status. **(D)** Patient survival of all kidney transplant recipients with a known post-transplant glycaemic status, including KTRs with pre-transplant diabetes (n = 781). IGT, impaired glucose tolerance; NODAT, new onset diabetes after transplant; DM, diabetes mellitus.

TABLE 5 | Univariate and multivariate Cox regression analysis of covariates associated with death post-transplant.

	Crude HR (95% CI)	P (Wald's Test)	Adjusted OR (95%CI)	P (Wald's Test)
NODAT[a]	2.29 (1.21–4.32)	0.024	1.37 (0.69–2.72)	0.369
Age at transplant	1.05 (1.02–1.08)	<0.001	1.03 (1.01–1.06)	0.034
Deceased donor	2.74 (1.42–5.28)	0.002	1.92 (0.97–3.81)	0.061
Prior vascular disease[b]	3.94 (2.08–7.46)	<0.001	2.65 (1.35–5.28)	0.006

NODAT, new onset diabetes after transplant.
[a]*normoglycaemia as reference group.*
[b]*coronary artery disease, peripheral vascular disease, or cerebrovascular disease).*

reported risk-factors, such as the type of calcineurin inhibitor (27) and early rejection events did not correlate with post-transplant dysglycaemia, and may be explained by the infrequent occurrence of both cyclosporine use and rejection events in our cohort. As a practice-derived cohort, it is also likely that the finding of dysglycaemia on pre-transplant OGTT may have influenced the choice of calcineurin inhibitor for some patients (40). In contrast to the findings of Caillard et al (13), we did not find ADPKD to be associated with an increased risk of NODAT, despite a similar incidence of ADPKD and NODAT across both cohorts.

The development of NODAT is associated with an increased risk of adverse events, particularly cardiovascular morbidity and mortality (1–3, 41, 42). IGT has also been shown to convey a similarly increased risk of cardiovascular events in both KTRs (2, 22, 43) and general populations (44), however its impact on overall mortality appears less clear (45). Neither NODAT or IGT were

independently associated with all-cause mortality in our cohort, and a number of factors may have contributed to these findings. Firstly, we recorded the incidence of dysglycaemia at 3 months post-transplant, and acknowledge that 20–30% of cases may revert to a normoglycaemic state within the first post-transplant year (22, 33). Whilst defining NODAT at an early timepoint may have reduced the sensitivity of our survival analysis, our approach of early NODAT detection is supported by previous studies which have associated early detection (<3 months) with an increased risk of future cardiovascular events and death (5). Secondly, previous studies reporting lower patient survival with NODAT have used varying diagnostic criteria or included patients manifesting NODAT up to several years post-transplantation. These studies, which exclude patients with occult NODAT only identifiable via an OGTT likely report on a cohort of KTRs with a more severe disease phenotype in whom clinical NODAT is readily apparent. Thirdly, we cannot exclude that the unchanged survival in our NODAT cohort may reflect the intended benefit derived from a program of early screening and subsequent initiation of management strategies. Lastly, our study may be underpowered to detect an independent association between glycaemic status and mortality.

This study presents the strongest evidence to date in support of the use of OGTTs to identify KTRs with or at risk of NODAT. However, there are certain limitations to our study. Firstly, we evaluated a predominantly Caucasian population, and caution should therefore be applied when extrapolating to other ethnicities. Secondly, the post-transplant glycaemic status could not be adequately ascertained for some patients. Whilst these patients did not have clinical NODAT, we cannot exclude the presence of occult dysglycaemia that would have been detected by an OGTT. Additionally, we were not able to report on the presence of some factors known to contribute to development of NODAT, such as hyperlipidaemia and a family history of diabetes. However, whilst these factors are no doubt important considerations in the assessment of risk, their absence does not detract from the utility presented by an OGTT.

Our findings, whilst supporting those of Caillard's data from the cyclosporine era (13), report on a significantly different cohort. Here, we demonstrate the utility of a pre-transplant OGTT in assessing the risk of future NODAT in the contemporary transplant era, in recipients of both deceased and living donor kidneys, treated predominantly with tacrolimus, mycophenolate and maintenance corticosteroids. Our findings clearly demonstrate the inadequacies of relying upon fasting glucose levels as a screening tool for abnormal glucose metabolism pre- and post-transplant. The benefits of performing an OGTT both prior to transplant, to inform risk of NODAT, and post-transplant, to detect NODAT and inform cardiovascular risk, are evident and in our opinion outweigh the modest associated economic costs and inconvenience. Ultimately, robust prospective trials are needed to determine whether various interventions, including choice of immunosuppression (40), alters the development of NODAT, major adverse cardiovascular events and mortality in high-risk individuals, such as those with pre-transplant IGT.

DATA AVAILABILITY STATEMENT

Deindetified data pertaining to this study will be made available to investigators upon reasonable request and submission of a research plan of sufficient scientific merit. Requests to access the datasets should be directed to steve.chadban@health.nsw.gov.au.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee Royal Prince Alfred Hospital Sydney Local Health District. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JS participated in the research design, data analysis and writing of the paper. LA participated in research design and data analysis. KW and DG participated in research design. TY participated in research design and data analysis, and SC participated in the study conception, design, analysis, and writing of the paper. All authors read and approved the final 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.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance provided by the Sydney Informatics Hub, a Core Research Facility of the University of Sydney. Data is in this manuscript was supplied by the Australia and New Zealand Dialysis and Transplant Registry (ANZDATA). The interpretation and reporting of these data are the responsibility of the authors and in no way should be seen as an official policy or interpretation of the Australia and New Zealand Dialysis and Transplant Registry.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10078/full#supplementary-material

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ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/ti.2022.10253

Impact of Size Matching Based on Donor-Recipient Height on Kidney Transplant Outcomes

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Transplantation of kidneys from shorter donors into taller recipients may lead to suboptimal allograft survival. The effect of discrepancy in donor and recipient heights (ΔHeight) on long term transplant outcomes is not known. Adult patients ≥18 years undergoing living or deceased donor (LD or DD) kidney transplants alone from donors ≥18 years between 2000 and 2016 in the United States were included in this observational study. The cohort was divided into three groups based on ΔHeight of 5 inches as 1) Recipient < Donor (DD: 31,688, LD: 12,384), 2) Recipient = Donor (DD: 84,711, LD: 54,709), and 3) Recipient > Donor (DD: 21,741, LD: 18,753). Univariate analysis showed a higher risk of DCGL and mortality in both DD and LD ($p < 0.001$ for both). The absolute difference in graft and patient survival between the two extremes of ΔHeight was 5.7% and 5.7% for DD, and 0.4% and 1.4% for LD. On multivariate analysis, the HR of DCGL for Recipient < Donor and Recipient > Donor was 0.95 ($p = 0.05$) and 1.07 ($p = 0.01$) in DD and 0.98 ($p = 0.55$) and 1.14 ($p < 0.001$) in LD. Similarly, the corresponding HR of mortality were 0.97 ($p = 0.07$) and 1.07 ($p = 0.003$) for DD and 1.01 ($p < 0.001$) and 1.05 ($p = 0.13$) for LD. For DGF, the HR were 1.04 ($p = 0.1$) and 1.01 ($p = 0.7$) for DD and 1.07 ($p = 0.45$) and 0.89 ($p = 0.13$) for LD. Height mismatch between the donor and recipient influences kidney transplant outcomes.

Keywords: kidney transplantation, size mismatch, height mismatch, weight mismatch, SRTR

INTRODUCTION

Differences in the size of the recipient and donor have been shown to influence kidney transplant outcomes. This difference in outcomes is postulated to be secondary to the individual's kidney size and the number of nephrons, which is proportional to the overall size of the individual. The deficit in nephron endowment at the time of birth is permanent and does not change with the increase in demand later in life (1). There is no consensus on the anthropometric measure that best correlates with an individual's kidney size and nephron mass. From a physiological standpoint, transplantation of a kidney with a smaller number of nephrons into a larger individual may cause the nephrons to undergo hypertrophy, hyperfiltration injury, and eventually, sclerosis, exhaustion, and fibrosis (1,2).

OPEN ACCESS

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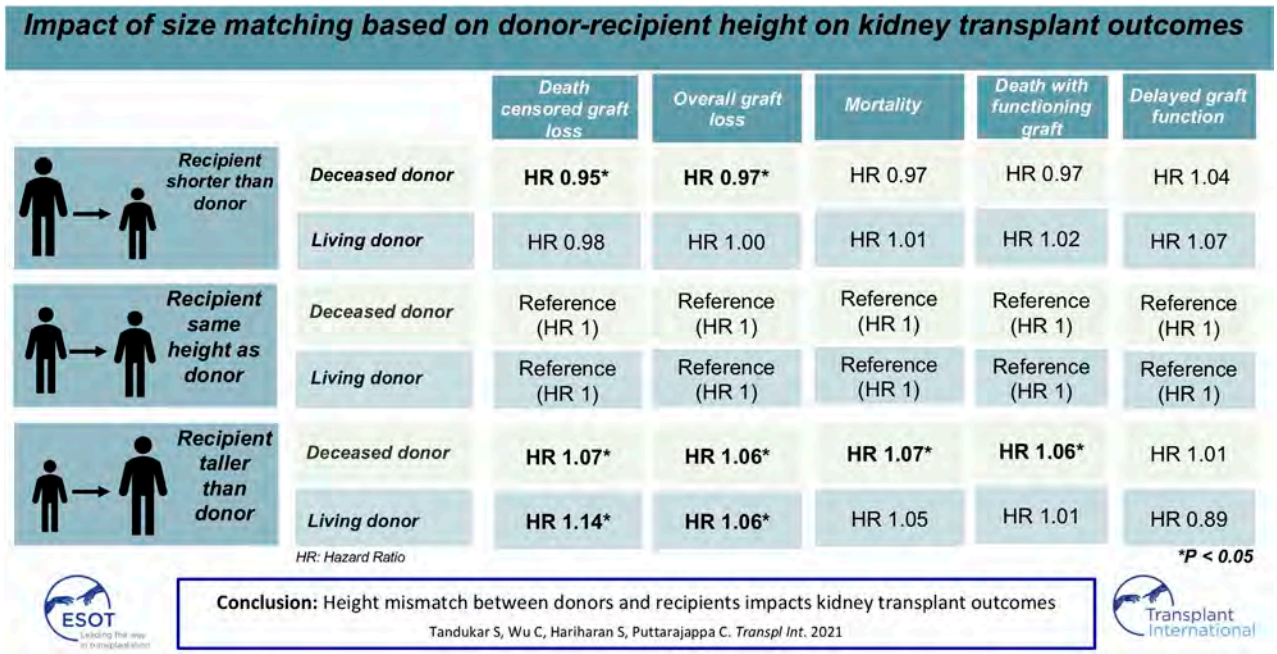
Received: *19 November 2021*
Accepted: *04 February 2022*
Published: *18 March 2022*

Citation:
Tandukar S, Wu C, Hariharan S and Puttarajappa C (2022) Impact of Size Matching Based on Donor-Recipient Height on Kidney Transplant Outcomes. Transpl Int 35:10253. doi: 10.3389/ti.2022.10253

Abbreviations: AIC, Akaike Information Criterion; ANOVA, Analysis of Variance; BMI, Body Mass Index; BSA, Body Surface Area; CI, Confidence Interval; DCGL, Death censored graft loss; DD, Deceased donor kidney transplant; DGF, Delayed graft function; ESRD, End Stage Renal Disease; ΔHeight, Difference in recipient and donor height; HLA, Human Leukocyte Antigen; HR, Hazard ratio; HRSA, Health Resources and Services Administration; KDPI, Kidney Donor Profile Index; LD, Living donor kidney transplant; OPTN, Organ Procurement and Transplantation Network; PRA, Panel Reactive Antibodies; SRTR, Scientific Registry of Transplant Recipients; ΔWeight, Difference in recipient and donor weight.



GRAPHICAL ABSTRACT |

Size mismatch between the donor and the recipient has been studied based on differences in their weight, body mass index (BMI), and body surface area (BSA) as surrogates for kidney size and nephron mass (2,3). These studies have shown conflicting results on the effect of these discrepancies on kidney transplant outcomes. In a population based study conducted on transplant patients in the UK Transplant Registry, there was no difference in graft survival and higher mortality in patients receiving kidneys from donors with a higher weight and BMI (2). In contrast, another study from the Scientific Registry of Transplant Recipients (SRTR) based on discrepancies of BSA showed that those receiving organs from smaller sized donors based on their BSA had increased risk of graft loss, an effect that was modulated by the recipient and donor ages (3).

We hypothesized that adult height may be a more optimal measure of nephron mass in an individual. The reasons for this are 1) adult height has a strong association with birth weight and length, which are known predictors of nephron mass (4–6), 2) adult height is strongly correlated with the length of the kidney (7), 3) adult height is less prone to distortion by an individual's lifestyle such as eating habits and physical activity, or by fluid balance in end stage renal disease (ESRD) patients, both of which may alter an individual's weight and composite anthropometric measures such as BMI and BSA, and 4) adult height is less likely to change once an individual enters adulthood, unlike weight, BMI and BSA, which may show wide temporal fluctuations within a person's lifespan.

This study aimed to determine whether height discrepancies in the donors and recipients predicted kidney transplant outcomes such as death censored graft survival, overall graft survival, patient survival, delayed graft function, and death with a functioning graft.

METHODS

Patient Population

This study used data from the Scientific Registry of Transplant Recipients (SRTR). The SRTR data system includes data on all donors, wait-listed candidates, and transplant recipients in the US, submitted by the members of the Organ Procurement and Transplantation Network (OPTN). The Health Resources and Services Administration (HRSA), U.S. Department of Health and Human Services provides oversight to the activities of the OPTN and SRTR contractors.

Adult patients above the age of 18 years, undergoing kidney transplants alone from donors, above the age of 18 years between January 1, 2000 and December 31, 2016, were selected. The algorithm for the derivation of the study cohort is presented in **Figure 1**.

Baseline characteristics included the recipient and donor's age, sex, ethnicity, dialysis vintage (for recipients), history of diabetes mellitus, hypertension, pre-emptive transplantation, height, weight, body mass index (BMI), and body surface area (BSA). BSA was calculated in m^2 using the Mosteller formula, $\sqrt{}$ (height (cm) × weight (kg)/3600). For deceased donors, data on history of hepatitis C infection, terminal donor creatinine, cause of death, donation after circulatory death, and cold ischemia time were also recorded. Other transplant

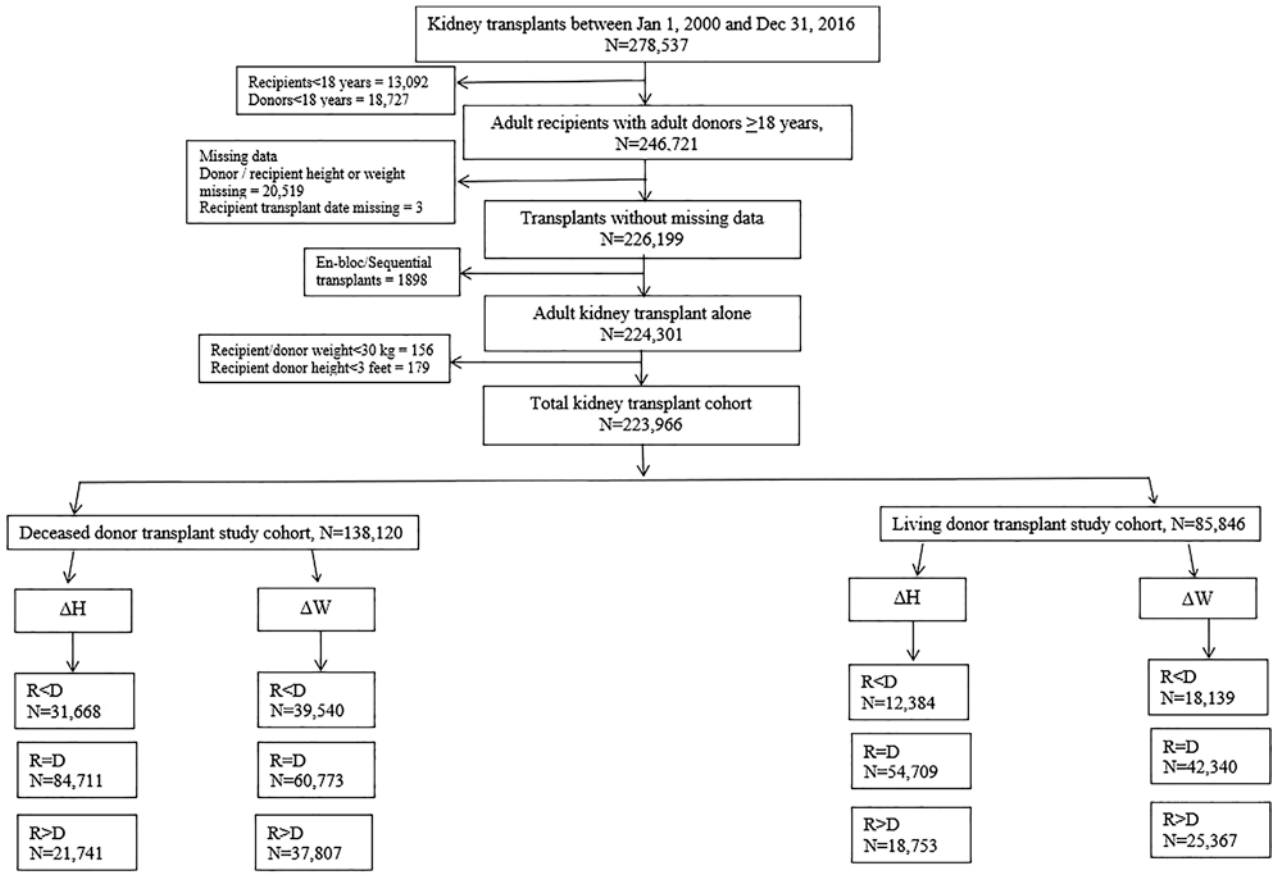


FIGURE 1 | Algorithm showing derivation of patient cohorts for analysis.

variables such as the number of HLA mismatches, peak panel reactive antibodies (PRA), acute rejection episodes, and the use of thymoglobulin for induction were also included (**Tables 1**, **2**).

Height and Weight Mismatch Between Donors and Recipients

The recipient and donor pairs were classified into 3 groups for DD and LD transplants separately based on height discrepancy (ΔHeight) as 1) Recipient >5 inches shorter than the donor (Recipient < Donor), 2) Recipient within 5 inches of donor's height (Recipient = Donor), and 3) Recipient >5 inches taller than the donor (Recipient > Donor).

The recipient and donor pairs were also classified for DD and LD transplants separately into 3 groups based on weight discrepancy (ΔWeight) as 1) Recipient >15 kg lighter than the donor (Recipient < Donor), 2) Recipient within 15 kg above or below donor's weight (Recipient = Donor), and 3) Recipient >15 kg heavier than the donor (Recipient > Donor).

A cut-off of 5 inches and 15 kg was chosen to create a well balanced sample for the groups, based on the distribution of height and weight discrepancies seen in the donor-recipient pairs (**Supplementary Figures S1, S2**).

Outcome Measures

The primary outcome was death censored graft loss (DCGL). DCGL was defined as a return to permanent long-term dialysis or repeat transplantation. Secondary outcomes were patient mortality, delayed graft function (DGF), overall graft loss, and death with a functioning graft. Overall graft loss was defined as graft loss occurring either due to graft failure with a return to permanent long-term dialysis or repeat transplantation or death. Death with a functioning graft was defined as death occurring in a patient whose graft was functioning at the time of the death.

Statistical Analysis

Continuous variables are reported as means and standard deviations for parametric, or medians and interquartile ranges for non-parametric data. Categorical variables are summarized as proportions. The baseline characteristics of the patients in different subgroups were analyzed using the Analysis of Variance (ANOVA) or Kruskal-Wallis test for continuous and chi squared test for categorical variables as appropriate. Kaplan

TABLE 1 | Baseline characteristics of patients stratified by differences in donor and recipient height for deceased donor kidney transplants.

Characteristic*	Recipient >5 inches shorter than donor (R < D)	Recipient up to 5 inches taller or shorter than donor (R = D)	Recipient >5 inches taller than donor (R > D)	Overall cohort	*p*-Value
N (%)	31668 (22.9%)	84711 (61.3%)	21741 (15.7%)	138120	
Recipient characteristics					
Age, years	51.4 (13.8)	52.7 (12.8)	52.9 (12.3)	52.4 (13.0)	<0.001
Gender					
Male	9162 (28.9%)	55676 (65.7%)	19887 (91.5%)	84725 (61.3%)	<0.001
Race					
White	19483 (61.5%)	51504 (60.8%)	12664 (58.2%)	83651 (60.6%)	<0.001
Black	8467 (26.7%)	27093 (32%)	8265 (38%)	43825 (31.7%)	
Asian	3024 (9.5%)	4509 (5.3%)	510 (2.3%)	8043 (5.8%)	
Others	694 (2.2%)	1605 (1.9%)	302 (1.4%)	2601 (1.9%)	
Dialysis vintage, days [Median (IQR)]	1423 (839, 2191)	1449 (859, 2240)	1397 (836, 2153)	1425 (843, 2196)	0.95
Hypertension	22601 (71.4%)	61478 (72.6%)	15800 (72.7%)	99879 (72.3%)	<0.001
Diabetes mellitus	10224 (32.3%)	29765 (35.1%)	7687 (35.4%)	47676 (34.5%)	<0.001
Height, inches	63.0 (3.1)	67.4 (3.5)	71.6 (3.2)	67.1 (4.3)	<0.001
Weight, kg	71.9 (16.7)	82.3 (18.3)	92.3 (19.2)	81.5 (19.2)	<0.001
BMI, kg/m^2	28 (5.9)	27.9 (5.5)	27.9 (5.4)	27.9 (5.6)	0.019
BSA, m^2	1.8 (0.2)	2.0 (0.2)	2.2 (0.3)	2.0 (0.3)	<0.001
Donor characteristics					
Age, years	39.3 (13.9)	41.4 (13.8)	43.1 (13)	41 (13)	<0.001
Gender					
Male	28128 (88.8%)	49316 (58.2%)	5289 (24.3%)	82733 (59.9%)	<0.001
Ethnicity					
White	26635 (84.1%)	71082 (83.9%)	18135 (83.4%)	115852 (83.9%)	<0.001
Black	4297 (13.6%)	10860 (12.8%)	2617 (12%)	17774 (12.9%)	
Asian	418 (1.3%)	1999 (2.4%)	768 (3.5%)	3185 (2.3%)	
Other	318 (1.0%)	770 (0.9%)	221 (1.0%)	1309 (0.9%)	
Height, inches	71.3 (2.8)	67.6 (3.4)	63.6 (3.2)	67.8 (3.9)	<0.001
Weight, kg	90.0 (20.3)	81.9 (20)	74.9 (19.8)	82.6 (20.6)	<0.001
BMI, kg/m^2	27.4 (5.8)	27.8 (6.4)	28.7 (7.5)	27.8 (6.5)	<0.001
BSA, m^2	2.1 (0.3)	2.0 (0.3)	1.8 (0.3)	2.0 (0.3)	<0.001
Diabetes mellitus	1967 (6.2%)	5870 (6.9%)	1801 (8.3%)	9638 (7%)	<0.001
Hypertension	8404 (26.5%)	24930 (29.4%)	7067 (32.5%)	40401 (29.3%)	<0.001
Hepatitis C virus	651 (2.1%)	2703 (3.2%)	763 (3.5%)	4117 (3.0%)	<0.001
Terminal donor creatinine					
Cr <=1.5 mg/dl	25153 (79.4%)	70894 (83.7%)	19029 (87.5%)	115076 (83.3%)	<0.001
Cr > 1.5 mg/dl	6501 (20.5%)	13789 (16.3%)	2707 (12.5%)	22997 (16.7%)	
Cr unknown	14 (<0.1%)	28 (<0.1%)	5 (<0.1%)	47 (<0.1%)	
Donation after circulatory death	4495 (14.2%)	10506 (12.4%)	2471 (11.4%)	17472 (12.6%)	<0.001
Transplant characteristics					
Cold ischemia time, hours	17.9 (8.8)	17.7 (9.0)	17.6 (8.8)	17.8 (8.9)	0.021
Number of HLA mismatches					
0	3740 (11.8%)	8632 (10.2%)	1978 (9.1%)	14350 (10.4%)	<0.001
1	442 (1.4%)	1016 (1.2%)	266 (1.2%)	1724 (1.2%)	
2	1593 (5.0%)	4128 (4.9%)	1057 (4.9%)	6778 (4.9%)	
3	4536 (14.3%)	11604 (13.7%)	2991 (13.8%)	19131 (13.9%)	
4	8068 (25.5%)	21923 (25.9%)	5658 (26.0%)	35649 (25.8%)	
5	8889 (28.1%)	24823 (29.3%)	6552 (30.1%)	40264 (29.2%)	
6/Unknown mismatches	4400 (13.9%)	12585 (14.9%)	3239 (14.9%)	20224 (14.6%)	
Thymoglobulin induction	14637 (46.2%)	38803 (45.8%)	10074 (46.3%)	63514 (46.0%)	0.238
Pre-emptive transplants	440 (1.4%)	1200 (1.4%)	317 (1.5%)	1957 (1.4%)	0.804
Cause of death					
Anoxia	6813 (21.5%)	19442 (23.0%)	5709 (26.3%)	31964 (23.1%)	<0.001
Cerebrovascular/Stroke	9388 (29.6%)	32158 (38.0%)	10018 (46.1%)	51564 (37.3%)	
Head trauma	14535 (45.9%)	30596 (36.1%)	5256 (24.2%)	50387 (36.5%)	
Others	932 (2.9%)	2515 (3.0%)	758 (3.5%)	4205 (3.0%)	
Acute rejection episodes	684 (2.2%)	1814 (2.1%)	449 (2.1%)	2947 (2.1%)	0.735
Peak PRA					
0	9828 (31%)	31884 (37.6%)	9094 (41.8%)	50806 (36.8%)	<0.001
>0	21840 (69%)	52827 (62.4%)	12647 (58.2%)	87314 (63.2%)	

**Data is presented in the format of mean (standard deviation) or N (%) unless stated otherwise.*

TABLE 2 | Baseline characteristics of patients stratified by differences in donor and recipient height for living donor kidney transplants.

Characteristic	Recipient >5 inches shorter than donor (R < D)	Recipient up to 5 inches taller or shorter than donor (R = D)	Recipient more than 5 inches taller than donor (R > D)	Overall cohort	*p*-Value
N (%)	12384 (14.4%)	54709 (63.7%)	18753 (21.8%)	85846	
Recipient characteristics					
Age, years	47 (15)	47 (14)	48 (13)	47 (14)	<0.001
Gender					
Male	2101 (17%)	32287 (59%)	17831 (95.1%)	52219 (60.8%)	<0.001
Race					
White	9800 (79.1%)	44091 (80.6%)	15339 (81.8%)	69230 (80.6%)	<0.001
Black	1727 (13.9%)	7442 (13.6%)	2595 (13.8%)	11764 (13.7%)	
Asian	667 (5.4%)	2398 (4.4%)	589 (3.1%)	3654 (4.3%)	
Others	190 (1.5%)	778 (1.4%)	230 (1.2%)	1198 (1.4%)	
Dialysis vintage, days	789 (857)	786 (805)	792 (810)	788 (814)	0.874
Hypertension	8726 (70.5%)	39387 (72%)	13612 (72.6%)	61725 (71.9%)	<0.001
Diabetes mellitus	3302 (26.7%)	15348 (28.1%)	5531 (29.5%)	24181 (28.2%)	<0.001
Height, inches	63 (3)	67 (3)	71.3 (3)	67 (4.2)	<0.001
Weight, kg	69.4 (17)	79.4 (18.7)	91.1 (18.5)	80.5 (19.6)	<0.001
BMI, kg/m^2	27 (6.1)	27.3 (5.6)	27.7 (5.2)	17.3 (5.6)	<0.001
BSA, m^2	1.8 (0.2)	1.9 (0.3)	2.1 (0.2)	1.9 (0.3)	<0.001
Donor characteristics					
Age, years	40.3 (11.7)	41.1 (11.5)	43.3 (11.4)	41.5 (11.6)	<0.001
Gender					
Male	10466 (84.5%)	21802 (39.9%)	1387 (7.4%)	33655 (39.2%)	<0.001
Ethnicity					
White	10190 (82.3%)	45472 (83.1%)	15821 (84.4%)	71483 (83.3%)	<0.001
Black	1652 (13.3%)	6516 (11.9%)	2034 (10.8%)	10202 (11.9%)	
Asian	378 (3.1%)	2010 (3.7%)	643 (3.4%)	3031 (3.5%)	
Other	164 (1.3%)	711 (1.3%)	255 (1.4%)	1130 (1.3%)	
Height, inches	70.9 (3.1)	66.7 (3.5)	63.6 (2.6)	66.5 (3.9)	<0.001
Weight, kg	88 (15.6)	77.4 (15.4)	70.3 (13.1)	77.4 (15.8)	<0.001
BMI, kg/m^2	27.1 (4.2)	26.9 (4.4)	26.9 (4.6)	26.9 (4.4)	<0.001
BSA, m^2	2.1 (0.2)	1.9 (0.2)	1.8 (0.2)	1.9 (0.2)	<0.001
Transplant characteristics					
Number of HLA mismatches					
0	1032 (8.3%)	4925 (9%)	1254 (6.7%)	7211 (8.4%)	<0.001
1	691 (5.6%)	2907 (5.3%)	763 (4.1%)	4361 (5.1%)	
2	2033 (16.4%)	9028 (16.5%)	2486 (13.3%)	13547 (15.8%)	
3	3228 (26.1%)	14793 (27%)	4484 (23.8%)	22505 (26.2%	
4	1901 (15.4%)	8081 (14.8%)	3300 (17.6%)	13282 (15.2%)	
5	2253 (18.2%)	9486 (17.3%)	4052 (21.6%)	15791 (18.4%)	
6/Unknown mismatches	1246 (10.1%)	5489 (10.0%)	2414 (12.9%)	9149 (10.7%)	
Thymoglobulin induction	4701 (38%)	20340 (37.2%)	6918 (36.9%)	31959 (37.2%)	0.148
Pre-emptive transplants	781 (6.3%)	3082 (5.6%)	966 (5.2%)	4829 (5.6%)	<0.001
Acute rejection episodes	199 (1.6%)	918 (1.7%)	361 (1.9%)	1478 (1.7%)	0.046
Peak PRA					
0	5547 (44.8%)	27090 (49.5%)	10087 (53.8%)	42724 (49.8%)	<0.001
>0	6837 (55.2%)	27619 (50.5%)	8666 (46.2%)	43122 (50.2%)	

**Data is presented in the format of mean (standard deviation) or N (%) unless stated otherwise.*

Meier analysis was done for univariate analysis of the impact of ΔHeight on the primary outcome of DCGL and secondary outcome of patient mortality and DGF. Multiple Cox regression analysis was performed utilizing covariates known to influence transplant outcomes. For LD transplants, the covariates included were differences in recipient and donor height and weight, recipient and donor ethnicity, age, gender, history of diabetes and hypertension, number of HLA mismatches, pre-emptive transplant, history of acute rejection, induction with thymoglobulin, and peak panel reactive antibody (PRA). For DD transplants, additional covariates included were terminal donor creatinine, donor cause of death, donation after circulatory death, and history of hepatitis C virus infection. Interactions between recipient-donor height and weight differences, and between height and gender differences were evaluated to assess if these factors modified the impact of height difference on outcomes. The best model fit was determined based on Akaike Information Criterion (AIC) scores and likelihood ratio tests. All analyses were conducted in R statistical software, version

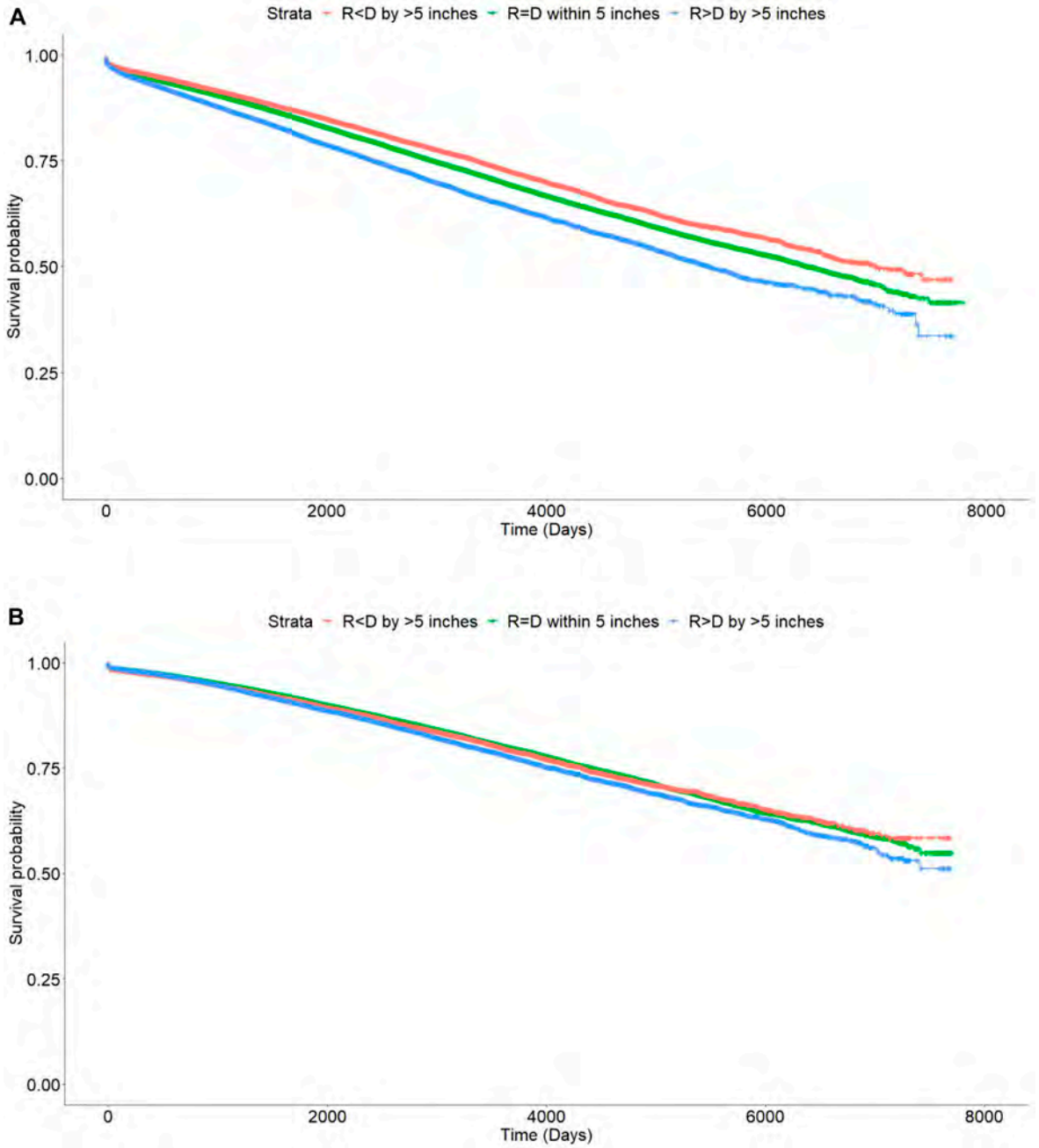


FIGURE 2 | Kaplan Meier curves showing differences in death censored graft loss with incremental difference between recipient and donor heights [Height of recipient (R) shorter than (<) or taller than (>) donor (D) by more than 5 inches, or within 5 inches (=)]. **(A)** Deceased donor, $p < 0.001$. **(B)** Living donor, $p < 0.001$.

4.1.0. (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient Population

A total of 278,537 kidney transplants alone were performed in the US between January 1, 2000 and December 31, 2016. After excluding recipients <18 years of age (n = 13,092), donors <18 years of age (n = 18,727), patients with missing donor/recipient height or weight (n = 20,519), missing transplant date (n = 3), en-bloc or sequential kidney transplants (n = 1,898), recipient/donor weight <30 kg (n = 156) and recipient/donor height <3 feet (n = 179), the total cohort of patients remaining for analysis was 223,966. In this cohort, 138,120 were DD transplants and 85,846 were living donor transplants (**Figure 1**).

TABLE 3 | Death censored graft and patient survival stratified by height differences.

	1 year (%)	3 years (%)	5 years (%)	Until last follow up (%)
Deceased donors				
Death censored graft survival				
R = D	94.9	90.5	86.2	76.8
R < D	95.7	91.5	87.7	78.8
R > D	93.8	88.0	82.8	73.1
Patient survival				
R = D	95.3	90.2	84.0	65.1
R < D	95.9	91.6	86.3	68.6
R > D	94.7	88.9	82.1	62.9
Living donors				
Death censored graft survival				
R = D	97.8	94.9	91.9	82.7
R < D	97.4	94.6	91.3	81.9
R > D	97.7	94.5	90.8	81.5
Patient survival				
R = D	98.4	95.8	92.4	76.7
R < D	98.4	95.7	92.4	77.3
R > D	98.3	95.3	91.8	75.9

Deceased Donor Cohort

The DD cohort was sub-divided into 3 categories based on ΔHeight as 1) Recipient < Donor (n = 31,668), 2) Recipient = Donor (n = 84,711), and 3) Recipient > Donor (n = 21,741) respectively (**Figure 1**).

The mean (SD) age of recipients and donors was 52.4 (13) and 41 (13) years respectively. There were more male recipients in the Recipient > Donor (91.5%) and Recipient = Donor (65.7%) groups and more female recipients in the Recipient < Donor (71.1%) group. The ethnic distribution in each of the ΔHeight categories (Recipient < Donor, Recipient = Donor and Recipient > Donor) corresponded to the overall transplant population in descending order of prevalence in Whites, Blacks, Asians, and other ethnic backgrounds (60.6, 31.7, 5.8, and 1.9% in recipients and 83.9, 12.9, 2.3, and 0.9% respectively in donors respectively). The terminal donor creatinine was >1.5 mg/dl in 20.5, 16.3, and 12.5% among the three height difference categories in the deceased donors. A larger proportion of donation after circulatory death (DCD) patients were in the Recipient < Donor group (14.2%), followed by Recipient = Donor (12.4%) and then Recipient > Donor (11.4%) groups. Other pertinent recipients, donor, and DD transplant-specific characteristics are summarized in **Table 1**.

The distribution of recipient-donor height differences at different categories of recipient weights and recipient-donor pairs with height differences greater than 10 inches stratified by weight quartiles of the recipient are shown in **Supplementary Figures S3, S4**.

Living Donor Cohort

The LD cohort was sub-divided into 3 categories based on ΔHeight as 1) Recipient < Donor (n = 12,384), 2) Recipient = Donor (n = 54,709), and 3) Recipient > Donor (n = 18,753) respectively (**Figure 1**).

The mean (SD) age of recipients and donors were 47 (14) and 41.5 (11.6) years respectively. There were larger proportions of male recipients in the Recipient > Donor (95.1%) and Recipient = Donor (59%) groups and more female recipients in the Recipient < Donor (83%) group. The ethnic distribution followed the same pattern of prevalence as the DD cohort with Caucasian donors/recipients comprising the highest proportion followed by Black, Asian, and donors/recipients from other ethnic groups in each sub-group (Recipient < Donor, Recipient = Donor, Recipient > Donor; 80.6, 13.7, 4.3, and 1.4% in recipients and 83.3, 11.9, 3.5, and 1.3% in donors respectively). Pre-emptive transplants occurred in 6.3, 5.6, and 5.2% respectively. The use of thymoglobulin was not different across the three groups (p = 0.148). The largest proportion of patients were mismatched at 3 HLA antigens (26.1, 27, and 23.8% respectively). The Recipient > Donor group had a higher proportion of non-sensitized (Peak PRA 0%) patients followed by Recipient = Donor and Recipient < Donor groups (53.8, 49.5, and 44.8% respectively). Other pertinent recipients, donors, and LD transplant specific characteristics are summarized in **Table 2**.

Primary Outcome

Death Censored Graft Loss

Deceased Donor Kidney Transplants

On Kaplan Meier analysis, there was a statistically significant difference in DCGL with incremental degrees of ΔHeight. The Recipient < Donor group had the lowest rates of DCGL followed by Recipient = Donor and Recipient > Donor groups ($p < 0.001$). In other words, the taller the recipient as compared to the donor, the worse the primary outcome of DCGL. The differences were more pronounced in the DD cohort compared to the LD cohort (**Figure 2A**).

The 1, 3, and 5 years graft survival for DD transplant recipients were 95.7, 91.5, and 87.7% for Recipient < Donor group, 94.9, 90.5, and 86.2% for Recipient = Donor group, and 93.8, 88, and 82.8% for Recipient > Donor group respectively. At last follow up, the graft survival rates were 78.8, 76.8, and 73.1% respectively. The absolute difference in graft survival rates between the two extremes of Recipient < Donor and Recipient > Donor groups was 5.7% (**Table 3**).

On Cox multivariate regression analysis using recipient-donor ΔHeight and ΔWeight along with other covariates as discussed above, the HR [95% confidence interval (CI)] of Recipient < Donor was lower at 0.95 (0.91–1.00; p = 0.05) and that of Recipient > Donor was higher at 1.07 (1.01–1.13; p = 0.01) compared to the reference group of Recipient = Donor n the DD cohort. The HR (95% CI) for the two extremes of ΔWeight were similarly lower for Recipient < Donor group at 0.95 (0.91–0.98; p = 0.004) and higher for Recipient > Donor group at 1.12 (1.08–1.16; $p < 0.001$) compared to the reference group of Recipient = Donor (**Figure 3A**).

Living Donor Kidney Transplants

On Kaplan Meier analysis, there was a statistically significant difference in DCGL with incremental degrees of ΔHeight. The Recipient < Donor group had the lowest rates of DCGL followed

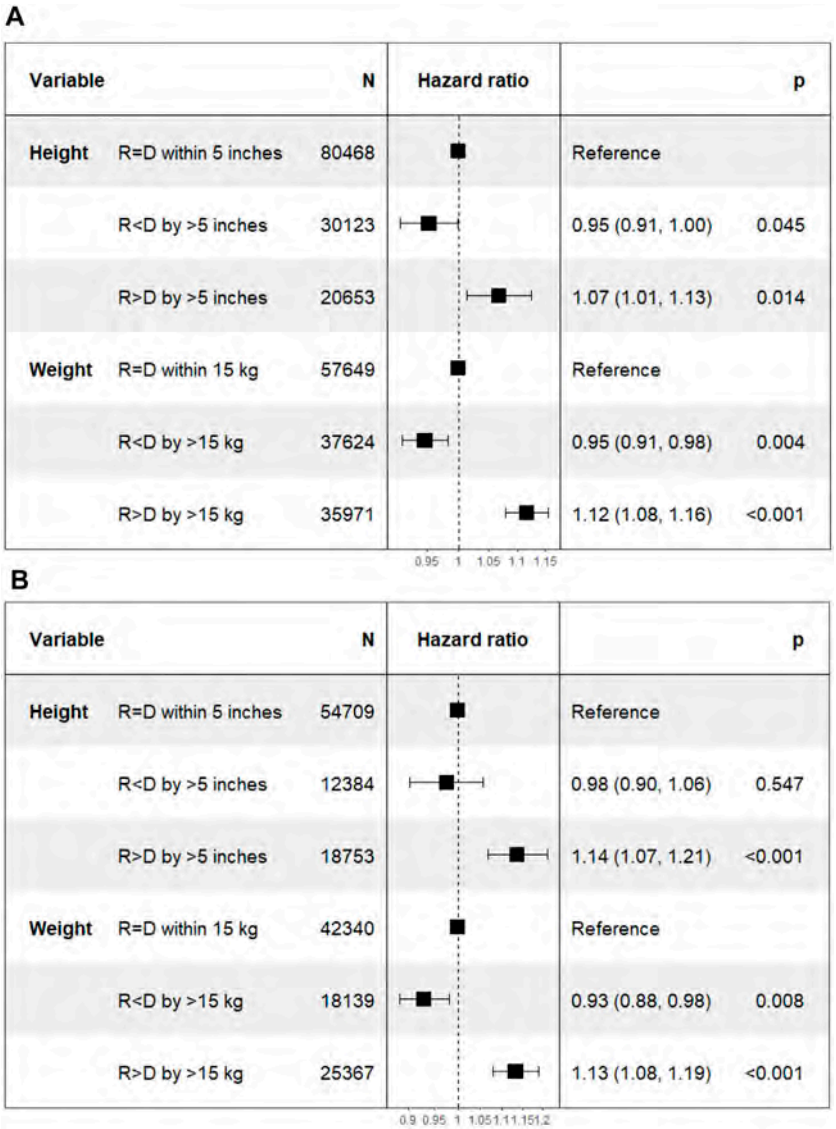


FIGURE 3 | Differences in death censored graft loss with incremental differences in recipient and donor heights. [Height of recipient (R) shorter than (<) or taller than (>) donor (D) by more than 5 inches, or within 5 inches (=)]. **(A)** Deceased donor kidney transplant. **(B)** Living donor kidney transplant.

by Recipient = Donor and Recipient > Donor groups ($p < 0.001$). (**Figure 2B**).

The 1, 3, and 5 years graft survival for LD transplant recipients were 97.4, 94.6, and 91.3% respectively for Recipient < Donor group, 97.8, 94.9, and 91.9% for Recipient = Donor group and 97.7, 94.5, and 90.8% for Recipient > Donor group. At last follow up, the graft survival rates were 81.9, 82.7, and 81.5% respectively. The absolute difference in graft survival rates between the two extremes of Recipient < Donor and Recipient > Donor groups was 0.4% (**Table 3**).

The HR (95% CI) of DCGL in Recipient < Donor group was lower at 0.98 (0.90–1.06; $p = 0.55$) for Recipient < Donor group and higher at 1.14 (1.07–1.21; $p < 0.001$) in Recipient > Donor group compared to Recipient = Donor group. In the ΔWeight categories, the HR (95% CI) of DCGL was lower at 0.93 (0.88–0.98; $p = 0.008$) in Recipient < Donor group and higher at 1.13 (1.08–1.19; $p < 0.001$) in Recipient > Donor group compared to the Recipient = Donor group (**Figure 3B**).

The model with height differences as a covariate performed better than the model without height differences with lower AIC scores for both DD and LD transplants (p value with likelihood ratio test <0.001) (**Supplementary Table S1**).

Secondary Outcomes

Mortality

Deceased Donor Kidney Transplants

On Kaplan Meier analysis, there was a statistically significant difference in patient survival between the different categories of ΔHeight (p value < 0.001) (**Figure 4A**).

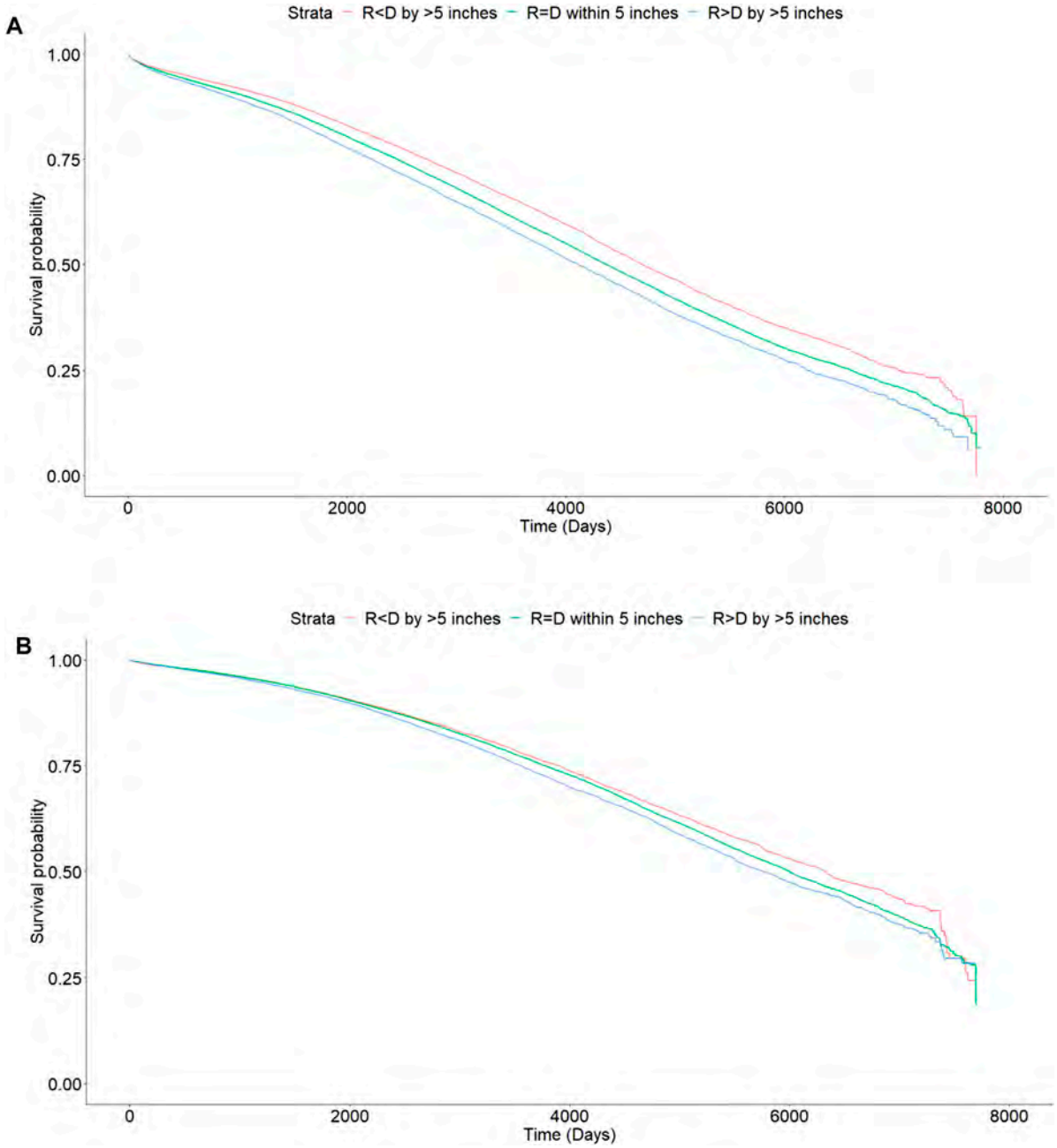


FIGURE 4 | Kaplan Meier curves showing differences in mortality with incremental difference between recipient and donor heights [Height of recipient (R) shorter than (<) or taller than (>) donor (D) by more than 5 inches, or within 5 inches (=)]. **(A)** Deceased donor transplants, $p < 0.001$. **(B)** Living donor transplants, $p < 0.001$.

The 1, 3, and 5 years patient survival for DD transplant recipients were 95.9, 91.6, and 86.3% for Recipient < Donor group, 95.3, 90.2, and 84.0% for Recipient = Donor group, 94.7, 88.9, and 82.1% for Recipient > Donor group respectively. At last follow up, the patient survival rates were 68.6, 65.1, and 62.9% respectively. The absolute difference in patient survival rates between the two extremes of Recipient < Donor and Recipient > Donor groups was 5.7% (**Table 3**).

On Cox multivariate analysis using recipient-donor ΔHeight and ΔWeight along with other covariates as discussed above, there was a statistically significant higher HR of mortality in Recipient > Donor group for both ΔHeight [1.07 (1.02–1.12); $p = 0.003$] and ΔWeight [1.04 (1.01–1.07); $p = 0.01$] categories using Recipient = Donor as the reference category in the DD cohort. However, the HR for Recipient < Donor were not significant for

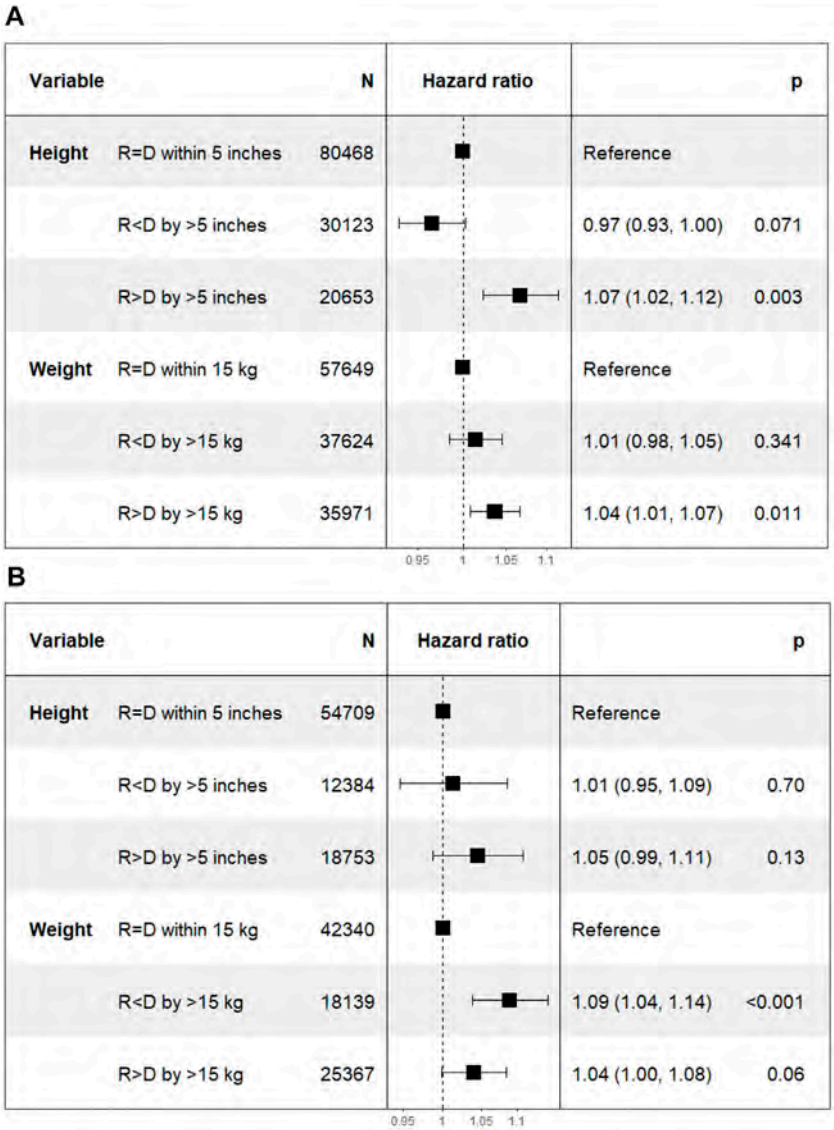


FIGURE 5 | Differences in mortality with incremental differences in recipient and donor height and weight. [Height of recipient (R) shorter than (<) or taller than (>) donor (D) by more than 5 inches, or within 5 inches (=)]. **(A)** Deceased donor transplant. **(B)** Living donor transplant.

either ΔHeight [0.97 (0.93–1.00); p = 0.07] or ΔWeight [1.01 (0.98–1.05); p = 0.34] categories (**Figure 5A**).

Living Donor Transplants

On Kaplan Meier analysis, there was a statistically significant difference in patient survival between the different categories of ΔHeight (p value < 0.001) (**Figure 4B**).

The 1, 3, and 5 years patient survival for LD transplant recipients were 98.4, 95.7, and 92.4% for Recipient < Donor group, 98.4, 95.8, and 92.4% for Recipient = Donor group, and 98.3, 95.3, and 91.8% respectively for Recipient > Donor group. At last follow up, the patient survival rates were 77.3, 76.7, and 75.9% respectively. The absolute difference in patient survival rates between the two extremes of Recipient < Donor and Recipient > Donor groups was 1.4% (**Table 3**).

The HR (95% CI) of mortality was 1.01 (0.95–1.09; p = 0.7) in Recipient < Donor group and 1.05 (0.99–1.11; p = 0.13) in Recipient > Donor group. Similarly, in the weight categories, the HR (95% CI) was 1.09 (1.04–1.14; p < 0.001) in Recipient < Donor and 1.04 (1.00–1.08; p = 0.06) in Recipient > Donor group (**Figure 5B**).

The model with height differences performed better than the one without height differences with lower AIC scores for both DD and LD transplants (p value with likelihood ratio test <0.001) (**Supplementary Table S1**).

Delayed Graft Function

Deceased Donor Kidney Transplants

In DD transplant recipients, multivariate logistic regression showed no statistical difference in DGF in Recipient < Donor [1.04 (0.99–1.1); p = 0.1] and Recipient > Donor [1.01 (0.95–1.08;

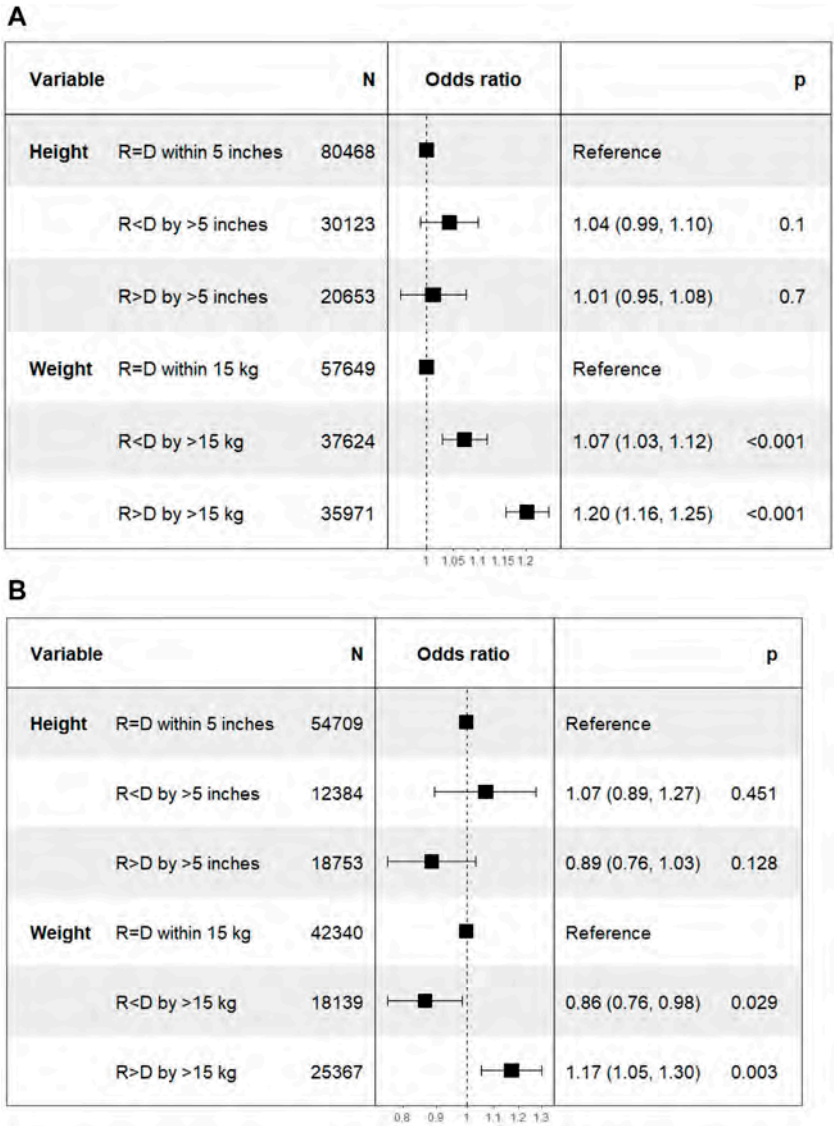


FIGURE 6 | Differences in delayed graft function with incremental differences in recipient and donor height and weight. [Height of recipient (R) shorter than (<) or taller than (>) donor (D) by more than 5 inches, or within 5 inches (=)]. **(A)** Deceased donor transplants. **(B)** Living donor transplants.

$p = 0.7$] groups stratified by ΔHeight. However, there was a higher HR of DGF with both Recipient < Donor [1.07 (1.03–1.12); $p < 0.001$] and Recipient > Donor [1.20 (1.16–1.25); $p < 0.001$] groups compared to the Recipient = Donor group stratified by ΔWeight.

Living Donor Kidney Transplants

In LD transplant recipients, the HR (95% CI) of DGF in Recipient < Donor and Recipient > Donor groups were 1.07 (0.89–1.27; $p = 0.45$) and 0.89 (0.76–1.03; $p = 0.13$) for ΔHeight categories. The corresponding HR for ΔWeight categories were 0.86 (0.76–0.98; $p = 0.03$) and 1.17 (1.05–1.30; $p = 0.003$) respectively (**Figure 6**).

Overall Graft Loss

Deceased Donor Kidney Transplants

In the deceased donor cohort, there was a statistically significant lower HR in the Recipient < Donor category of 0.97 (0.94–1; $p = 0.04$) and higher HR in the Recipient > Donor category of 1.06 (1.02–1.1; $p = 0.002$).

Living Donor Kidney Transplants

In the living donor cohort, there was an HR of 1.00 (0.95–1.06; $p = 0.88$) in the Recipient < Donor category and a higher HR of 1.06 (1.01–1.11; $p = 0.02$) in the Recipient > Donor group (**Supplementary Figure S5**).

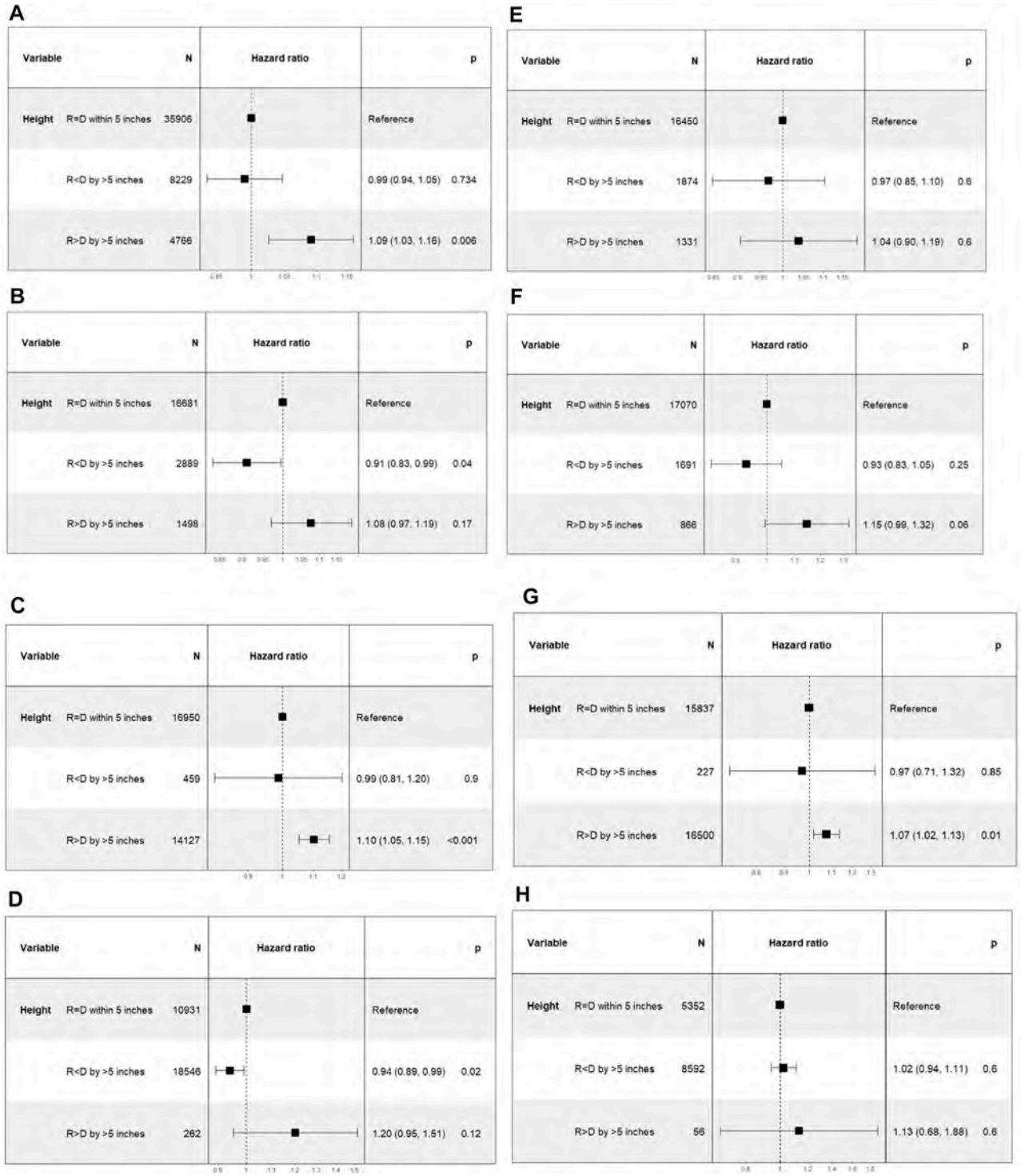


FIGURE 7 | Differences in death censored graft loss with incremental differences in recipient and donor heights, categorized on the basis of gender differences between the donor-recipient pairs. [Height of recipient (R) shorter than (<) or taller than (>) donor (D) by more than 5 inches, or within 5 inches (=)]. **(A)** Deceased donor male to male transplant. **(B)** Deceased donor female to female transplant. **(C)** Deceased donor female to male transplants. **(D)** Deceased donor male to female transplant. **(E)** Living donor male to male transplants. **(F)** Living donor female to female transplant. **(G)** Living donor female to male transplant. **(H)** Living donor male to female transplant.

Death With a Functioning Graft

Deceased Donor Kidney Transplants

In the deceased donor cohort, there was a lower HR of 0.97 (0.93–1.01; p = 0.19) in the Recipient < Donor category and a higher HR of 1.06 (1.01–1.12; p = 0.02) in the Recipient > Donor category.

Living Donor Kidney Transplants

In living donor cohort, there was a higher HR of 1.02 (0.94–1.11; p = 0.6) and 1.01 (0.95–1.08; p = 0.7) in Recipient < Donor and Recipient > Donor categories respectively (**Supplementary Figure S6**).

Interaction Between Differences in Donor-Recipient Height and Weight

Model fit improved with the addition of height differences and weight differences separately. Including both height and weight differences resulted in the best model fit. However, the inclusion of an interaction term between height difference and weight difference did not improve the model fit as evidenced by higher AIC scores and statistically non-significant p values (**Supplementary Table S1**).

Donor-Recipient Gender Combination

As a sub-group analysis, the DD and LD cohorts were divided into four categories of male to male, female to female, male to female and female to male transplants. Within each of these categories, a consistent trend of a lower hazard ratio for Recipient < Donor and a higher hazard ratio for Recipient > Donor was seen in both DD and LD transplants—among DD, male to male transplants Recipient > Donor group [n = 4766; 1.09 (1.03–1.16); p = 0.006], female to female transplants Recipient < Donor group [n = 2889; 0.91 (0.83–0.99); p = 0.04], female to male transplants Recipient > Donor group [n = 14127; 1.10 (1.05–1.15); p < 0.001], male to female transplants Recipient < Donor group [n = 18546; 0.94 (0.89–0.99); p = 0.02], and among LD, female to male transplants Recipient > Donor group [n = 16,500; 1.07 (1.02–1.13); p = 0.01] (**Figure 7**).

Inclusion of height and gender pair differences led to better model fit but the inclusion of an interaction term between height differences and gender pair differences did not improve the model fit as evidenced by higher AIC scores (**Supplementary Table S1**).

DISCUSSION

Size mismatch between the recipient and donor is known to influence transplant outcomes. However, the impact of height mismatch specifically is not known. In this analysis of a large database of deceased and living donor kidney transplant patients from SRTR, we found that height mismatch between the recipient and donor is an independent factor predicting kidney transplant outcomes.

Prior studies have evaluated size mismatch between the recipient and donor on the basis of body surface area (BSA), body mass index (BMI), and weight (2,3,8,9). These parameters have been used as surrogates for discrepancies in kidney size and/or nephron mass in the recipient-donor pair. We found that there is a poor correlation between an individual's height and weight. At a given weight, there was a wide variation of heights in the population (**Supplementary Figures S3, S4**). Similarly, despite the derivation of BSA from height and weight of the individual, we only found a modest correlation of BSA with height (r = 0.69 for DD, r = 0.7 for LD), but a high correlation with weight (r = 0.98 for DD, r = 0.98 for LD).

Height discrepancy in the recipient-donor pairs has not been studied rigorously as a predictor of outcomes in kidney transplant patients without being included in a composite measure such as BSA or BMI. In a study done by Vinson et al, their risk prediction model showed a statistically significant lower hazard ratio with increasing donor-recipient height difference in DD transplants [0.726 (0.664–0.794)] (10). Our study had similar findings, with a more robust categorization of height discrepancies, and included DD and LD cohorts separately. Donor-recipient height ratios have also been included in a kidney graft survival calculator that showed a lower hazard ratio for a height ratio >1.06 [0.94 (0.91–0.98)] and a higher hazard ratio for a height ratio <0.94 [1.05 (1.02–1.09)] (11).

It is not clear from the literature which anthropometric measurement is the best surrogate for nephron mass. One of the predictors of nephron mass is birth weight and length (1,4–6), which has a strong association with adult height (12). It is well known that nephron endowment at the time of birth is final and any deficit due to pre-maturity exposes the individual to a higher likelihood of developing kidney disease during their lifetime, due to the increased demands placed on the lower number of nephrons. Although these do influence adult weight as well, it is prone to be modified by an individual's lifestyle such as dietary habits and physical activity, and fluid balance. Higher weight has been shown to be associated with larger nephron size, but not necessarily with a higher number of nephrons (4). A larger nephron size may be a reflection of increased metabolic demand on a limited number of nephrons. Higher recipient BMI has been shown to be associated with increased morbidity and graft loss after kidney transplantation (13,14).

In their population based study from the UK Transplant Registry, Arshad et al did not find any differences in DGF or DCGL due to donor-recipient weight differences but they did find increased mortality in patients receiving kidneys from donors whose weights were over 25% of recipient weight (2). Miller et al found that a concurrent mismatch in donor-recipient weight and donor-recipient sex was associated with a higher risk of DCGL (8). This finding could be secondary to a higher weight in the donor being a reflection of other co-morbidities that accompany obesity such as diabetes mellitus and hypertension. In addition, obesity could lead to hyperfiltration injury in the donor kidney that would likely not be the case in non-obese donors. Lepeytre et al reported that the effect of donor-recipient size mismatch based on their BSA on long-term transplant outcomes is modulated by the recipient and donor age in their population based study from SRTR (3). This is not surprising as a serial decline in the number of nephrons with age parallels the progressive decline in glomerular filtration rate with age (4,15,16). Instead of using a composite measure such as BSA or BMI in prior studies, we evaluated the individual components of these measures to reconcile the discrepant results in prior studies.

Gender differences are also an important factor to consider when assessing size mismatch (8,9,17,18). We found that the influence of height discrepancies persisted within different combinations of donor-recipient genders in a predictable pattern just as in the overall cohort of DD and LD transplants. In subgroups that contained a large number of patients, the trends were statistically significant. However, we suspect some categories failed to show statistical significance due to the relatively fewer number of patients in the category. Regardless, the trends show that Recipient < Donor have better outcomes and Recipient > Donor have poorer outcomes compared to the Recipient = Donor group (**Figure 7**). Inclusion of height differences to a model including gender pair differences improved the model fit for DCGL and mortality but the interaction effect was not significant, suggesting that the height differences do not impact the outcomes differently among those with different donor-recipient gender combinations.

Our study has several strengths. It is based on a large database of transplant populations from SRTR in the modern era of tacrolimus-based maintenance immunosuppression and transplant care. Our study utilizes height as an anthropometric measure to assess size mismatch, which likely correlates best with nephron mass and is less likely to be influenced by an individual's lifestyle choices. The use of height mismatch is also simpler to use compared to other composite anthropometric measures such as BMI and BSA. Although donor height is incorporated into the allocation system as a part of the KDPI score, height mismatch between the donor-recipient pair may be a more important factor to consider in terms of transplant outcomes. Our study also incorporates analyses of multiple models with interactions between donor-recipient height differences and differences in weight and gender to determine the differential impact of these co-occurring donor-recipient mismatches.

The results of this population-based study must be interpreted within the limitations of the design of these studies. While we made efforts to minimize bias by incorporating multiple covariates that could influence transplant outcomes, it is not possible to include all the variables. We acknowledge that there could be residual confounding resulting from variables that could not be incorporated into our analysis. We could not assess the degree of proteinuria in the patients in the different subgroups as this information was not available in the database. This would have allowed us to see the impact of height mismatch on downstream physiologic effects such as progressive glomerular sclerosis and resultant urinary protein excretion. The results of our study are based on data from the US population and may not be generalizable to other populations.

In conclusion, our study finds that transplantation of kidneys from individuals of shorter stature into taller recipients leads to worse transplant outcomes. This effect appeared more pronounced in deceased donors than in living donors. This information may be used while counseling living donors and their recipients that height mismatch may not have a major influence in determining post-transplant outcomes, especially when multiple donors are available. The quality of a living donor kidney and recipient comorbidities likely supersede the influence of height mismatch in living donor transplantation. Size mismatch in the donor-recipient pair based on discrepancies in their heights may be more reliable compared to other anthropometric measures in determining post-transplant outcomes.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: A research proposal is required to be submitted to SRTR to have access to the dataset. Requests to access these datasets should be directed to https://srtr.org/requesting-srtr-data/data-requests/.

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

ST conceived the idea of the project, participated in research design, writing of the manuscript, performance of the research and data analysis. CW, SH, and CP reviewed and edited the manuscripts and provided feedback for revisions and further analysis.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

The authors would like to thank Bryn Thompson, Senior Project Manager at SRTR, for assistance with providing the datasets for this study. The data reported here have been supplied by the Hennepin Healthcare Research Institute (HHRI) as the contractor for the Scientific Registry of Transplant Recipients (SRTR). 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 SRTR or the U.S. Government.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2022.10253/full#supplementary-material

Supplementary Figure 1 | Histogram showing distribution of height differences **(A)** Deceased donor kidney transplant **(B)** Living donor kidney transplant.

Supplementary Figure 2 | Histogram showing distribution of weight differences **(A)** Deceased donor kidney transplant **(B)** Living donor kidney transplant.

Supplementary Figure 3 | Differences in recipient-donor heights at different categories of recipient weights for deceased donor kidney transplants.

Supplementary Figure 4 | Donor-recipient pairs with height differences greater than 10 inches stratified by weight quartiles of the deceased donor transplant recipients.

Supplementary Figure 5 | Cox multivariate analysis for overall graft loss with differences in donor and recipient height **(A)** Deceased donor kidney transplant **(B)** Living donor kidney transplant.

Supplementary Figure 6 | Cox multivariate analysis for death with a functioning graft with differences in donor recipient height **(A)** Deceased donor kidney transplant **(B)** Living donor kidney transplant.

Supplementary Table 1 | Comparison of different models with various interaction terms with differences in donor-recipient height.

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ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/ti.2022.10279

Post-Transplantation Early Blood Transfusion and Kidney Allograft Outcomes: A Single-Center Observational Study

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The association between blood transfusion and the occurrence of *de novo* HLA donor specific antibodies (DSA) after kidney transplantation remains controversial. In this single-center observational study, we examined the association between early blood transfusion, i.e. before 1-month post-transplantation, and the risk of DSA occurrence, using Luminex based-methods. In total, 1,424 patients with a minimum of 1-month follow-up were evaluated between January 2007 and December 2018. During a median time of follow-up of 4.52 years, we observed 258 recipients who had at least one blood transfusion during the first month post-transplantation. At baseline, recipients in the transfused group were significant older, more sensitized against HLA class I and class II antibodies and had a higher 1-month serum creatinine. Cox proportional hazards regression analyses did not show any significant association between blood transfusion and the risk of *de novo* DSA occurrence (1.35 [0.86–2.11], $p = 0.19$), the risk of rejection (HR = 1.33 [0.94–1.89], $p = 0.11$), or the risk of graft loss (HR = 1.04 [0.73–1.50], $p = 0.82$). These data suggest then that blood transfusion may not be limited when required in the early phase of transplantation, and may not impact long-term outcomes.

Keywords: kidney transplantation, allograft failure, graft loss, donor specific antibody, blood transfusion

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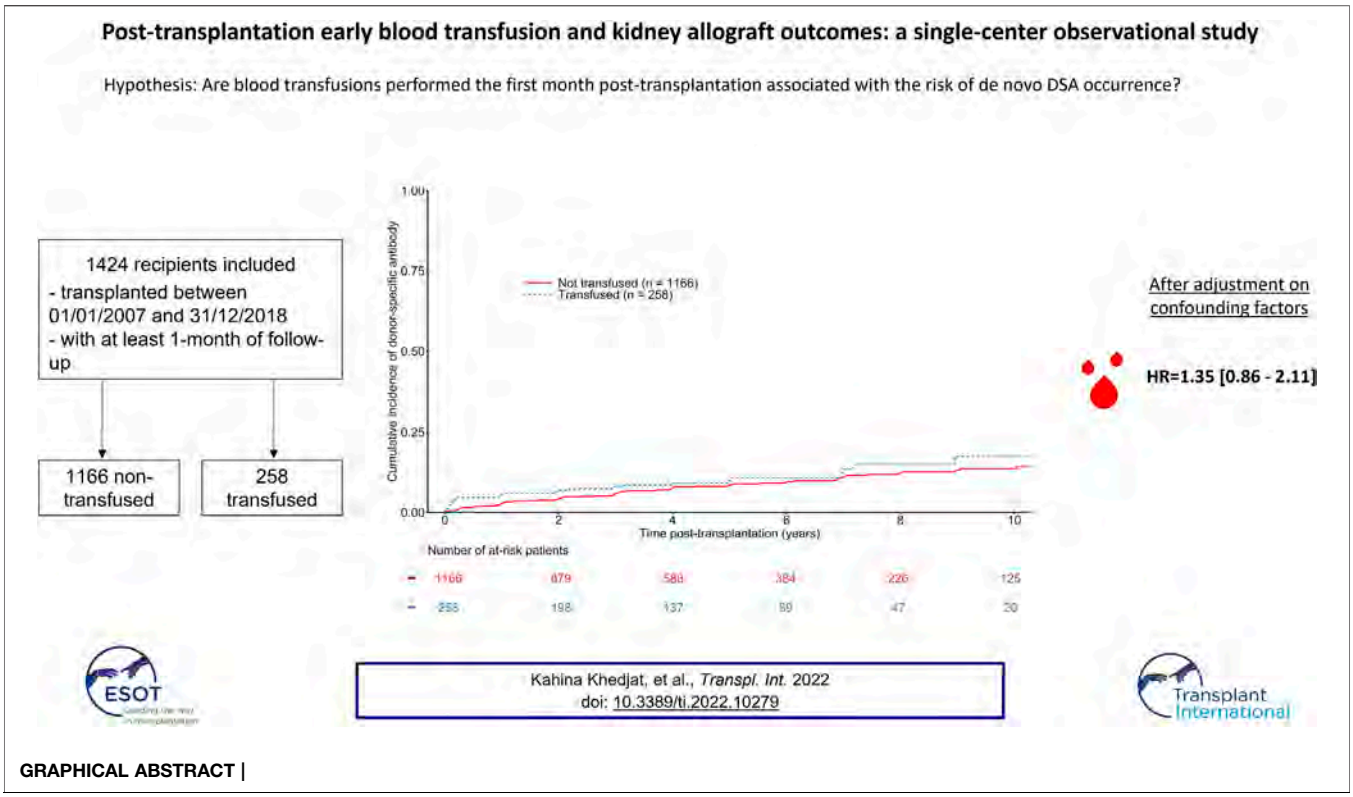
Received: 02 December 2021
Accepted: 07 February 2022
Published: 18 March 2022

Citation:
Khedjat K, Lenain R, Hamroun A, Baes D, Top I, Labalette M, Lopez B, Van Triempont M, Provôt F, Frimat M, Gibier J-B, Hazzan M and Maanaoui M (2022) Post-Transplantation Early Blood Transfusion and Kidney Allograft Outcomes: A Single-Center Observational Study. Transpl Int 35:10279. doi: 10.3389/ti.2022.10279

Abbreviations: ABMR, Antibody-Mediated Rejection; BMI, Body-Mass Index; BPAR, Biopsy-Proven Acute Rejection; DBD, Donation after Brainstem Death; DCD, Donor after Circulatory Death; DSA, Donor Specific Antibodies; ESKD, End-Stage Kidney Disease; HLA, Human Leukocyte Antigen; HPM, Hypothermic Perfusion Machine; HR, Hazard-Ratio; IQR, Interquartile Range; KT, Kidney Transplantation; RBC, Red Blood Cell; TSA, Transfusion Specific Antibodies; WBC, White Blood Cells.



GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney transplantation (KT) is currently the best treatment option, considering quality of life, life expectancy and cost-effectiveness in end-stage renal disease (1–3). However, there is an increased risk of death during the first months post-transplantation compared to dialysis, owing to surgical and infectious complications (2). This sensitive early post-transplant period brings along increased risks of bleedings due to surgery and anemia of multifactorial origins (infections, inflammation, medications, . . .) (4). Early blood transfusion is therefore often necessary, in an era where the age of recipients is constantly increasing, as well as the use of anticoagulant drugs (5).

Blood transfusions are a well-known cause of allogenic sensitization, especially before transplantation. Even though red blood cells are reputed to carry only low levels of Human Leukocyte Antigens (HLA) antigens, blood transfusions also bring few lymphocytes or platelets which may carry class I or class II HLA molecules (6). This antigen exposure to the immune system causes the generation of long-lived alloantibody-producing memory B cells (7) and anti-HLA antibodies. This process is dose-dependent, as the level of pre-transplant sensitization is correlated with the number of pre-transplant transfusions (8). Early blood transfusion is frequent after kidney transplantation, and concerns up to 40–60% among recipients (9–12). Considering its impact, the interrelationship between early blood transfusion allogenic exposure, *de novo* Donor Specific Antibodies (DSA) formation and allograft outcomes is less clear as previous reported cohorts provide contradictory results (9–11). Furthermore, the detection of DSA has greatly evolved over time thanks to Luminex-based methods, and there is a lack of large-scaled studies which examined the link between transfusion and *de novo* DSA occurrence using Luminex.

Our objective was then to examine the impact of post-KT early blood transfusions on *de novo* DSA formation, using Luminex-based methods, in a large cohort of renal transplant recipients. We also evaluated the impact of post-KT blood transfusions on the risk of biopsy-proven acute rejection (BPAR) and graft failure.

METHODS

Data Source and Ethical Statement

This single-center observational study was performed according to Istanbul Declaration, as well as the Helsinki Declaration ethical guidelines. The study data were collected from *Agence de la Biomédecine*—a state agency that coordinates and administers organ procurement in France—and completed with the recipient medical records. No organs were procured from prisoners. The French legislation stipulates that registry-based research is an integral part of outcome assessment for solid organ transplantation and is exempt from Institutional Review Board approval. All participants provided their informed consent. Patients and laboratory data were pseudonymized and registered according to the French data protection registry (CNIL), referenced #DEC19-054.

Population

This study included all consecutive adult recipients who had undergone kidney transplantation from January 2007 to December 2018, at the Lille University hospital (Lille, France), with at least 1 month of follow-up. Follow-up terminated in December 2019. Recipients with active or passive desensitization protocol before transplantation were not included as well as patients who previously received transplantation from another organ or a combined transplantation. Patients with lack of information regarding post-transplantation HLA antibody testing were excluded.

Exposure

Blood transfusions were exhaustively registered thanks to the eTRACELINE software (Mak-System®), which identifies the number, the nature and the time of every blood transfusion at the Lille University Hospital. Only ABO-compatible transfusions were performed in the cohort. No information regarding other blood group systems were collected (e.g., rhesus, MNS system, Kell system or others). Only leukocyte-depleted packed red cells were transfused, according to French Laws regarding the risk of infectious agents' transmission. Early-blood transfusion was then defined as any recipient who benefitted from at least one blood transfusion before 1-month post-transplantation.

Post-Transplantation Management

The immunosuppressive regimen consisted in an induction therapy (basiliximab or thymoglobulin) and a maintenance triple drug treatment (tacrolimus, mycophenolate mofetil and steroids) for all recipients. Tacrolimus was started at 0.15 mg/kg/d, then adapted to tacrolimus trough level with a target of 10–15 ng/ml up to day-15, and 6–8 ng/ml thereafter. Daily doses of mycophenolate mofetil were 750 mg twice a day. Steroids were withdrawn at day-7 in first-transplant non-sensitized recipient and progressively tapered to 0.1 mg/kg per day in others. Valganciclovir was administered during the first 6 months post transplantation in cytomegalovirus seronegative patients who received a kidney from a cytomegalovirus seropositive donor. A prophylaxis against Pneumocystis jirovecii (trimethoprim-sulfamethoxazole) was prescribed the first 3 months post transplantation.

Data Collection and Outcomes

The following donors' parameters were collected: age, sex, blood type, Body Mass-Index (BMI), living donor, cause of death, cold ischemia time, conservation modality (hypothermic perfusion machine (HPM) or static cold storage), donation after brainstem death (DBD) or after circulatory death (DCD). The following recipients' baseline parameters were collected: age, sex, body mass index (BMI), blood type, cause of end stage kidney disease (ESKD), type of dialysis, time on dialysis, time on the waiting list, previous transplantation, induction therapy, HLA sensitization, number of HLA mismatches, number of blood transfusion, time of blood transfusion, serum creatinine values, time of graft failure defined as the return to dialysis or pre-emptive retransplantation, time of BPAR, time of death.

Anti-HLA antibodies were routinely tested for every recipient at 3 months, 1 year and every year post-KT. Class I and II anti-HLA antibodies were defined by the presence of class I and II anti-HLA antibodies by the LABScreen Mixed Luminex flow bead assay (One Lambda). In case of positivity, specificities were determined according to the LABScreen Single Antigen Luminex flow bead assay (One Lambda). DSA targeting the A, B, Cw, DR, DQ, and DP antigens, were considered as significant if a minimum of mean fluorescence intensity of 1,000 was reached.

In recipients who required blood transfusion, our local protocol involves additional anti-HLA antibodies testings at Day 15, 21, and 28.

The primary outcome of this study was to determine the association between post-KT blood transfusions and the emergence of *de novo* DSA. Secondary outcomes included the association between blood transfusions and one/the risk of BPAR and two/death-censored graft survival. BPAR was determined according to the Banff classification system at the time of kidney biopsy.

Statistical Analysis

Baseline variables were compared between transfused and non-transfused patients by chi-square (categorical data) or Student's t-tests (continuous data). The Aalen-Johansen estimator was used to analyze the cumulative incidence of DSA, BPAR and death-censored graft failure accounting for the competing risk of graft loss and death. Hazard ratios (HRs) and 95% confidence intervals associated with transfusion status were estimated using Cox proportional hazards modeling. A multivariate backward selection procedure was implemented, with a univariate threshold $p < 0.20$ for inclusion. Characteristics known to be associated with graft survival were selected *a priori* to be included in the final model even if not significant (cold ischemia time). Log-linearity and the proportional hazards assumption were tested using a graphical method. Sensitivity analyses included the evaluation of the transfusion status on short term outcomes, i.e. the risk of *de novo* DSA occurrence, death-censored graft loss and rejection at 1-year post-transplantation using logistic regression. A multivariate backward selection procedure was implemented, with a univariate threshold of $p < 0.20$ for inclusion. All analyses were carried out in R, version 3.6.3. Statistical significance was determined by a two-tailed p value < 0.05.

RESULTS

Study Population and Baseline Characteristics

In total, 1,620 recipients underwent kidney transplantation between January 2007 and December 2018. Among these, 1,424 recipients met the criteria of inclusion and had a functional graft at 1-month post-KT (See Flowchart in **Supplementary Figure S1**). The median time of follow-up was 4.52 years (first–third quartile: 2.41–7.56 years). Overall, 258 recipients (18% of the cohort), benefitted from at least one transfusion before 1-month post-KT, with a median number of two transfusions (first–third quartile: 2–2). Forty recipients benefitted from more than three transfusions. Transfused recipients were significantly older, sensitized in class I and class II HLA antibodies, and had a longer time on the waiting list compared to non-transfused recipients. Donors from transfused

TABLE 1 | Baseline characteristics between transfused and non-transfused recipients.

	Non-transfused (*n* = 1,166)	Transfused (*n* = 258)	*p*-value
Donor			
Age (years), median (IQR)	52.00 (41.00–62.00)	56.00 (46.00–65.00)	0.001
Living donor, *n* (%)	103 (8.83)	3 (1.16)	<0.001
Sexe (female), *n* (%)	506 (43.40)	90 (34.88)	0.015
BMI (kg/m^2), median (IQR)	25.46 (22.58–28.54)	26.10 (23.53–29.41)	0.007
Blood type, *n* (%)			0.659
A	473 (40.57)	111 (43.02)	
AB	36 (3.09)	9 (3.49)	
B	107 (9.18)	18 (6.98)	
O	550 (47.17)	120 (46.51)	
Recipient			
Age (years), median (IQR)	51.89 (39.19–60.47)	56.25 (45.26–62.80)	<0.001
First kidney transplantation, *n* (%)	986 (84.56)	204 (79.07)	0.039
Sexe (female), *n* (%)	389 (33.36)	132 (51.16)	<0.001
BMI (kg/m^2), median (IQR)	24.52 (21.63–27.54)	24.78 (21.75–28.70)	0.137
Blood type, *n* (%)			0.557
A	493 (42.28)	117 (45.35)	
AB	49 (4.20)	12 (4.65)	
B	121 (10.38)	20 (7.75)	
O	503 (43.14)	109 (42.25)	
Type of dialysis			0.380
Hemodialysis, *n* (%)	909 (77.96)	208 (80.62)	
Peritoneal dialysis, *n* (%)	133 (11.41)	30 (11.63)	
Preemptive transplantation, *n* (%)	124 (10.63)	20 (7.75)	
Cause of ESKD			0.818
Glomerulonephritis, *n* (%)	153 (13.12)	40 (15.50)	
Vascular nephropathy, *n* (%)	333 (28.56)	72 (27.91)	
Undetermined, *n* (%)	93 (7.98)	23 (8.91)	
Diabetes, *n* (%)	148 (12.69)	32 (12.40)	
ADPKD, *n* (%)	73 (6.26)	20 (7.75)	
Tubulo-interstitial nephritis, *n* (%)	230 (19.73)	44 (17.05)	
Others, *n* (%)	136 (11.66)	27 (10.47)	
Waiting time on dialysis, median (IQR)	2.11 (1.12–3.71)	2.58 (1.44–4.25)	0.003
HLA sensitization class I, *n* (%)	187 (16.04)	61 (23.64)	0.008
HLA sensitization class II, *n* (%)	203 (17.41)	71 (27.52)	0.001
Transplantation			
Cold ischemia time (h), median (IQR)	15.83 (11.68–20.67)	18.27 (14.08–23.42)	<0.001
Hypothermic perfusion machine, *n* (%)	243 (20.84)	59 (22.87)	0.321
ABDR mismatches, median (IQR)	4.00 (3.00–5.00)	4.00 (3.00–4.00)	0.665
Induction therapy (Thymoglobulin), *n* (%)	695 (59.61)	158 (61.24)	0.008
1-month baseline serum creatinine (mg/L), median (IQR)	17.00 (13.00–21.85)	21.00 (15.00–27.50)	<0.001
Number of transfusions			
1 or 2	—	218 (84.50)	
Over 3	—	40 (15.50)	

ADPKD, autosomal dominant polycystic kidney disease; BMI, body mass index; ESKD, end-stage kidney disease HLA, human leukocyte antigen; IQR, InterQuartile Range.

recipients were also significantly older, with higher BMI and longer cold ischemia times. Regarding post-transplant characteristics, thymoglobulin induction was more frequent in transfused recipients and baseline median 1-month serum creatinine was significantly higher in transfused recipients (21.00 mg/L [15.00–27.50] vs 17.00 mg/L [13.00–21.85], $p < 0.001$) (**Table 1**).

Association Between Post-KT Blood Transfusions and Emergence of *De Novo* DSA

The median time to *de novo* DSA occurrence was 731 days (first–third quartile: 173–1,461 days). The mean number of HLA measurements during follow-up was 5.71 (±3.14) in transfused recipients and 5.3 (±3.36) in non-transfused recipients. A total of 124 patients developed *de novo* DSA, including 28 in transfused recipients. The overall estimated probability of DSA occurrence was 3.22% (CI 95% 2.41–4.29), 6.04% (CI 95% 4.87–7.49), 8.20% (CI 95% 6.75–9.93) at 1, 3, and 5 years respectively. The estimated probabilities of DSA occurrence at 1, 3, and 5 years were 2.73% [CI 95% 1.93–3.86], 5.74% [CI 95% 4.48–7.34], and 8.03% [CI 95% 6.44–9.98] in non-transfused recipient, versus 5.43% [CI 95% 3.25–8.99], 7.49% [CI 95% 4.84–11.50], and 9.09% [CI 95% 6.05–13.54] in transfused recipients (See **Figure 1**). Multivariable Cox regression models did not show any association between transfusion and *de novo* DSA occurrence (HR = 1.35 [0.86–2.11], $p = 0.19$) (See **Table 2**). Being highly transfused (i.e. over three transfusions) was also not associated with an increased risk of *de*

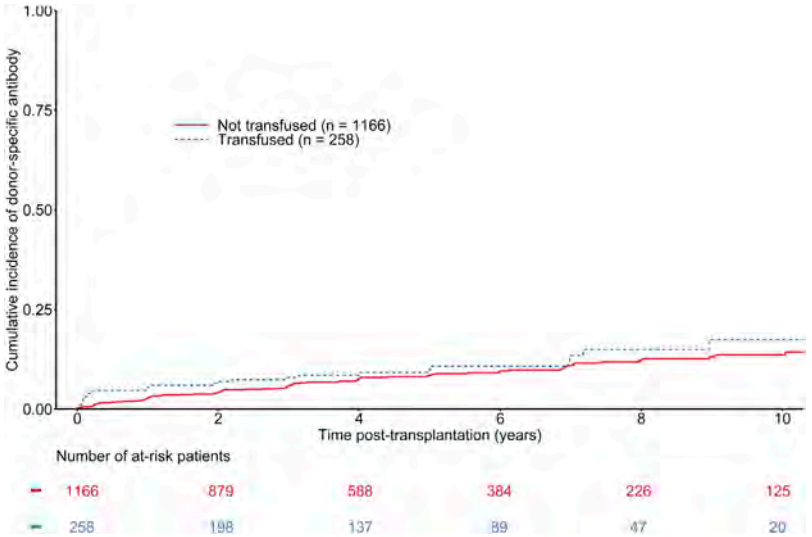


FIGURE 1 | Cumulative incidence of *de novo* donor specific antibodies according to the transfusion status of kidney transplant recipients. Gray-test: $p = 0.32$.

novo DSA occurrence (HR = 0.93 [0.29–2.97], $p = 0.90$). Furthermore, we did not find any significant difference regarding the nature of DSA (Class I or Class II DSA) according to the transfusion status (**Supplementary Table S1**). Other independent predictors of *de novo* DSA occurrence included recipient and donor age, HLA class II sensitization, and the number of HLA ABDR mismatches (See **Table 2**). As a sensitivity analysis, we analyzed the short-term effects of early blood transfusion on *de novo* DSA occurrence at 1-year post-transplantation. Forty-five recipients presented with *de novo* DSA at 1-year post-transplantation. Multivariate logistic regression did not show any association between transfusion and *de novo* DSA occurrence at 1-year (OR = 1.58 [0.79–3.18], $p = 0.20$) (See **Supplementary Table S2**).

TABLE 2 | Multivariate Cox regression model for the risk of development of *de novo* DSA.

	***de novo* DSA**	
	Multivariate HR [95% CI]	***p*-value**
Blood transfusion post-KT (yes vs. no)	1.35 [0.86–2.11]	0.19
1 or 2 blood transfusions	1.43 [0.89–2.30]	0.13
Over 3 blood transfusions	0.93 [0.29–2.97]	0.90
Recipient age (per year)	0.95 [0.93–0.97]	< 0.01
Donor age (per year)	1.03 [1.01–1.05]	< 0.01
HLA sensitization class II (yes vs. no)	1.81 [1.18–2.80]	0.01
ABDR mismatches (>4 vs. ≤ 4)	1.33 [1.14–1.55]	< 0.01

HLA, human leukocyte antigen; KT, kidney transplantation.

Association Between Post-KT Blood Transfusions and Biopsy-Proven Acute Rejection

The median time to BPAR onset was 94 days (first–third quartile: 17–475 days). A total of 189 patients were diagnosed with BPAR, including 49 in the group of transfused recipients. The overall estimated probability of BPAR was 7.80% [CI 95% 6.39–9.50], 10.42% [CI 95% 8.76–12.38] at 1 and 3 years respectively. The estimated probabilities of BPAR at 1 and 3 years were 7.80% [CI 95% 6.39–9.50] and 10.42% [CI 95% 8.76–12.38] vs. 15.52% [CI 95% 11.63–20.55] in non-transfused recipient and 17.21% [CI 95% 13.10–22.43] in transfused recipients (See **Figure 2**). Even though univariate analyses suggested a significant difference between transfused and non-transfused recipients (See **Figure 2**), adjusted multivariable Cox regression models did not show any association between transfusion and BPAR (HR = 1.33 [0.94–1.89], $p = 0.11$) (See **Table 3**). Other independent predictors of BPAR involved donor sex, HLA class II sensitization, and 1-month serum creatinine (See **Table 3**). As a sensitivity analysis, we analyzed the short-term effects of early blood transfusion at 1-year post-transplantation on the risk of BPAR. One hundred and thirty recipients presented with BPAR at 1-year post-transplantation. On the contrary to the long-term analysis, multivariate logistic regression showed an association between transfusion and BPAR at 1-year (OR = 1.63 [1.05–2.52], $p = 0.03$). Other independent variables associated with the risk of BPAR at 1-year remained the same than presented in the Cox model (See **Supplementary Table S3**).

Association Between Post-KT Blood Transfusions and Graft Loss

The median time to graft failure was 973 days (first–third quartile: 336–1925 days). A total of 170 patients experienced graft failure during follow-up, including 52 in the group of transfused recipients. The overall estimated probability of death-censored graft failure at 1, 3, and 5 years was 3.15% [CI 95% 2.36–4.22], 7.17% [CI 95% 5.87–8.73], and 10.70% [CI 95% 9.02–12.67], respectively. The estimated probabilities of death-censored graft failure at 1, 3, and 5 years were 2.03% [CI 95% 1.35–3.04], 5.68% [CI 95% 4.42–7.30], 9.44% [CI 95% 7.66–11.61] in non-transfused recipient versus 8.16%

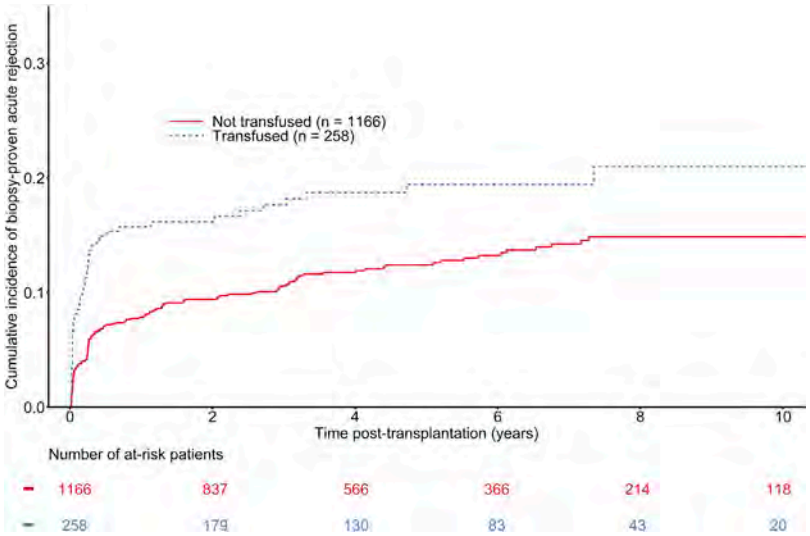


FIGURE 2 | Cumulative incidence of biopsy-proven rejection according to the transfusion status of kidney transplant recipients. Gray-test: $p = 0.004$.

TABLE 3 | Multivariate Cox regression model for the risk of biopsy-proven acute rejection.

	BPAR	
	Multivariate HR [95% CI]	***p*-value**
Post-KT blood transfusion (yes vs. no)	1.33 [0.94–1.89]	0.11
Male donor	0.75 [0.56–1.00]	0.05
HLA sensitization class II	1.83 [1.32–2.52]	<0.01
1-month serum creatinine (per 0.1 mg/dl)	1.02 [1.01–1.03]	<0.01

BPAR, biopsy-proven acute rejection; HLA, human leukocyte antigen; KT, kidney transplantation.

TABLE 4 | Multivariate Cox regression model for death-censored graft loss.

	Graft loss	
	Multivariate HR [95% CI]	***p*-value**
Post-KT blood transfusion (yes vs. no)	1.04 [0.73–1.50]	0.82
Recipient age (per year)	0.98 [0.97–1.00]	0.03
Donor age (per year)	1.03 [1.02–1.05]	<0.01
Waiting time on dialysis (per year)	1.07 [1.04–1.11]	<0.01
Cold ischemia time (per hour)	1.02 [1.00–1.04]	0.09
1-month serum creatinine (per 0.1 mg/dl)	1.06 [1.05–1.06]	<0.01

KT, kidney transplantation.

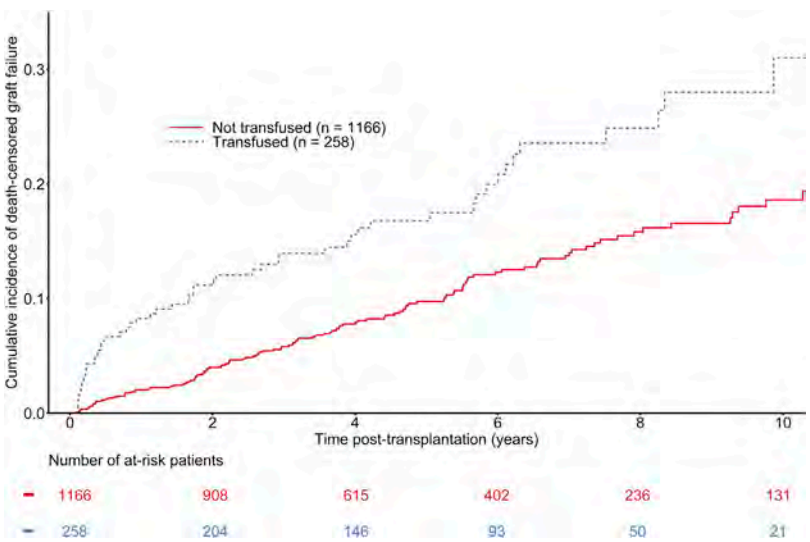


FIGURE 3 | Cumulative incidence of kidney graft failure according to the transfusion status of kidney transplant recipients. Gray-test: $p < 0.001$.

[CI 95% 5.40–12.23], 13.61% [CI 95% 9.91–18.54], and 16.25% [CI 95% 12.10–21.63] in transfused recipients (See **Figure 3**). Even though univariate analyses suggested a significant difference between transfused and non-transfused recipients (See **Figure 3**), adjusted multivariable Cox regression models did not show any association between transfusion and graft loss (HR = 1.04 [0.73–1.50], $p = 0.82$) (See **Table 4**). Other independent predictors of graft failure included recipient and donor age, waiting time on dialysis, and 1-month serum creatinine. As a sensitivity analysis, we analyzed the short-term effects of early blood transfusion at 1-year post-transplantation. Forty-four recipients presented with graft failure. Multivariate logistic regression showed a trend of association between transfusion and BPAR at 1-year (OR = 2.02 [0.93–4.40], $p = 0.08$). The other independent variables significantly associated with the risk of death-censored graft failure at 1-year remained the same than presented in the Cox model, except for the waiting time on dialysis (See **Supplementary Table S4**).

DISCUSSION

In this single-center study comprising a large number of KT and a median time of follow-up of 4.52 years, we did not show any association between post-KT early blood transfusions and the occurrence of *de novo* DSA, BPAR or graft failure.

Allorecognition leads to the generation of alloantibodies targeting non-self antigens, i.e. *de novo* DSA (7), which are associated with an increased risk of antibody-mediated rejection and allograft failure (13). As far as blood transfusions are concerned, the risk of induced-alloimmunization seems to have decreased over the last decades (14). A blood product is composed of three distinct parts (1): the desired product, such as red blood cells (RBCs) or platelets (2); excipients (e.g. anticoagulant or residual plasma) (3); residual leukocytes that carry HLA antigens, and in rare cases unexpected residual cells, such as platelets in red blood cells products (15). Leukocytes carry most of the antigenic load in a blood unit, yet the systematic use of leukoreduction process implemented in the late 90's to fight against Creutzfeldt-Jakob disease transmission dramatically reduced the amount of WBC into blood units (16, 17). As a consequence, the rate of post-transfusion HLA sensitization decreased from nearly 30% of transfused patients to 10–20% depending on studies (18–21). However, even with leukoreduction, the risk of sensitization still persists as erythrocytes constitutively express HLA class I molecules at low levels (22). The risk of transfusion-related sensitization also depends on the immunological history of the recipient. Indeed, transfused kidney transplant candidates with a history of pregnancy or previous transplantation have a higher risk of sensitization after transfusion compared to kidney transplant candidates with a sole history of blood transfusion (23). Moreover, there seems to be a dose-effect, as the level of immunogenicity correlates with the number of administered units (6, 24).

Even though blood transfusion seems to be clearly associated with the risk of HLA sensitization, its impact on allograft outcomes remains unclear. Paradoxically, throughout the early beginnings of solid organ transplantation and before the implementation of cyclosporine, pre-transplant blood transfusion was supposed to be associated with immunomodulatory properties and benefits on renal allograft outcomes (25, 26). Donor-specific transfusion in KT has long been part of routine practices for its supposed ability to prevent post-transplant rejection (27, 28). Animal models also provided evidence of transfusion-related immunomodulation properties owing to the generation of alloreactive $CD25^{+}CD4^{+}$ regulatory T cells that prevent graft rejection (29), from both related and unrelated donor blood. Nowadays, even if donor-specific transfusion is no longer used, the potential immunomodulatory properties of blood transfusion question the impact of early blood transfusion after KT.

In our study, we did not find any association between early blood transfusion post-KT and the further risk of *de novo* DSA development, on a large-scaled cohort using Luminex-based methods to identify DSA. On the contrary, HLA mismatches and HLA sensitization were significantly associated with *de novo* DSA and are a well-known risk factors of post-transplantation allosensitization (30, 31). Aging was also associated with the risk of *de novo* DSA occurrence. On the one hand, aging in recipients was associated with a lower risk of allosensitization, which may reflect the aging-related immunosenescence in recipients. On the other hand, aging in donors was associated with an increased risk of *de novo* DSA occurrence, which exhibits the aging-related immunogenicity of kidney donors (32). The transfusion status was also not associated with secondary outcomes such as the long-term risks of rejection or graft failure. Considering the literature, Scornik et al. (9) reported 746 patients transplanted followed for 6 years, including 45% transfused-recipients with 79% of blood transfusions performed during the first month post-KT. There was no significant difference regarding the incidence of rejection episodes or graft loss according to the transfusion status. There was also no difference regarding the frequency of *de novo* DSA between transfused and non-transfused recipients (17% vs. 15%, $p = 0.67$). Verghese et al. (12) reported then a pediatric study of 482, including 44% transfused patients. Among these, 134 recipients could be tested for HLA antibodies using solid-phase based methods, including 82 transfused recipients. In their study, blood transfusion was also not associated with the risks of *de novo* DSA after KT (HR 0.9; 95% CI 0.6–1.4; $p = 0.65$), rejection or graft failure. In the same way, Daloul et al. recently reported their experience of 273 recipients, including 127 transfused recipients before 1-month post-KT. They did not find any difference at 1-year post-KT regarding the incidence of *de novo* DSA using solid-phase based methods (12.8% in transfused recipients and 10.9% in non-transfused recipients, $p = 0.48$) (33), as well as with the risk of rejection or graft loss.

Conversely, Ferrandiz et al. showed opposite results regarding the association between blood transfusion and *de novo* DSA, with one of the largest cohorts studying HLA antibodies using Luminex. Three hundred and ninety non-sensitized kidney transplant recipients were included, of which 250 were transfused during the first year post-KT. 94.8% of them were transfused during the first month post-KT. During the first-year post-transplantation, 18 recipients (7.2%) in the transfusion group developed *de novo* DSA, compared to only one (0.7%) in the nontransfusion group ($p < 0.0001$). This higher prevalence of *de novo* DSA was also associated with a higher incidence of ABMR (15 transfused-recipients (6%) vs two non-transfused recipients (1.4%), $p = 0.04$). However, baseline

characteristics significantly differed regarding notably the immunosuppressive regimen, with a higher proportion of transfused recipients treated with cyclosporine. Furthermore, they examined early outcomes as logistic regression at 1-year post-KT revealed that both the use of cyclosporin and blood transfusions were associated with the risk of DSA formation. In our study, both the evaluation of early and long-term outcomes did not find any association with the transfusion status. Yet, we acknowledge that it may be difficult to compare these two monocentric studies, as far as local practices, e.g. regarding the immunosuppressive regimen management, may influence the results. Recently, Hassan et al. reported a cohort of 1,104 recipients including 667 transfused recipients. 88.9% of blood transfusions were performed before 1-month post-KT. Blood transfusion was significantly associated with the development of *de novo* DSA (transfusion received: HR = 1.49 [1.10–2.04], $p = 0.01$) and graft failure (transfusion received: HR = 1.85 [1.19–2.77], $p = 0.005$). However, the prevalence of blood transfusion was surprisingly high, which could be linked to the baseline characteristics of the overall cohort (not provided). Nevertheless, they provided novel data dealing with the analysis of shared transfusion and kidney donors' alloantibodies in transfused recipients. They analyzed a subgroup of 86 transplant recipients who received transfusion from 244 blood donors. Overall, 61.5% of transfused recipients developed *de novo* transfusion specific antibodies (TSA), of which 46.7% shared HLA antibody specificity with a DSA response in the recipient (DSA+/TSA+). DSA+/TSA + recipients had an increased risk of allograft loss or rejection compared to recipients with only TSA or DSA. This may suggest a need of blood donor HLA matching in kidney transplant recipients.

Compared to the existing literature, our study has two main strengths. First, we provide one of the largest cohort of recipients screened for HLA antibodies during their whole follow-up, combined with their transfusion status. Second, this cohort benefitted from a long-term follow-up with the evaluation of reliable time-dependent outcomes. Still, our findings need to be interpreted in the context of some caveats. Indeed, the retrospective nature of the study could be associated with information bias. Then, it is also limited by the lack of information regarding the hemoglobin levels. Post-KT anemia is indeed known to be associated with mortality and graft loss (34, 35). Yet, our primary outcome is focused on the emergence of *de novo* DSA and no association between anemia and *de novo* DSA has been reported to date. Thus, it does not seem to constitute a confounding factor. Finally, there are significant baseline differences between transfused and non-transfused recipients that should be considered to interpret our results. Donors from transfused recipients were significantly older, with longer cold ischemia times. Transfused recipients were significantly older, sensitized against HLA antibodies, more frequently treated with thymoglobulin induction and had a significant worse graft function at 1-month post-transplantation. These baseline differences may explain why univariate and short-term analyses revealed differences concerning rejection and graft failure. However, after adjusting on confounding factors on long-term analyses, transfusion was no longer associated with any of those outcomes. To be noted, as far as our primary criteria of judgement is concerned, none of our analyses, i.e. short-term or long-term, univariate and multivariate, revealed an association between transfusion and *de novo* DSA occurrence.

Ultimately, even if on a global scale we did not find any association between transfusion and the development of *de novo* DSA, it does not mean that this correlation does not exist at an individual level, as suggested by Hassan et al. The risk of allosensitization should be kept in mind, and strategies of HLA matching between blood and kidney donors may be of interest in the next few years. However, our data provide evidence that transfusion should not be limited in the early period post-KT when required.

CONCLUSION

In our large-scaled cohort of kidney transplant recipients, we did not find any association between post-KT early blood transfusions and the development of *de novo* DSA, nor the risks of rejection and graft failure.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was not provided for this study on human participants because French legislation stipulates that registry-based research is an integral part of outcome assessment for solid organ transplantation and is exempt from Institutional Review Board approval. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design: KK, RL, AH, IT, MH, MM. Acquisition of data and data management: KK, RL, AH, DB, IT, BL, J-BG, MM. Drafting of the manuscript: KK, RL, AH, DB, ML, FP, MF, MV, MM. Critical Revision: KK, RL, AH, DB, IT, ML, BL, MT, FP, MF, J-BG, MH, MM. All authors approved the final 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.2022.10279/full#supplementary-material

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ORIGINAL RESEARCH
published: 18 March 2022
doi: 10.3389/ti.2022.10240

High Plasma Oxalate Levels Early After Kidney Transplantation Are Associated With Impaired Long-Term Outcomes

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Background: Elevated levels of oxalate are common in renal failure patients and non-hyperoxaluria disease, and may cause damage after transplantation. We examined outcomes after 15 years for 167 kidney transplant recipients who had plasma oxalate measured early after transplantation. Analyses included plasma oxalate, recipient age, donor age, live donor, HLA-DR mismatch, mGFR, and smoking.

Results: Median age was 52 years (range 18–81), 63% were male and 38% had live donors. Median plasma oxalate concentration 10 weeks after transplantation was 9.0 µmol/L (range 2.7–53.0), one third above the upper reference limit (11.0 µmol/L). Multivariable analysis revealed upper quartile plasma oxalate (>13.0 µmol/L, $p = 0.008$), recipient age ($p < 0.001$), deceased donor ($p = 0.003$), and current smoking ($p < 0.001$) as significant factors associated with patient survival. Upper quartile plasma oxalate ($p = 0.021$), recipient age ($p = 0.001$), deceased donor kidney ($p = 0.001$), HLA-DR mismatch ($p = 0.015$), and current smoking ($p = 0.014$) were also associated with graft loss. Factors associated with death censored graft losses were donor age ($p = 0.012$), deceased donor ($p = 0.032$), and HLA-DR mis-matched kidneys ($p = 0.005$) but plasma oxalate was not ($p = 0.188$).

Conclusions: Plasma oxalate in the upper quartile early after transplantation was significantly associated with impaired long-term patient survival and graft losses, but not when censored for death.

Keywords: kidney transplantation, patient survival, graft loss, oxalate, long term outcomes, prospective follow-up

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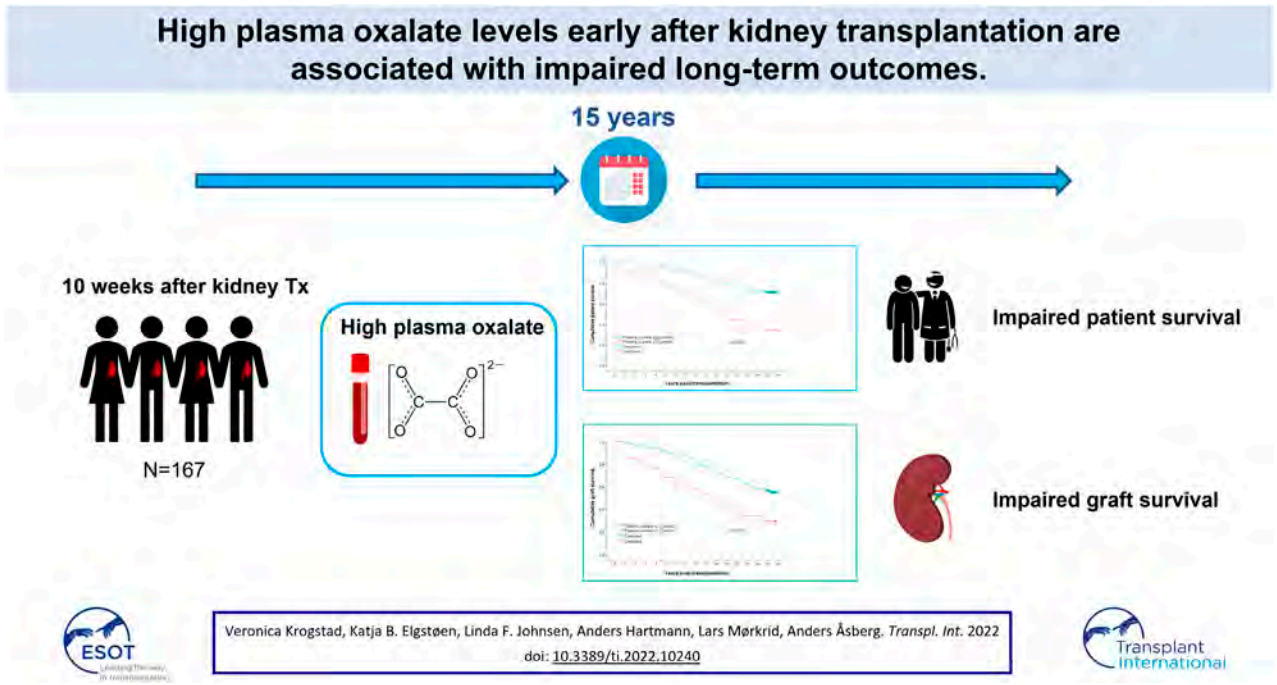
Received: *15 November 2021*
Accepted: *04 February 2022*
Published: *18 March 2022*

Citation:
Krogstad V, Elgstøen KBP, Johnsen LF, Hartmann A, Mørkrid L and Åsberg A (2022) High Plasma Oxalate Levels Early After Kidney Transplantation Are Associated With Impaired Long-Term Outcomes. Transpl Int 35:10240. doi: 10.3389/ti.2022.10240

INTRODUCTION

Hyperoxalemia/-oxaluria may cause kidney failure. In typical example cases, it often affects primary hyperoxaluria patients leading to terminal kidney failure at a young age (1). Secondary forms of hyperoxaluria also occur with intestinal disease or following bariatric surgery, which are well recognized to harm the kidneys (2). Another major cause of oxalate retention is kidney failure since



GRAPHICAL ABSTRACT |

the main excretion route for oxalate is glomerular filtration and tubular secretion (3). When patients with kidney failure are successfully treated with a kidney transplant, excess oxalate is excreted by the transplanted kidney and may potentially cause damage.

The retention of oxalate in end-stage renal failure patients without a primary defect in oxalate metabolism has not been well studied. Almost 2 decades ago we started a single center prospective study to assess the outcomes of kidney transplant patients related to levels of plasma oxalate in the perioperative phase (4). We found that more than a third of the patients still had plasma levels of oxalate above the upper reference limit 10 weeks after transplantation and oxalate plasma levels were inversely correlated to kidney graft function. There is growing evidence that oxalate may seriously harm transplanted kidneys (5–7) and possibly also affect mortality (8). The original protocol of our study outlined long-term follow-up of these patients to assess outcomes including patient survival and graft loss. The present study describes the long-term outcomes over 15 years for a cohort of 167 patients who had valid measurements of plasma oxalate 10 weeks after kidney transplantation (4).

MATERIALS AND METHODS

Study Design

In this single-center prospective study, we measured plasma oxalate in kidney transplant recipients in a stable phase, on average 10 weeks after kidney transplantation, consecutively between February 2004 and May 2005. The present study is a long-term follow-up of outcomes in 167 patients that was part of the original protocol, none were lost to follow-up. The design of the single-center prospective study has been previously described in detail (4). Long-term follow-up data on mortality and graft losses were retrieved from the Norwegian Renal Registry until December 2019.

The protocol was approved by the Regional Ethics Committee in South-East Norway and the biobank was approved by the Data Inspectorate. All patients signed informed consent for both the initial study and for biobanking of plasma samples. The study was conducted in accordance with the Declaration of Helsinki.

Bioanalysis

Plasma oxalate was measured with a validated method as previously described (9). In short, fresh plasma samples were subject to solid-phase extraction followed by derivatization of oxalate and analysis with liquid chromatography-tandem mass spectrometry. All samples were analyzed in duplicate, and the method showed an average CV of 6.9%. Glomerular filtration rate (GFR) at 10 weeks was measured by plasma disappearance of ^{51}Cr-EDTA (10). For 10 patients (five patients in the Q1-Q3 group and five patients in the Q4 group), measurement of GFR was not performed, and GFR was estimated for these patients using the MDRD-4 equation (11).

Statistics

A potential harmful effect of plasma oxalate is only expected at high values. We, therefore, examined the upper quartile versus the other quartiles of plasma oxalate values as predictors for outcomes. The upper quartile had values above 13.0 µmol/L,

TABLE 1 | Demographic and transplant data according to quartiles of plasma oxalate. Data presented as median (total range) and number (%).

	All patients (n = 167)	Upper quartile (n = 41)	Other quartiles (n = 126)	p
Plasma oxalate 10 weeks after Tx (μmol/L)	9.0 (2.7–53)	16.0 (13.1–53.0)	7.7 (2.7–13.0)	NA
Age (years)	52 (18–81)	59 (22–79)	50 (18–81)	0.002[a]
Male sex	105 (62.9)	30 (73.2)	75 (59.5)	0.116[b]
Preemptive Tx	39 (23.4)	8 (19.5)	31 (24.6)	0.503[b]
Retransplanted patients	27 (16.2)	5 (12.2)	22 (17.5)	0.426[b]
Dialysis time (months)[c]	14 (1–71)	15 (1–71)	12 (1–60)	0.107[a]
Donor beyond 60 years	20 (12.0)	9 (22.0)	11 (8.7)	0.024[b]
Living donor	63 (37.7)	13 (31.7)	50 (39.7)	0.360[b]
HLA-DR mismatch (1 or 2)	108 (64.7)	31 (75.6)	77 (61.1)	0.092[b]
PRA positive	12 (7.2)	3 (7.3)	9 (7.1)	0.970[b]
Cold ischemia time (hours)	7.7 (0.0–24.0)	9.0 (0.8–20.2)	7.0 (0.0–24.0)	0.870[a]
Acute rejection first 10 weeks after Tx	67 (40.1)	19 (46.3)	48 (38.1)	0.349[b]
mGFR 10 weeks after Tx (ml/min)[d]	61 (16–135)	49 (16–90)	64 (30–135)	<0.001[a]
Current smoker	28 (16.8)	8 (19.5)	20 (15.9)	0.588[b]

Abbreviations: HLA-DR, Human Leukocyte Antigen-DR; PRA, Panel Reactive Antibody; Tx, transplantation; mGFR, measured glomerular filtration rate.
[a]*Mann-Whitney U test.*
[b]*Chi-square test.*
[c]*Excluding patients with preemptive transplantation.*
[d]*mGFR missing for five patients in the Q1-Q3 group and five patients in the Q4 group. For these patients, eGFR was calculated using the MDRD-4 equation.*

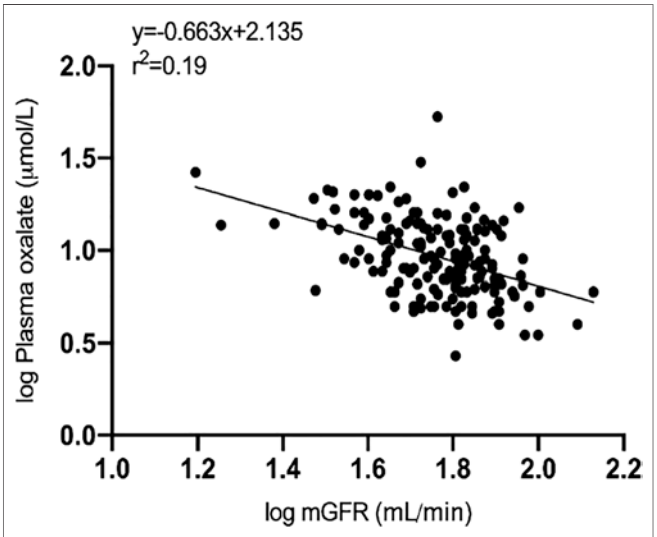


FIGURE 1 | Simple linear regression analysis of logarithmic values of plasma oxalate (μmol/L) as a function of logarithmic values of measured GFR (ml/min) 10 weeks after transplantation (y = −0.663x + 2.135, r^2 = 0.19, p < 0.001).

which is close to the upper reference limit with the present method (11.0 μmol/L).

Kaplan-Meier analyses with a log-rank test were performed to compare patient survival, graft survival, and death-censored graft survival in patients with upper quartile plasma oxalate concentrations (Q4) and the remaining patients (Q1-Q3). Furthermore, univariate and multivariable Cox regression analyses were performed to evaluate the independent effect of post-transplantation plasma oxalate concentration and other clinically relevant risk factors on long-term outcomes. Variables in the univariate analysis with a p-value lower than 0.10 for the outcomes and clinically plausible variables were included in the multivariable regression model. Proportional hazards were checked with log-minus-log plots as well as partial residual plots against time rank variables. p-values below 0.05 were considered statistically significant. All analyses were performed with SPSS software (IBM, version 26.0.0.1).

RESULTS

Demographic and transplantation-related baseline data are given for all patients in **Table 1**, which also includes specified data for the upper quartile of plasma oxalate patients versus the other quartiles of patients combined.

The 41 patients in the upper quartile with plasma oxalate values above 13.0 μmol/L had a median plasma oxalate concentration of 16.0 μmol/L, while 126 patients in the other quartiles combined with plasma oxalate concentrations at or below 13.0 μmol/L, had a median plasma oxalate concentration of 7.7 μmol/L.

The upper quartile patients were significantly older and had older donors. They also had substantially lower mGFR compared with the other patients at 10 weeks after transplantation. Plasma oxalate was inversely correlated with mGFR (**Figure 1**). Transplant demographic data were not different between recipients of a kidney in 2004 and 2005 in which oxalate were measured (n = 167) or not (n = 326, data not shown).

The median observation time was 15.0 years (range 0.7–15.8). Early rejection episodes were not different between the quartile groups (p = 0.35). In the observation period, 64 (38%) patients died and the median time from transplantation to death was 8.2 years (range 0.7–14.8). Uncensored graft loss occurred in 85 patients with a median time from transplantation of 8.2 years

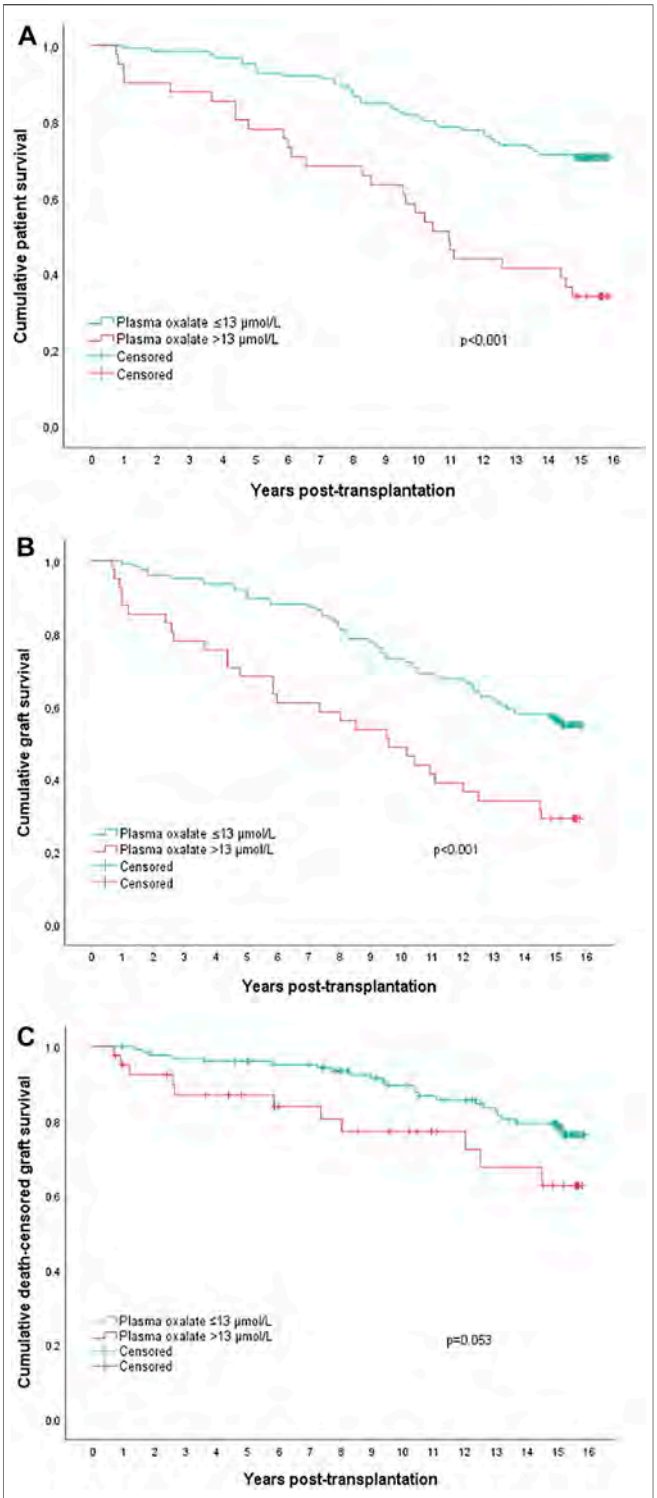


FIGURE 2 | Actuarial **(A)** patient survival, **(B)** overall graft survival, and **(C)** death censored graft survival in kidney transplanted patients with upper quartile plasma oxalate concentrations (>13.0 µmol/L, $n = 41$) versus all other patients ($n = 126$). p-values by Log-rank test.

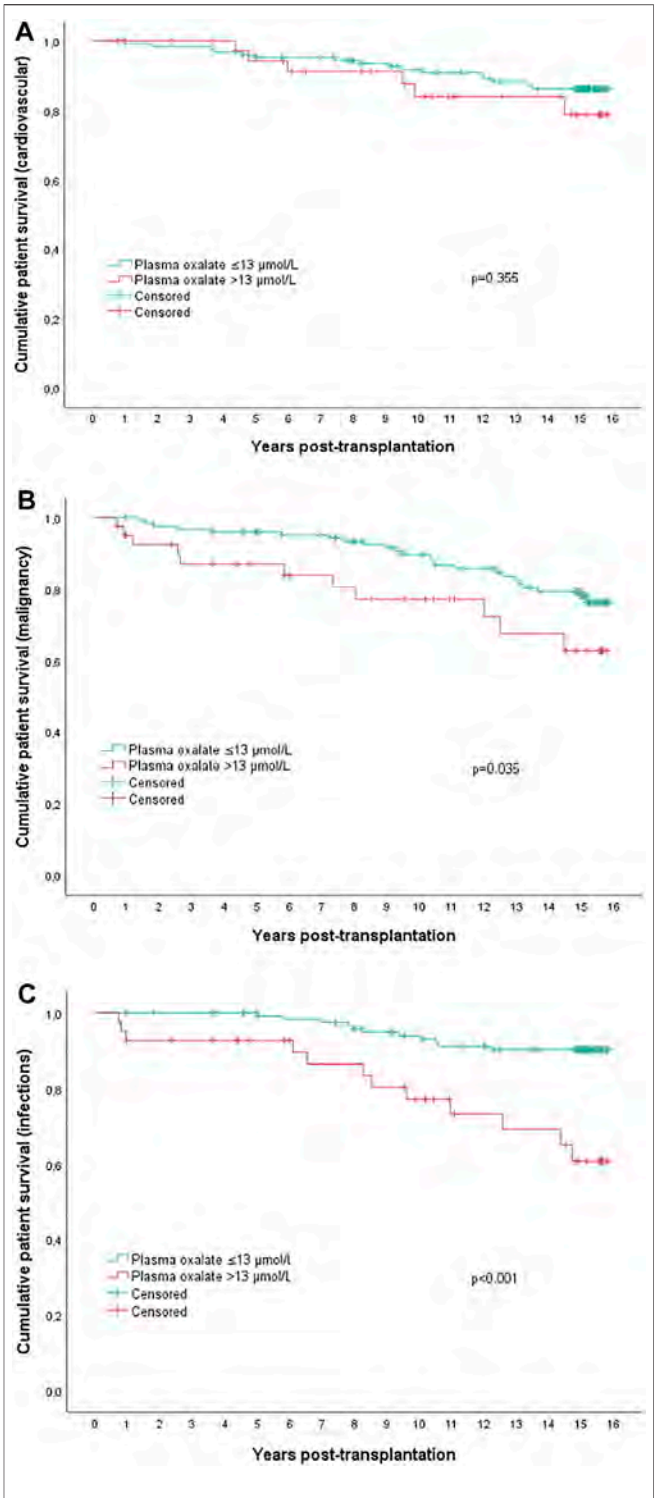


FIGURE 3 | Actuarial **(A)** cardiovascular survival, **(B)** malignancy survival and **(C)** infectious survival in kidney transplanted patients with upper quartile plasma oxalate concentrations (>13.0 µmol/L, $n = 41$) versus all other patients ($n = 126$). p-values by Log-rank test.

(range 0.7–15.1). Death-censored graft loss occurred in 35 (21%) patients, and the median time from transplantation to death-censored graft loss was 9.0 years (range 0.7–15.1).

Kaplan-Meier analysis survival plots are shown in **Figure 2**. The upper panel shows that estimated patient survival was shorter in the upper quartile patients ($p < 0.0001$), with a 15-year survival

TABLE 2 | Univariate Cox regression analysis of risk factors associated with death, graft loss, or death-censored graft loss.

	Death		Graft loss		Death-censored graft loss	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***	**HR (95%CI)**	***p***
Plasma oxalate >13.0 μmol/L after Tx	**3.05 (1.86–5.02)**	**<0.001**	**2.26 (1.44–3.54)**	**<0.001**	2.00 (0.98–4.09)	0.058
Recipient age (years)	**1.09 (1.06–1.11)**	**<0.001**	**1.04 (1.03–1.06)**	**<0.001**	1.00 (0.98–1.02)	0.962
Male sex	1.63 (0.95–2.81)	0.078	1.28 (0.81–2.01)	0.292	1.08 (0.54–2.14)	0.829
Donor > 60 years	1.75 (0.91–3.35)	0.092	**2.03 (1.15–3.61)**	**0.015**	**3.08 (1.40–6.80)**	**0.005**
Living donor	**0.27 (0.14–0.52)**	**<0.001**	**0.42 (0.26–0.68)**	**<0.001**	0.62 (0.31–1.24)	0.176
HLA-DR mismatch (1 or 2)	1.04 (0.62–1.73)	0.882	1.45 (0.91–2.29)	0.117	**3.06 (1.27–7.37)**	**0.013**
Preemptive Tx	0.72 (0.39–1.35)	0.310	0.73 (0.43–1.24)	0.237	0.60 (0.25–1.45)	0.258
PRA positive	1.07 (0.43–2.67)	0.881	1.08 (0.50–2.33)	0.852	0.74 (0.18–3.09)	0.681
mGFR at 10 weeks (ml/min)	**0.98 (0.97–1.00)**	**0.013**	**0.98 (0.97–1.00)**	**0.014**	0.98 (0.96–1.00)	0.117
Current smoker at 10 weeks	1.77 (0.99–3.16)	0.053	1.50 (0.89–2.52)	0.129	0.53 (0.16–1.74)	0.295

Abbreviations: HLA-DR, Human Leukocyte Antigen-DR; PRA, Panel Reactive Antibody; Tx, transplantation; mGFR, measured glomerular filtration rate. Bold data indicate statistical significant findings.

TABLE 3 | Multivariable Cox regression model of risk factors associated with death, graft loss, or death-censored graft loss.

	Death		Graft loss		Death-censored graft loss	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***	**HR (95%CI)**	***p***
Plasma oxalate >13.0 μmol/L	**2.23 (1.24–4.01)**	**0.008**	**1.80 (1.09–2.97)**	**0.021**	1.68 (0.78–3.64)	0.188
Recipient age (years)	**1.08 (1.06–1.11)**	**<0.001**	**1.03 (1.01–1.05)**	**0.001**		
Donor > 60 years	1.13 (0.55–2.32)	0.731	1.50 (0.80–2.81)	0.205	**3.00 (1.27–7.08)**	**0.012**
Living donor	**0.36 (0.18–0.70)**	**0.003**	**0.43 (0.26–0.72)**	**0.001**	**0.45 (0.21–0.93)**	**0.032**
HLA-DR mismatch (1 or 2)	1.10 (0.65–1.85)	0.718	**1.81 (1.12–2.93)**	**0.015**	**3.64 (1.47–9.01)**	**0.005**
mGFR at 10 weeks (ml/min)	1.00 (0.99–1.02)	0.727	0.99 (0.98–1.01)	0.401	0.99 (0.97–1.01)	0.471
Current smoker at 10 weeks	**3.10 (1.69–5.68)**	**<0.001**	**1.96 (1.15–3.35)**	**0.014**	0.67 (0.20–2.25)	0.516

Abbreviations: HLA-DR, Human Leukocyte Antigen-DR; mGFR, measured glomerular filtration rate. Bold data indicate statistical significant findings.

TABLE 4 | Multivariable Cox regression model of risk factors associated with death, graft loss or death-censored graft loss-excluded mGFR.

	Death		Graft loss		Death-censored graft loss	
	HR (95%CI)	***p***	**HR (95%CI)**	***p***	**HR (95%CI)**	***p***
Plasma oxalate >13.0 μmol/L	**2.14 (1.24–3.68)**	0.006	**1.91 (1.18–3.08)**	0.008	1.83 (0.87–3.84)	0.109
Recipient age (years)	**1.08 (1.06–1.11)**	<0.001	**1.03 (1.02–1.05)**	<0.001		
Donor > 60 years	1.10 (0.55–2.18)	0.794	1.60 (0.86–2.95)	0.135	**3.29 (1.44–7.52)**	**0.005**
Living donor	**0.36 (0.19–0.71)**	0.003	**0.43 (0.26–0.71)**	0.001	**0.44 (0.21–0.91)**	**0.027**
HLA-DR mismatch (1 or 2)	1.11 (0.66–1.86)	0.697	**1.76 (1.09–2.82)**	0.020	**3.50 (1.43–8.61)**	**0.006**
Current smoker at 10 weeks	**3.10 (1.69–5.69)**	<0.001	**1.92 (1.12–3.27)**	0.017	0.61 (0.19–2.01)	0.421

Bold data indicate statistical significant findings.

rate of 34% (95% CI 20–49%) compared with 71% (95% CI 63–79%) for the other patients. Similarly, the uncensored graft survival rate was also shorter in the upper quartile group ($p < 0.001$, middle panel); 15-year graft survival rates of 29% (95% CI 15–43%) and 56% (95% CI 48–64%), respectively. In the lower panel, death-censored graft survival rate is shown, which also tended to be shorter in the upper quartile group ($p = 0.053$). The 15-year death censored graft survival was 63% (95% CI 44–81%) and 78% (95% CI 70–86%) in the respective group.

Kaplan-Meier analyses were also performed to compare mortality due to cardiovascular, malignant, and infectious causes in the upper quartile plasma oxalate patients versus the other patients (**Figure 3**). Twenty-two patients died from cardiovascular causes; six patients were in the upper quartile group (15%), not significantly different from 16 deaths in the other patient groups combined (13%) ($p = 0.355$). Eleven patients died from malignancy, five patients in the upper quartile group (12%), significantly more than six among the other patients (5%) ($p = 0.035$). Finally, 23 patients died from infectious causes, 12 in the upper quartile group (29%), significantly more than 11 among the other patients (9%) ($p < 0.001$).

Univariate and multivariable Cox regression models for patient survival, graft survival, and death-censored graft survival are shown in **Tables 2** and **3**. The upper quartile of plasma oxalate along with recipient age, deceased donor kidneys,

and current smoking were independently associated with mortality in the multivariable model. Upper quartile plasma oxalate levels, recipient age, deceased donor kidney, and current smoking were also associated with graft loss and in addition also HLA-DR mismatches. The multivariable model for death-censored graft loss revealed donor age over 60 years, deceased donor kidneys, and any HLA-DR mismatch as independent factors. Neither plasma oxalate (p = 0.19) nor mGFR (p = 0.47) were independently associated with long-term graft loss censored for death. A sensitivity analysis excluding mGFR from the above-mentioned Cox regression showed similar results (**Table 4**).

DISCUSSION

Patient Survival

The main finding of the present study is that hyperoxalemia in an early stable post-transplant phase is associated with impaired long-term survival for 15 years. This is a novel finding. A previous retrospective study of 67 patients with calcium oxalate deposits in biopsies taken early after transplantation showed that such deposits were associated with impaired outcomes after 5 years (8). The outcome was a composite end-point of death and graft loss but graft loss was a major contributor to the combined end-point. They did not address mortality *per se*. In fact, in our study, we also found a significant effect of hyperoxalemia on uncensored graft losses, i.e., the combination of deaths and graft losses.

One might question the reason for the association between hyperoxalemia and impaired long-term patient survival as demonstrated in the present study. The patients with hyperoxalemia in the upper quartile were older, more of them also had donors beyond 60 years and their graft function was significantly lower at baseline, i.e., 10 weeks after transplantation. These are well-acknowledged risk factors for patient and graft survival. Nevertheless, in the multivariable analysis including these covariates, the effect of hyperoxalemia on mortality remained strong. The risk of dying was twice as high for the patients with hyperoxalemia in the upper quartile. Due to covariation between renal function and plasma oxalate concentrations the inclusion of both mGFR and plasma oxalate in the same multivariable Cox-analysis may be questioned. However, the results outlined above also hold true when mGFR is left out of the analysis (**Table 4**). Although the most common cause of death was cardiovascular, we did not find a significant effect of hyperoxalemia on cardiovascular mortality. On the other hand, the effect on malignancy deaths and particularly infectious deaths were markedly increased. These associations are hard to explain. The number of events is limited in these analyses, and one may only speculate whether hyperoxalemia has any causal relation to the cause of death. However, in kidney transplanted patients in general there is an increased risk for both malignancies and infectious deaths due to obligatory immunosuppressive therapy and also due to previous long-term kidney failure.

The immunosuppressive regimen during follow-up after transplantation is standardized on a national level and should not be different between the upper quartile plasma oxalate patients and the other patients. Rejection episodes are treated with steroids and often the immunosuppressive regimen is strengthened, but there was no significant difference in rejection episodes between the groups that could explain the different infectious death outcomes.

In a recent study, the effect of hyperoxaluria on mortality was addressed in a cohort of stable transplanted patients more than a year after transplantation (12). During 7 years of follow-up there was a significant reduction in mortality among patients with hyperoxaluria, mainly driven by a reduction of infectious disease related deaths. We did not measure urinary excretion but addressed plasma levels of oxalate in an early phase after transplantation that may be more relevant to early harmful effects. In any case, the reason for the difference in outcomes between the present study and the study by Tubben et al. (12) remains speculative.

Graft Survival

As mentioned above we found an association of hyperoxalemia and graft loss when including mortality, but when patients who died with functioning grafts were censored, the association was no longer significant. The only significant factors for such an association were HLA-DR mismatch, deceased donor kidney, and high donor age, as would be expected. The lack of associations to graft outcomes in the present study may be surprising since numerous other publications have shown kidney damage related to hyperoxaluria and calciumoxalate deposits (2,5-7) In the kidney, oxalate microcrystals may cause programmed inflammation and necrosis and also mitochondrial damage leading to necrosis in distal tubular cells and acute kidney injury (13,14). Hyperoxaluria in kidney transplant patients in the study from Tubben et. al. did not reveal any effect on graft loss during 7 years follow-up (12). However, a much larger study in more than 3,000 chronic kidney disease patients with similar observation time found that hyperoxaluria was associated with a 30% increase in the progression of kidney disease and also end-stage kidney disease (15). There may be differences between these patients and kidney transplanted patients but the kidney function was similar in the studies.

Oxalate deposits are shown to have an impact on kidney graft outcomes. One biopsy study examined renal outcomes in 67 patients who had oxalate deposits in early biopsies with 70 control patients. Those who had deposits in the biopsies had worse kidney function at one, but not at 2 years, but with significantly more interstitial scarring than controls (6). Also two other biopsy studies revealed that calcium oxalate deposits showed association to impaired graft function and long-term graft loss during up to 12 years (5,6). We did not examine oxalate deposits in biopsies but oxalate deposits are associated with high plasma levels, leading to increased filtration and probably also secretion of oxalate in a functioning kidney, leading to hyperoxaluria (3).

The number of death censored graft losses in the present study was only 35, limiting the possibility to reveal any association to hyperoxalemia.

The strengths of the present analysis are the prospective design with long-term outcomes included in the original protocol. The

cohort of transplant patients was unselected, and none of them was lost to follow-up. It was, however, a weakness that no urine samples were obtained for oxalate data, and no biopsies were obtained for oxalate deposition or nephrocalcinosis. The time course of the covariates after 10 weeks might also be an unknown modifying factor.

In conclusion, plasma oxalate concentration in the upper quartile early after transplantation is significantly associated with impaired long-term patient and graft survival but not when graft losses were censored for death with functioning grafts.

DATA AVAILABILITY STATEMENT

The data underlying this article will be shared on reasonable request to the corresponding author. A specific ethical approval may be needed before data sharing is possible according to Norwegian law.

ETHICS STATEMENT

The protocol was approved by the Regional Ethics Committee in South-East Norway and the biobank approved by the Data Inspectorate. All patients have signed informed consent for both the initial study and for biobanking of plasma samples. The study was conducted in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KE, LJ, LM and AH were authors of the previous baseline study and hence contributed to conception and design of the present follow-up study. VK, AH and AÅ performed the current analyses and all authors interpreted the results. VK, AH and AÅ drafted the manuscript and all authors contributed in the revision phase with critical discussions. All authors approved the final version submitted.

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
published: 21 March 2022
doi: 10.3389/ti.2021.10225

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

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Received: *12 November 2021*
Accepted: *27 December 2021*
Published: *21 March 2022*

Citation:
Budde K, Rostaing L, Maggiore U, Piotti G, Surace D, Geraci S, Procaccianti C, Nicolini G, Witzke O, Kamar N, Albano L, Büchler M, Pascual J, Gutiérrez-Dalmau A, Kuypers D, Wekerle T, Głyda M, Carmellini M, Tisone G, Midtvedt K, Wennberg L and Grinyó JM (2022) Prolonged-Release Once-Daily Formulation of Tacrolimus Versus Standard-of-Care Tacrolimus in de novo Kidney Transplant Patients Across Europe. Transpl Int 35:10225. doi: 10.3389/ti.2021.10225

Background: Tacrolimus is the calcineurin inhibitor of choice for preventing acute rejection episodes in kidney transplant patients. However, tacrolimus has a narrow therapeutic range that requires regular monitoring of blood concentrations to minimize toxicity. A new once-daily tacrolimus formulation, LCP-tacrolimus (LCPT), has been developed, which uses MeltDose™ drug-delivery technology to control drug release and enhance overall bioavailability. Our study compared dosing of LCPT with current standard-of-care tacrolimus [immediate-release tacrolimus (IR-Tac) or prolonged-release tacrolimus (PR-Tac)] during the 6 months following *de novo* kidney transplantation. Comparisons of graft function, clinical outcomes, safety, and tolerability for LCPT versus IR-Tac/PR-Tac were also performed.

Abbreviations: AE, adverse event; ANOVA, analysis of variance; CI, confidence interval; ECG, electrocardiogram; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen; IR-Tac, intermediate-release tacrolimus; LCPT, LCP-tacrolimus; LS, least squares; MMRM, mixed model for repeated measures; mITT, modified intent-to-treat; NE, not estimable; PR-Tac, prolonged-release tacrolimus; PRA, panel reactive antibody; SAE, serious adverse event; SD, standard deviation; STRATO, switching STudy of kidney tRansplant pAtients with Tremor to LCP-TacrO; TDD, total daily dose; TEAE, treatment-emergent adverse event.

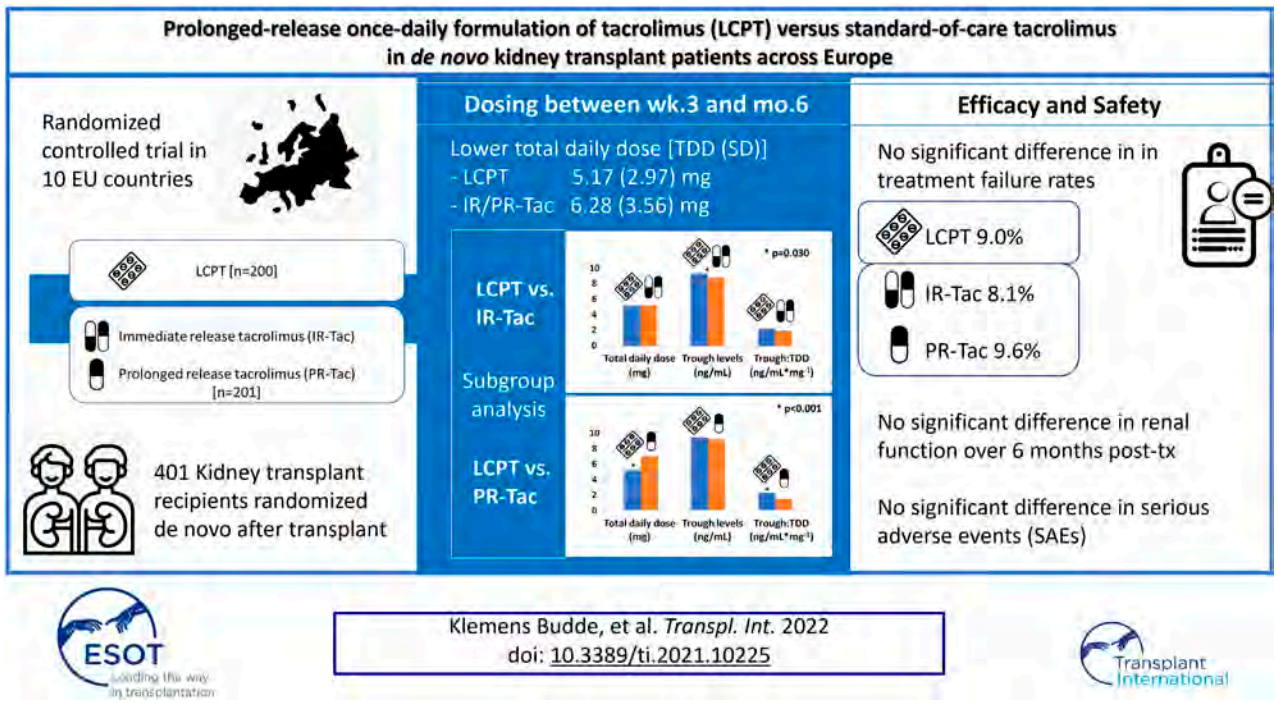
Methods: Standard immunological risk patients with end-stage renal disease who had received a *de novo* kidney transplant were randomized (1:1) to LCPT (N = 200) or IR-Tac/PR-Tac (N = 201).

Results: Least squares (LS) mean tacrolimus total daily dose from Week 3 to Month 6 was significantly lower for LCPT than for IR-Tac/PR-Tac. Although LS mean tacrolimus trough levels were significantly higher for LCPT than IR-Tac/PR-Tac, tacrolimus trough levels remained within the standard reference range for most patients. There were no differences between the groups in treatment failure measures or safety profile.

Conclusion: LCPT can achieve similar clinical outcomes to other tacrolimus formulations, with a lower daily dose.

Clinical Trial Registration: https://clinicaltrials.gov/, identifier NCT02432833.

Keywords: kidney, transplantation, immunosuppression, tacrolimus, pharmacokinetics, LCPT



GRAPHICAL ABSTRACT |

INTRODUCTION

Tacrolimus is the calcineurin inhibitor of choice in the prevention of acute rejection episodes in kidney transplant patients (1). It has a primary role in immunosuppressive regimens and is associated with improved outcomes owing to its efficacy and beneficial effect on renal allograft function (2). There may, however, be complexities with respect to regimen optimization due to the variability of tacrolimus exposure, which is partly a function of its low bioavailability (3, 4). In addition, tacrolimus has a narrow therapeutic range that imposes regular monitoring of blood drug concentrations to maintain therapeutic target levels and minimize toxicity (5, 6). Exposure below the minimum therapeutic level puts patients at risk of graft rejection and graft failure (and indeed, recent trends for tacrolimus minimization are still producing unsatisfying results) (7), whilst overexposure is associated with increased toxicity, including development of delayed graft function and post-transplant diabetes mellitus (8).

Two formulations of tacrolimus have been available for some time: an immediate-release formulation (IR-Tac), which is dosed twice daily (3), and a prolonged-release formulation (PR-Tac),

which is dosed once daily (4). These formulations exhibit considerable inter- and intra-patient variability in absorption and metabolism, affected by multiple factors including the patient's *CYP3A5* phenotype, sex, age, concomitant medication, and diet (9–11). Therapeutic drug level monitoring is therefore mandatory, and trough levels are concentration-controlled in clinical practice (as they correlate with systemic exposure as indicated by the area under the blood drug concentration–time curve). The benefits of once-daily administration of PR-Tac must be balanced against delayed achievement of, or change in, therapeutic trough levels and the higher dose needed to achieve similar trough levels to IR-Tac (12).

A new once-daily formulation of tacrolimus is now available [Envarsus®, LCP-tacrolimus (LCPT)] (13). LCPT was developed using MeltDose™ drug-delivery technology in order to enhance overall bioavailability (14). This technology controls the release of the drug mainly through a more distal distribution of tacrolimus within the gut, with the potential of being less affected by first-pass metabolism due to CYP3A activity along the proximal gut wall (15, 16). Compared with IR-Tac and PR-Tac, LCPT has higher bioavailability and a flatter time concentration curve in stable and *de novo* kidney transplant recipients (17, 18), even at very low trough levels (19). LCPT demonstrated non-inferiority in clinical outcomes and similar safety profiles to twice-daily tacrolimus in both *de novo* and stable kidney transplant patients (14, 20, 21).

The present study compared LCPT with current standard-of-care tacrolimus (IR-Tac or PR-Tac according to local clinical practice) during the 6 months following *de novo* kidney transplant in a series of European centers. Because dosing may affect drug exposure, in turn impacting graft function and drug side effects, the primary objective was to compare dosing of LCPT with standard-of-care tacrolimus. Clinical outcomes, safety, and tolerability were also evaluated.

METHODS AND MATERIALS

This was a Phase IV, randomized, open-label, parallel group study, conducted in 10 European countries. The study was conducted according to the current International Council for Harmonisation Good Clinical Practice guidelines, any local guidelines, and the Declaration of Helsinki, and the study protocol was approved by Independent Ethics Committees in accordance with local requirements. All patients provided written informed consent. The study was sponsored by Chiesi Farmaceutici (NCT02432833).

Study Population

Adults (≥18 years of age) with end-stage renal disease who received a *de novo* kidney transplant from a living or deceased donor were eligible. Patients with a known contraindication for tacrolimus or other macrolides were excluded. Key exclusion criteria included receipt of any other transplanted organ; receipt of a previous kidney transplant or of a kidney from a donor following cardiac death; receipt of a kidney with cold ischemia time of ≥30 h; receipt of a kidney from positive cross-match or ABO-incompatible donor; and current anti-human leukocyte antigen panel reactive antibody levels of >30%.

Design and Study Drugs

Fifteen study visits were scheduled over the 6-months study period: screening [0–28 days before transplantation if possible (e.g., in the case of a living donor)]; Day 0 (kidney transplantation); Day 1 (first administration of study drug); and Days 3, 5, 7, 10, 14, 21, 28, 60, 90, 120, 150, and 180. Baseline assessments were performed at the screening visit. If this was not possible (e.g., in the case of a deceased donor), they were performed on the day of transplantation.

Patients were randomized (1:1) to receive either LCPT or standard-of-care tacrolimus according to local practice, i.e., IR-Tac (Prograf®; Astellas Ireland Co., Ltd., Killorglin, Ireland) or PR-Tac (Advagraf®; Astellas Ireland Co., Ltd., Killorglin, Ireland). A balanced, blocked, randomization scheme, stratified by study site, was prepared by the study sponsor using a computerized system. Randomization was performed using an interactive web response system after baseline assessments were complete. Randomization took place preferably after transplantation, although it was allowed before transplantation once it was certain the patient would receive the kidney. At latest, randomization took place on the day following transplantation prior to the first administration of study drug.

In accordance with the prescription insert, the starting doses of study drug were 0.17 mg/kg/day once daily in the morning for LCPT, 0.20 mg/kg/day in two divided doses (morning and evening) for IR-Tac, and 0.20 mg/kg/day once daily in the morning for PR-Tac. The first dose was administered within 24 h after surgery. All study drugs were given orally. Doses were adjusted to maintain tacrolimus whole blood trough levels within the standard reference range, i.e., 5–15 ng/ml during the first 3 months following transplantation and 5–10 ng/ml thereafter.

Permitted concomitant immunosuppressive drugs included basiliximab, mycophenolate mofetil, and corticosteroids; treatment for acute rejection included corticosteroids, T-cell and B-cell depleting antibodies, plasma exchange, and intravenous immunoglobulin.

Endpoints and Assessments

The primary endpoint was the tacrolimus total daily dose (TDD) from Week 3 to Month 6. Secondary dosage endpoints over the whole study period were 1) tacrolimus TDD overall, by visit and by period (weekly during the first month, 1–3 months, and 3–6 months); 2) TDD normalized for weight; 3) tacrolimus trough levels overall, by visit, and by period; 4) number of times the trough level was within the standard reference range; 5) ratio of trough level to TDD (trough:TDD) overall, by visit, and by period; 6) number of dose adjustments. Pre-specified exploratory dosage endpoints included separate comparisons of LCPT with each of the other Tac formulations, (LCPT vs. IR-Tac and LCPT vs. PR-Tac) for TDD from Week 3 to Month 6, trough levels, and trough:TDD over the same period.

Secondary clinical endpoints were 1) treatment failure (composite endpoint comprising death, graft failure, biopsy-proven acute rejection, and loss to follow-up); 2) treatment discontinuation; 3) delayed graft function (defined as dialysis in the first week); 4) local diagnosis of acute rejection requiring treatment (classified as acute by the investigator and requiring

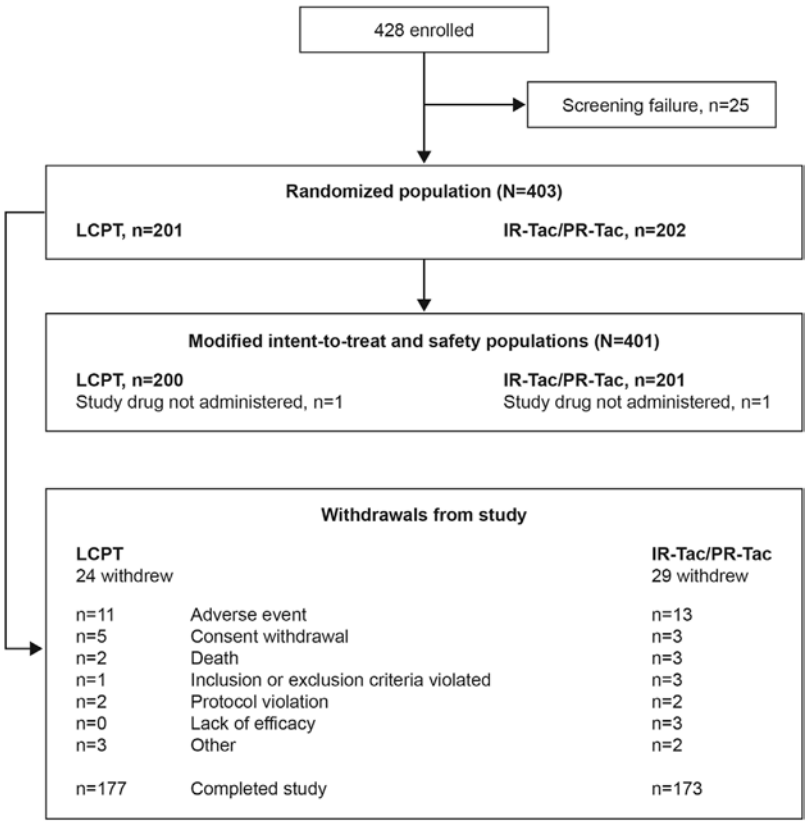


FIGURE 1 | Patient disposition. IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; PR-Tac, prolonged release tacrolimus.

additional immunosuppressive medications); 5) concomitant immunosuppressive medications. Safety assessments included adverse events (AEs), clinical laboratory tests (including for cytomegalovirus and urinary tract infections), 12-lead electrocardiogram (ECG), and vital signs.

Data Analysis

All efficacy endpoints were analyzed in the modified intent-to-treat (mITT) population (all randomized patients who received at least one dose of study treatment and had at least one available evaluation of efficacy after baseline). The safety population included all randomized patients who received at least one dose of study drug.

All statistical tests were carried out using 2-sided 0.05 significance levels. Differences between the treatment groups were estimated with the associated 2-sided 95% confidence intervals (CI). The exact Clopper-Pearson method was used to produce the 95% CI for individual proportions (rates) that corresponded to treatment groups. Fisher's exact test was used to compare the proportions between treatment groups. The difference in proportions between treatment groups was estimated and the associated 95% CIs provided were based on the Newcombe-Wilson method.

The primary endpoint was the average tacrolimus TDD from Week 3 to Month 6 and was compared between the two groups by applying an analysis of variance (ANOVA) model with treatment group and country as fixed effects. The adjusted least squares (LS) means in each treatment group and the adjusted LS mean difference between treatment groups were calculated with the corresponding 2-sided 95% CIs. The overall TDD (average over the whole treatment period) was analyzed in the same way.

For specific endpoints collected at several timepoints, a mixed model for repeated measures (MMRM) was performed. The model includes treatment arm, country, period, and a term for the interaction between treatment and period. Where specified, the baseline value was added as a covariate. The adjusted means in each treatment group and the adjusted mean difference between treatment groups were displayed with the corresponding 2-sided 95% CIs.

A sample size of 180 patients per study arm was planned to achieve a power of 80% to demonstrate a difference between LCPT and IR-Tac/PR-Tac of approximately −14% at a 2-sided significance level of 0.05, assuming an average TDD of 6.3 mg [standard deviation (SD) 3.0 mg] in both study arms. Assuming screening failure and discontinuation rates of 10%, 445 patients needed to be enrolled to achieve 400 patients randomized and 360 patients completing the study.

RESULTS

Patient Characteristics

A total of 401 patients were included in the mITT and safety populations: 200 in the LCPT group and 201 in the IR-Tac/PR-Tac

TABLE 1 | Baseline demographic and transplant characteristics (mITT population).

Characteristic	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	IR-Tac (N = 86)	PR-Tac (N = 115)
Age				
Mean (SD), years	53.8 (14.2)	54.8 (14.2)	53.4 (15.1)	55.8 (13.4)
<65 years, n (%)	147 (73.5)	147 (73.1)	63 (73.3)	84 (73.0)
Male sex, n (%)	146 (73.0)	136 (67.7)	59 (68.6)	77 (67.0)
Race, n (%)				
White	195 (97.5)	192 (95.5)	84 (97.7)	108 (93.9)
Asian	2 (1.0)	3 (1.5)	1 (1.2)	2 (1.7)
Black	1 (0.5)	1 (0.5)	0	1 (0.9)
Other	2 (1.0)	5 (2.5)	1 (1.2)	4 (3.5)
Body mass index, mean (SD), kg/m^2	26.8 (4.6)	26.0 (4.6)	25.6 (4.6)	26.4 (4.6)
Weight, mean (SD), kg	78.33 (15.07)	75.51 (14.64)	75.2 (16.2)	75.8 (13.4)
Diabetes pre-transplantation, n (%)	37 (18.5)	42 (20.9)	15 (17.4)	27 (23.5)
Time from transplant to first dose, mean (SD), hours	18.3 (8.2)	17.7 (7.5)	17.0 (8.7)	18.2 (6.5)
Pre-emptive transplantation, n (%)				
Yes	28 (14.0)	31 (15.4)	15 (17.4)	16 (13.9)
No	172 (86.0)	170 (84.6)	71 (82.6)	99 (86.1)
Type of dialysis, n (%)[a, b]				
Hemodialysis	138 (80.2)	139 (81.8)	66 (93.0)	73 (73.7)
Peritoneal dialysis	33 (19.2)	29 (17.1)	5 (7.0)	24 (24.2)
Missing	1 (0.6)	2 (1.2)	0	2 (2.0)
Time from first dialysis to transplant, median (range), months	29.3 (0, 152)	26.7 (0, 166)	32.9 (29.0)	38.7 (28.9)
Donor type				
Living	40 (20.0)	38 (18.9)	18 (20.9)	20 (17.4)
Deceased	160 (80.0)	163 (81.1)	68 (79.1)	95 (82.6)
HLA-A mismatch, n (%)				
0	31 (15.5)	33 (16.4)	18 (20.9)	15 (13.0)
1	98 (49.0)	94 (46.8)	47 (54.7)	47 (40.9)
2	66 (33.0)	68 (33.8)	20 (23.3)	48 (41.7)
HLA-B mismatch, n (%)				
0	21 (10.5)	24 (11.9)	15 (17.4)	9 (7.8)
1	88 (44.0)	99 (49.3)	41 (47.7)	58 (50.4)
2	86 (43.0)	72 (35.8)	29 (33.7)	43 (37.4)
HLA-DR mismatch, n (%)				
0	37 (18.5)	50 (24.9)	26 (30.2)	24 (20.9)
1	126 (63.0)	99 (49.3)	40 (46.5)	59 (51.3)
2	32 (16.0)	46 (22.9)	19 (22.1)	27 (23.5)
Maximum PRA, n (%)				
0%	171 (85.5)	182 (90.5)	73 (84.9)	109 (94.8)
≥1%	19 (9.5)	8 (4.0)	7 (8.1)	1 (0.9)

[a]*Percentage was based on the number of subjects with pre-emptive transplantation answered as "no".*

[b]*Type of dialysis has been derived for subjects with pre-emptive transplantation answered as "no"*

HLA, human leukocyte antigen; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; PRA, panel reactive antibody; PR-Tac, prolonged release tacrolimus; SD, standard deviation.

group (IR-Tac: 86; PR-Tac: 115), and 350 (86.8%) patients completed the study (**Figure 1**). Demographic and patient characteristics were similar in the LCPT and IR-Tac/PR-Tac groups; most patients were white men and the mean age was 54.3 years (**Table 1**).

Efficacy—Tacrolimus Dosage

Mean (SD) tacrolimus TDD, trough levels and trough:TDD are presented for the LCPT group and IR-Tac/PR-Tac groups, as well as for each tacrolimus formulation separately (**Table 2**).

TDD

The mean (SD) tacrolimus TDD from Week 3 to Month 6 after transplant (primary endpoint) was lower in the LCPT group than in the IR-Tac/PR-Tac group: 5.17 (2.97) mg versus 6.28 (3.56) mg, respectively [IR-Tac: 5.54 (2.91) mg; PR-Tac: 6.81 (3.88) mg] (**Table 2**). The LS mean tacrolimus TDD from Week 3 to Month 6 after transplant was significantly lower in the LCPT group (5.14 mg) than in the IR-Tac/PR-Tac group (6.24 mg): −1.11 (LS mean difference, LCPT-IR-Tac/PR-Tac), −1.76, −0.45 (95% CI) ($p < 0.001$, **Table 3**).

Similar results were observed across the whole study period, overall and at each study visit (**Table 2**; **Figure 2**). At each time period, except Week 2, the LS mean TDD was significantly lower in the LCPT group than the IR-Tac/PR-Tac group (**Table 3**). Mean TDD normalized for weight was lower in the LCPT group than the IR-Tac/PR-Tac group (**Table 3**).

TABLE 2 | Tacrolimus TDD, trough levels and trough:TDD by period (mITT population).

TDD, mean (SD), mg	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	IR-Tac (N = 86)	PR-Tac (N = 115)
Week 3 to Month 6	5.17 (2.97)	6.28 (3.56)	5.54 (2.91)	6.81 (3.88)
Overall	5.85 (3.08)	6.96 (3.65)	6.33 (3.24)	7.43 (3.88)
Week 1	10.96 (3.08)	11.72 (3.16)	11.34 (3.02)	12.01 (3.26)
Week 2	8.75 (4.01)	9.54 (4.55)	8.76 (3.94)	10.10 (4.87)
Week 3	8.07 (4.20)	9.20 (4.86)	8.17 (3.78)	9.93 (5.40)
Week 4	7.41 (4.02)	8.57 (4.64)	7.47 (3.66)	9.36 (5.11)
Months 1–3	5.80 (3.27)	7.00 (3.80)	6.23 (3.37)	7.56 (4.01)
Months 3–6	4.45 (2.87)	5.44 (3.23)	4.82 (2.74)	5.91 (3.50)
Trough Levels, Mean (SD), ng/ml				
Week 3 to Month 6	9.40 (1.72)	9.00 (1.67)	8.86 (1.51)	9.11 (1.78)
Overall	10.69 (2.58)	10.11 (2.12)	10.60 (2.46)	9.76 (1.76)
Week 1	13.96 (5.91)	13.07 (5.05)	14.59 (5.22)	11.94 (4.63)
Week 2	10.65 (3.67)	9.66 (3.60)	10.24 (3.54)	9.24 (3.60)
Week 3	10.70 (4.42)	9.91 (3.38)	10.45 (3.11)	9.52 (3.52)
Week 4	10.47 (3.52)	9.96 (3.04)	9.76 (2.54)	10.12 (3.38)
Months 1–3	9.69 (2.22)	9.36 (2.42)	9.23 (2.70)	9.45 (2.27)
Months 3–6	8.37 (1.87)	8.04 (1.78)	7.84 (1.89)	8.21 (1.69)
Trough:TDD Mean (SD), ng/ml mg^{-1}				
Week 3 to Month 6	2.26 (1.38)	1.69 (0.85)	1.90 (0.97)	1.54 (0.73)
Week 1	1.22 (0.69)	1.09 (0.63)	1.29 (0.69)	0.94 (0.54)
Week 2	1.46 (0.99)	1.26 (0.91)	1.38 (0.70)	1.18 (1.03)
Week 3	1.68 (1.14)	1.33 (0.86)	1.58 (1.07)	1.16 (0.62)
Week 4	1.77 (1.08)	1.44 (0.93)	1.66 (1.13)	1.27 (0.69)
Months 1–3	2.23 (1.51)	1.68 (0.96)	1.91 (1.09)	1.51 (0.82)
Months 3–6	2.62 (1.80)	1.87 (0.95)	2.06 (1.05)	1.72 (0.83)

IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; PR-Tac, prolonged release tacrolimus; SD, standard deviation; TDD, total daily dose.

TABLE 3 | Tacrolimus TDD (mITT).

TDD	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	Difference (LCPT−IR-Tac/PR-Tac) LS mean (95% CI)	*p*-value
Week 3 to Month 6 (primary endpoint)				
LS mean, mg[a]	5.14	6.24	−1.11 (−1.76, −0.45)	<0.001
Whole study period				
LS mean, mg				
Overall[a]	5.82	6.92	−1.11 (−1.77, −0.45)	0.001
Week 1[b]	10.91	11.67	−0.75 (−1.35, −0.16)	0.013
Week 2[b]	8.71	9.50	−0.79 (−1.62, 0.05)	0.064
Week 3[b]	8.04	9.12	−1.08 (−1.98, −0.19)	0.018
Week 4[b]	7.35	8.52	−1.18 (−2.05, −0.31)	0.008
Months 1–3[b]	5.71	6.91	−1.20 (−1.91, −0.49)	0.001
Months 3–6[b]	4.39	5.37	−0.98 (−1.60, −0.36)	0.002
Week 3 to Month 6 normalized for weight				
Mean (SD), mg/kg	0.07 (0.04)	0.09 (0.05)		

[a]*ANOVA model including treatment and country as fixed effects.*

[b]*MMRM model including treatment, period, treatment by period interaction, and country as fixed effects.*

Week 3 to Month 6: mean calculation normalized for weight, n = 186 (LCPT) and 187 (IR-Tac/PR-Tac).

Whole study period: mean calculation, n = 200 (LCPT) and 201 (IR-Tac/PR-Tac); LS mean calculation, n = 401 (overall), 401 (Week 1), 391 (Week 2), 388 (Week 3), 384 (Week 4), 378 (Months 1–3), and 365 (Months 3–6).

ANOVA, analysis of variance; CI, confidence interval; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; LS, least squares; mITT, modified intent-to-treat; MMRM, mixed model for repeated measures; PR-Tac, prolonged release tacrolimus; SD, standard deviation; TDD, total daily dose.

Trough Levels

Mean tacrolimus trough levels were higher in the LCPT group compared with the IR-Tac/PR-Tac group at each visit, except for Day 60 (**Table 2**; **Figure 3A**). LS mean tacrolimus trough levels were significantly higher in the LCPT group than the IR-Tac/PR-Tac group from Week 3 to Month 6: 0.41 (LS mean difference, LCPT-IR-Tac/PR-Tac), 0.08, 0.74 (95% CI) ($p = 0.016$, **Table 4**), and overall 0.62 (LS mean difference, LCPT-IR-Tac/PR-Tac),

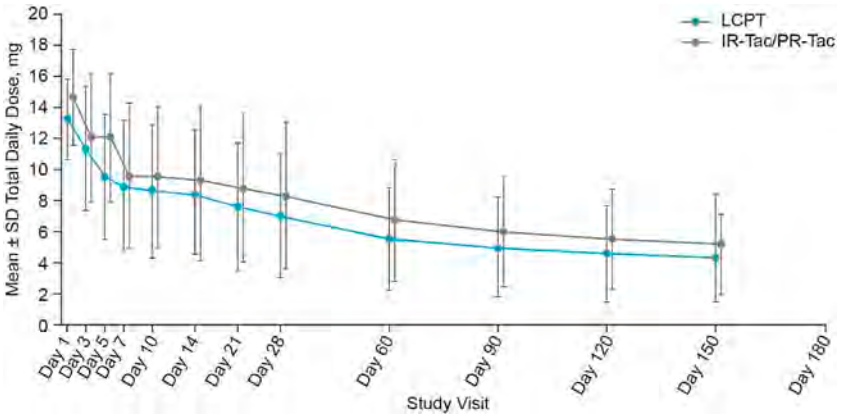


FIGURE 2 | Tacrolimus total daily dose at each study visit (mean ± SD, mITT). Mean daily dose data was not collected at Day 180. IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; PR-Tac, prolonged release tacrolimus; SD, standard deviation.

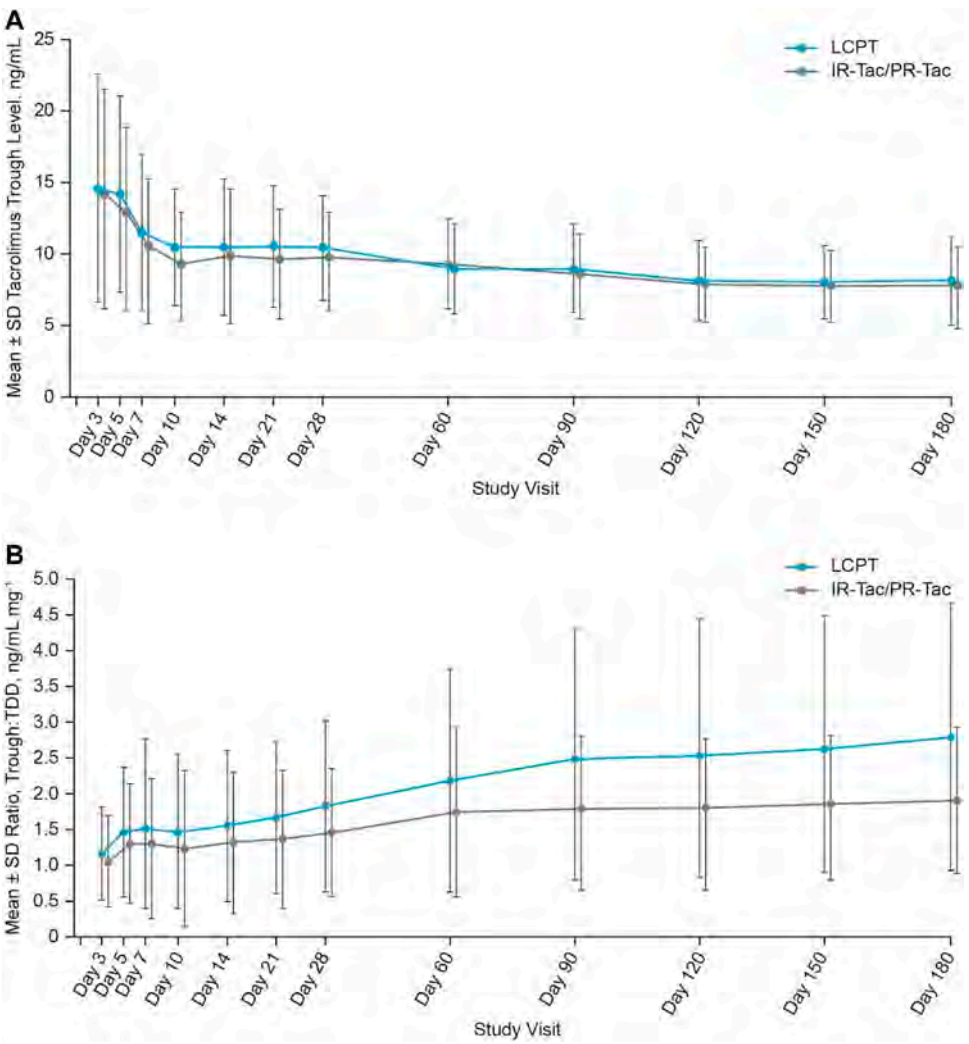


FIGURE 3 | Tacrolimus trough levels **(A)** and trough:TDD **(B)** at each study visit (mean ± SD, mITT). IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; PR-Tac, prolonged release tacrolimus; SD, standard deviation; TDD, total daily dose.

TABLE 4 | Tacrolimus trough levels (mITT).

	No. patients	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	Difference (LCPT—IR-Tac/PR-Tac)	
		LS mean, ng/ml	LS mean, ng/ml	LS mean (95% CI), ng/ml	*p*-value
Week 3 to Month 6	385	9.43	9.02	0.41 (0.08, 0.74)	0.016
Overall[a]	398	10.73	10.12	0.62 (0.17, 1.06)	0.007
Week 1[b]	397	13.99	13.06	0.93 (–0.14, 2.00)	0.090
Week 2[b]	389	10.68	9.68	1.01 (0.29, 1.72)	0.006
Week 3[b]	352	10.76	9.95	0.81 (–0.00, 1.61)	0.050
Week 4[b]	334	10.51	9.91	0.60 (–0.09, 1.29)	0.090
Months 1–3[b]	376	9.71	9.36	0.36 (–0.11, 0.82)	0.132
Months 3–6[b]	364	8.34	8.04	0.30 (–0.07, 0.67)	0.112

[a]*ANOVA model including treatment and country as fixed effects.*
[b]*MMRM model including treatment, period, treatment by period interaction, and country as fixed effects.*
ANOVA, analysis of variance; CI, confidence interval; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; LS, least squares; mITT, modified intent-to-treat; MMRM, mixed model for repeated measures; PR-Tac, prolonged release tacrolimus.

TABLE 5 | Tacrolimus trough:TDD (mITT).

	No. patients	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	Difference (LCPT—IR-Tac/PR-Tac)	
		LS mean, ng/ml mg^{-1}	LS mean, ng/ml mg^{-1}	LS mean (95% CI), ng/ml mg^{-1}	*p*-value
Week 3 to Month 6[a]	385	2.27	1.70	0.57 (0.34, 0.80)	<0.001
Week 1[b]	396	1.22	1.09	0.14 (0.01, 0.26)	0.034
Week 2[b]	389	1.47	1.26	0.21 (0.02, 0.40)	0.030
Week 3[b]	352	1.67	1.32	0.34 (0.14, 0.54)	<0.001
Week 4[b]	334	1.82	1.46	0.36 (0.15, 0.57)	<0.001
Months 1–3[b]	376	2.27	1.70	0.57 (0.31, 0.82)	<0.001
Months 3–6[b]	364	2.65	1.89	0.76 (0.47, 1.06)	<0.001

[a]*ANOVA model including treatment and country as fixed effects.*
[b]*MMRM model including treatment, period, treatment by period interaction, and country as fixed effects.*
ANOVA, analysis of variance; CI, confidence interval; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; LS, least squares; mITT, modified intent-to-treat; MMRM, mixed model for repeated measures; PR-Tac, prolonged release tacrolimus; TDD, total daily dose.

TABLE 6 | Exploratory dosage endpoints: LCPT vs. IR-Tac (mITT).

Exploratory endpoints	LCPT	IR-Tac	Difference (LCPT—IR-Tac)	
Week 3 to Month 6	(N = 200)	(N = 86)	LS mean (95% CI)	*p*-value
Tacrolimus TDD				
LS mean, mg[a]	5.19	5.28	–0.09 (–0.91, 0.73)	0.825
Tacrolimus trough levels				
LS mean, ng/ml	9.4	8.9	0.50 (0.05, 0.95)	0.030
Ratio of tacrolimus trough level over TDD				
LS mean, ng/ml mg^{-1}	2.25	2.00	0.25 (–0.11, 0.60)	0.172

[a]*ANOVA model including treatment and country as fixed effects. Difference in LS means calculated by [(LCPT)–(IR-Tac or PR-Tac)].*
ANOVA, analysis of variance; CI, confidence interval; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; LS, least squares; mITT, modified intent-to-treat; MMRM; PR-Tac, prolonged release tacrolimus; TDD, total daily dose.

0.17, 1.06 (95% CI) (p = 0.007, **Table 4**). The proportion of patients with trough levels within the standard reference range (5–15 ng/ml within the first 3 months after transplantation and 5–10 ng/ml thereafter) rose at each study visit from approximately 50% at Day 3 to >80% by Day 10. The proportion of trough level assessments within the standard range was similar in the LCPT and IR-Tac/PR-Tac groups (74.1 and 77.9%, respectively).

The LS mean ratios of tacrolimus trough:TDD were significantly higher in the LCPT group than the IR-Tac/PR-Tac group at each study visit and during each period [**Table 5**; mean (SD) data is shown in **Figure 3B**].

TABLE 7 | Exploratory dosage endpoints: LCPT vs PR-Tac (mITT).

Exploratory endpoints	LCPT	PR-Tac	Difference (LCPT—PR-Tac)	
Week 3 to Month 6	**(N = 200)**	**(N = 115)**	**LS mean (95% CI)**	***p*-value**
Tacrolimus TDD				
LS mean, mg[a]	5.15	7.04	−1.89 (−2.68, −1.10)	<0.001
Tacrolimus trough levels				
LS mean, ng/ml	9.4	9.2	0.21 (−0.19, 0.62)	0.298
Ratio of tacrolimus trough level over TDD				
LS mean, ng/ml mg^{-1}	2.26	1.49	0.78 (0.5, 1.06)	<0.001

[a]*ANOVA model including treatment and country as fixed effects. Difference in LS means calculated by [(LCPT)–(IR-Tac or PR-Tac)].*
ANOVA, analysis of variance; CI, confidence interval; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; LS, least squares; mITT, modified intent-to-treat; MMRM; PR-Tac, prolonged release tacrolimus; TDD, total daily dose.

TABLE 8 | Patients with treatment failure (mITT).

	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	Difference (LCPT—IR-Tac/PR-Tac)	
	n (%)	**n (%)**	**Estimate (95% CI), %**	***p*-value**
Overall treatment failure	18 (9.0)	18 (9.0)	0.0 (−5.7, 5.8)	>0.999
Death	4 (2.0)	4 (2.0)	0.0 (−3.2, 3.3)	>0.999
Graft failure	4 (2.0)	4 (2.0)	0.0 (−3.2, 3.3)	>0.999
Biopsy-proven acute rejection	12 (6.0)	10 (5.0)	1.0 (−3.7, 5.8)	0.668
Loss to follow-up	0	0	NE	NE

Two patients in the LCPT group experienced two events each (graft failure and biopsy-proven acute rejection).
p-value based on 2-sided Fisher's exact test; 95% CI based on the Newcombe-Wilson method.
CI, confidence interval; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; NE, not estimable; PR-Tac, prolonged release tacrolimus.

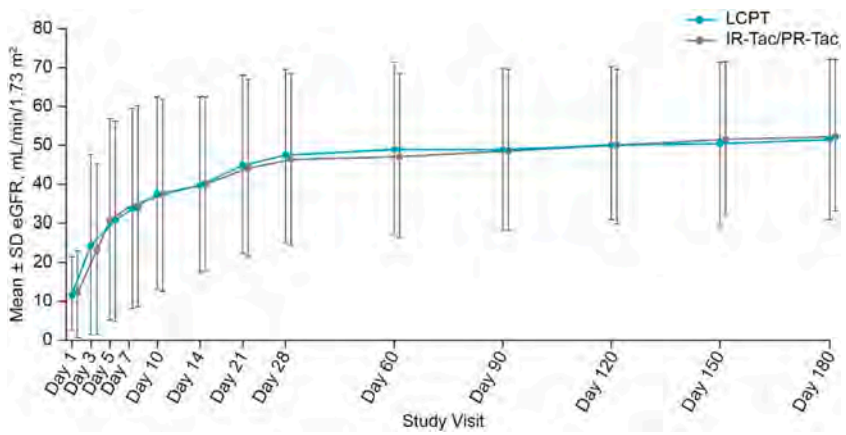


FIGURE 4 | Estimated glomerular filtration rate at each study visit (mean ± SD, mITT). eGFR, estimated glomerular filtration rate; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; PR-Tac, prolonged release tacrolimus; SD, standard deviation.

Dose Adjustments

With the exception of 2 subjects each in both the LCPT and IR-Tac/PR-Tac groups, all subjects had dose adjustments. For all time periods, the mean number of dose adjustments was <3 for patients in both the LCPT and IR-Tac/PR-Tac groups, with no notable differences between treatment groups at each period.

Exploratory Dosage Endpoints

Compared with IR-Tac, a similar dose of LCPT resulted in statistically higher tacrolimus trough levels. The LS mean tacrolimus TDD from Week 3 to Month 6 after transplant was similar: 5.19 and 5.28 mg respectively for LCPT and IR-Tac; 0.092 (LS mean difference, LCPT-IR-Tac), -0.91, 0.73 (95% CI) (p = 0.825, **Table 6** and **Supplementary Figure S1A**). LS

TABLE 9 | Concomitant immunosuppressant medications (mITT).

Subjects, n (%)	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)
Glucocorticoids and corticosteroid NOS	193 (96.5)	194 (96.5)
Immunosuppressants	155 (77.5)	166 (82.6)
Antithymocyte immunoglobulin	1 (0.5)	1 (0.5)
Belatacept	1 (0.5)	1 (0.5)
Everolimus	4 (2.0)	2 (1.0)
Mycophenolate mofetil and sodium	167 (83.5)	175 (87.1)
Basiliximab	117 (58.5)	121 (60.2)
Ciclosporin	1 (0.5)	1 (0.5)
Azathioprine	0	2 (1.0)

Subjects may have more than one medication. Concomitant medications were coded with the WHO Drug dictionary dated December 2014.
IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; mITT, modified intent-to-treat; NOS, not otherwise specified; PR-Tac, prolonged release tacrolimus; WHO, World Health Organization.

mean tacrolimus trough levels were significantly higher with LCPT than IR-Tac from Week 3 to Month 6: 9.4 and 8.9 ng/ml respectively for LCPT and IR-Tac; 0.50 (LS mean difference, LCPT-IR-Tac), 0.05, 0.95 (95% CI) (p = 0.030, **Table 6** and **Supplementary Figure S1A**). The LS mean ratios of tacrolimus trough:TDD were numerically, but not statistically, higher with LCPT compared with IR-Tac from Week 3 to Month 6: 2.25 vs 2.0 ng/ml mg^{-1} respectively for LCPT and IR-Tac, 0.25 (LS mean difference, LCPT-IR-Tac), −0.11, 0.60 (95% CI) (p = 0.172, **Table 6** and **Supplementary Figure S1A**).

Compared with PR-Tac, a significantly lower dose of LCPT was required to achieve similar tacrolimus trough levels. LS mean tacrolimus TDD from Week 3 to Month 6 after transplant was significantly lower with LCPT: 5.15 and 7.04 mg respectively for LCPT and PR-Tac; −1.89 (LS mean difference, LCPT-PR-Tac), −2.68, −1.10 (95% CI) (p < 0.001, **Table 7** and **Supplementary Figure S1B**). LS mean tacrolimus trough levels were similar from Week 3 to Month 6: 9.4 and 9.2 ng/ml respectively for LCPT and PR-Tac; 0.21 (LS mean difference, LCPT-PR-Tac), −0.19, 0.62 (95% CI) (p = 0.298, **Table 7** and **Supplementary Figure S1B**). The LS mean ratios of tacrolimus trough:TDD were significantly higher with LCPT compared with PR-Tac from Week 3 to Month 6: 2.26 vs 1.49 ng/ml mg^{-1} respectively for LCPT and PR-Tac, 0.78 (LS mean difference, LCPT-PR-Tac), 0.50, 1.06 (95% CI) (p < 0.001, **Table 7** and **Supplementary Figure S1B**).

Efficacy—Clinical Outcomes

There were no statistically significant differences between the LCPT and IR-Tac/PR-Tac groups overall or in any measure of treatment failure (death, graft failure, biopsy-proven acute rejection, or loss to follow-up; **Table 8**). Eighteen patients in each group (9.0%) experienced treatment failure, mainly biopsy-proven acute rejection [occurring in 12 (6.0%) patients in the LCPT group and 10 (5.0%) in the IR-Tac/PR-Tac group]. There were no statistically significant differences between the LCPT and IR-Tac/PR-Tac groups in time to treatment failure or time to treatment discontinuation (log-rank p = 0.965 and p = 0.461, respectively). Overall, the number (%) of subjects with treatment failure was 18 (9.0%) for LCPT, 7 (8.1%) for IR-Tac and 11 (9.6%) for PR-Tac; no significant difference was detected between the LCPT and IR-Tac subgroup (estimate 0.9; 95% CI: −7.5, 7.2; p-value: >0.999) or between the LCPT and PR-Tac subgroup (estimate −0.6; 95% CI: −8.1, 5.8; p-value: 0.843; **Supplementary Tables S1A,B**).

There were no statistically significant differences observed between the LCPT and IR-Tac/PR-Tac groups in the number of patients who experienced delayed graft function [23 (11.5%) and 22 (10.9%), respectively, p = 0.876] or the number of patients with rejection assessed as acute by the investigator [7 (3.5%) and 6 (3.0%), respectively, p = 0.787]. Biopsy-proven acute rejection was the reason for treatment failure in 12 (6.0%) patients in the LCPT group and 10 (5.0%) patients in the IR-Tac/PR-Tac group. In addition, no statistically significant differences in estimated glomerular filtration rates (eGFR) were shown between LCPT and IR-Tac/PR-Tac treatment groups at any post-baseline visit (**Figure 4**). The number (%) of subjects with delayed graft function was 23 (11.5%) for LCPT, 4 (4.7%) for IR-Tac and 18 (15.7%) for PR-Tac. No significant difference was detected between the LCPT and IR-Tac subgroups (estimate 6.8; 95% CI: −0.8, 12.7; p-value: 0.079) or between the LCPT and PR-Tac subgroups (estimate −4.2; 95% CI: −12.7, 3.4; p-value: 0.301) (**Supplementary Tables S1A,B**). The number (%) of subjects with local diagnosis of acute rejection requiring treatment was 7 (3.5%) for LCPT, 2 (2.3%) for IR-Tac and 4 (3.5%) for PR-Tac. No significant difference was detected between the LCPT and IR-

TABLE 10 | Treatment-emergent adverse events (TEAE) in the safety population.

Subjects (%) [E]	LCPT (N = 200)	IR-Tac/PR-Tac (N = 201)	IR-Tac (N = 86)	PR-Tac (N = 115)
Any TEAE	195 (97.5) [1704]	192 (95.5) [1546]	82 (95.3) [637]	110 (95.7) [909]
Any treatment-emergent ADR	73 (36.5) [164]	77 (38.3) [141]	43 (50.0) [86]	34 (29.6) [55]
Any serious TEAE	99 (49.5) [185]	93 (46.3) [178]	40 (46.5) [68]	53 (46.1) [110]
Any serious TEADR	26 (13.0) [34]	23 (11.4) [28]	13 (15.1) [18]	10 (8.7) [10]
Any severe TEAE	48 (24.0) [92]	59 (29.4) [97]	29 (33.7) [46]	30 (26.1) [51]
Any TEAE leading to discontinuation	12 (6.0) [15]	16 (8.0) [16]	8 (9.3) [8]	8 (7.0) [8]
Any treatment-emergent ADR leading to discontinuation	3 (1.5) [3]	4 (2.0) [4]	2 (2.3) [2]	2 (1.7) [2]
Any AE leading to death	4 (2.0) [6]	4 (2.0) [4]	1 (1.2) [1]	3 (2.6) [3]

E, number of events; ADR, adverse drug reaction; AE, adverse event; IR-Tac, immediate release tacrolimus; LCPT, LCP tacrolimus; PR-Tac, prolonged release tacrolimus; TEAE, treatment emergent AE.

Tac subgroup (estimate 1.2; 95% CI: −4.9, 5.1; *p*-value: 0.729) or between the LCPT and PR-Tac subgroup (estimate 0.0; 95% CI: −5.4, 4.2; *p*-value: >0.999) (**Supplementary Tables S1A,B**).

The most common concomitant immunosuppressants were glucocorticoids [taken by 193 (96.5%) and 194 (96.5%) patients in the LCPT and IR-Tac/PR-Tac groups, respectively], and basiliximab [117 (58.5%) and 121 (60.2%) patients; **Table 9**]. Mycophenolate, either mofetil or sodium, was used by 167 (83.5%) and 175 (87.1%) patients in the LCPT and IR-Tac/PR-Tac groups, respectively.

Safety

The safety profile of LCPT was similar to that of IR-Tac/PR-Tac and to that of the two formulations separately, and no new unexpected safety warnings were observed (**Table 10**). The most commonly reported AEs considered possibly related to treatment were tremor (13.5 and 9.0% in the LCPT and IR-Tac/PR-Tac groups, respectively), cytomegalovirus infection (4.5 and 3.5%), urinary tract infection (3.0 and 2.5%), and post-transplant diabetes mellitus (2.0 and 4.0%, defined as the need for any antidiabetic agent and/or HbA_{1c} >6.5% at Months 3 and 6). BK virus infections occurred in 11 (5.5%) and 12 (6.0%) of patients in the LCPT and IR-Tac/PR-Tac groups, respectively. A total of 99 patients (49.5%) in the LCPT group and 93 (46.3%) in the IR-Tac/PR-Tac groups experienced a serious adverse event (SAE). In the LCPT group, the most common SAEs were complications of the transplanted kidney (6.0%), raised blood creatinine (5.0%), transplant rejection (4.5%), and urinary tract infection (3.0%). In the IR-Tac/PR-Tac group, the most common SAEs were urinary tract infection (5.0%), transplant rejection (4.0%), and diarrhea (3.5%). Four (2%) patients in each study group died. Events leading to death in the LCPT group were duodenal ulcer, pancreatitis and sepsis (in one patient), intestinal ischemia, sequelae of a complicated mycotic aneurysm of the graft artery, and multi-organ failure. Events leading to death in the IR-Tac/PR-Tac group were acute respiratory distress syndrome, cardiac arrest, multi-organ failure, and myocardial infarction. There were no notable differences in the effects of LCPT and IR-Tac/PR-Tac on vital signs, ECG, or clinical laboratory results, including lipid profiles and blood pressure parameters.

DISCUSSION

This is the first study comparing LCPT versus tacrolimus standard-of-care in *de novo* kidney transplant recipients in real-life clinical practice across Europe. The results showed that LCPT can achieve similar clinical outcomes to other tacrolimus formulations, with a lower daily dose. The study met its primary objective by demonstrating a significantly lower mean tacrolimus TDD with LCPT than with IR-Tac/PR-Tac from Week 3 to Month 6. The 6-months timeframe for this study was chosen to be in line with similar studies assessing biopsy-proven acute rejection following transplantation, and with the assumption that it would take 3 weeks to stabilize tacrolimus dose levels post-transplantation (22–24).

TDD was significantly lower with LCPT than with IR-Tac/PR-Tac throughout the study period, and when normalized for weight. Despite the lower dose required, patients receiving LCPT maintained significantly higher tacrolimus trough levels than those receiving standard-of-care while importantly remaining within the standard reference range, leading to a higher ratio of tacrolimus trough:TDD in the LCPT group.

For all other secondary efficacy endpoints, there were no notable differences between the two treatment groups. The overall number of treatment failures and rejections was low; approximately 9% of patients in each treatment group experienced treatment failure (a composite of death, graft failure, biopsy-proven acute rejection, or loss to follow-up), approximately 6% had biopsy-proven acute rejection, and approximately 11% experienced delayed graft function. These results are in line with the low treatment failure rates seen in *de novo* kidney recipients receiving LCPT or IR-Tac in a 12-months study (14).

The safety profiles of LCPT and tacrolimus standard-of-care were similar, and no new unexpected safety warnings were observed. The most common treatment-related AEs in both treatment groups were tremor, cytomegalovirus infection, urinary tract infection, and diabetes mellitus.

Previous studies have also reported a lower TDD with LCPT compared with IR-Tac or PR-Tac (14, 17, 20, 21), in addition to lower rates of efficacy failure among high-risk subgroups, including black recipients and recipients ≥65 years of age (25). Non-inferiority of LCPT versus IR-Tac with respect to treatment failure has been previously shown in stable kidney transplant patients who converted from IR-Tac to LCPT (20). Non-inferiority of LCPT in *de novo* transplant patients has also been demonstrated at 1 year after transplantation (14) with similar efficacy and safety maintained over 2 years (21). The present study extends the existing knowledge to include comparison with PR-Tac in *de novo* patients, demonstrating that LCPT has similar efficacy to both IR-Tac and PR-Tac in this population.

The lower dose and higher trough levels observed with LCPT in the present study may be attributed to improved bioavailability resulting from controlled release of tacrolimus. This study did not assess bioavailability directly, however previous studies have demonstrated significantly higher bioavailability and lower peak-to-trough fluctuation with LCPT compared with PR-Tac (18). Lower tacrolimus bioavailability has been reported in women and African Americans, largely due to variations in hepatic CYP3A4 content and *CYP3A5* gene expression (26–29). It has also been suggested that elderly transplant recipients may have greater variability in tacrolimus levels compared with younger patients (30); therefore, elderly patients may particularly benefit from the improved pharmacokinetic profile of LCPT, as previously indicated by a subgroup analysis (25).

Given the different immunosuppressive regimens available, there is a need to increase the use of support systems and biomarkers to help improve clinical decision making and to monitor outcomes. Although recent pharmacokinetic studies have highlighted the major influence of CYP3A genotype on tacrolimus exposure (31–33), CYP3A phenotype did not explain

all pharmacokinetic variability, perhaps because multiple factors drive inter-individual variability in tacrolimus metabolism (11, 31–33). Continued investigation of optimal management algorithms is needed, and accordingly, a potential tool to assess risk factors for poor long-term outcomes has been proposed based on the concept of individual metabolic rates. This tool showed that fast tacrolimus metabolism, defined as having a low ratio of tacrolimus trough:TDD, associates with reduced survival rates of patients, lower renal function, and infection, suggesting that some patients may benefit from alternative immunosuppressive regimens or concepts (34–36).

Once-daily dosing may represent a further advantage of LCPT and PR-Tac over IR-Tac. Transplant recipients are often reported to be non-adherent to immunosuppressive therapy (37, 38), and once-daily tacrolimus has been shown to be associated with improved adherence (39, 40). This is key for successful treatment outcomes, particularly for therapies such as tacrolimus that have a narrow therapeutic window. Improvements in adherence with once-daily dosing could not be evaluated in the present study, because the tacrolimus standard-of-care control arm allowed use of both twice-daily IR-Tac (86 patients) and once-daily PR-Tac (115 patients). The prespecified subgroup analysis confirmed that LCPT has a clinically relevant greater bioavailability compared to the other oral formulations of tacrolimus, and that this difference in bioavailability of LCPT is particularly significant in comparison with PR-Tac.

A key strength of the study is that it reflected real-life conditions across a number of different countries for *de novo* kidney transplant patients, in that investigators were free to choose IR-Tac or PR-Tac for the comparator arm according to their usual clinical practice. The results therefore provide a representative picture of the potential benefits of LCPT compared with tacrolimus standard-of-care as routinely implemented in transplant centers across Europe. A limitation is that the study included mainly white, middle-aged men with standard immunological risk for graft rejection, and the results may not be generalizable to the overall kidney transplant population.

In conclusion, the study demonstrated that LCPT, when administered to *de novo* kidney transplant patients, allows a lower TDD than current standard-of-care tacrolimus, while maintaining gold-standard levels of clinical outcomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available. Chiesi access criteria and complete process for clinical data sharing is available on the Chiesi Group website. Requests to access the datasets should be directed to https://www.chiesi.com/en/chiesi-clinical-trial-data-request-portal/.

ETHICS STATEMENT

This was a Phase IV, randomized, open-label, parallel group study, conducted in 10 European countries. The study was conducted according to the current International Council for Harmonisation Good Clinical Practice guidelines, any local guidelines, and the Declaration of Helsinki. The study protocol was approved by the Ethics Commission of the State of Berlin for the site of the principal investigator, and by the appropriate Ethics Committees in accordance with local requirements for the other centers participating in the clinical trial. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article and had full access to all the data in this study and take complete responsibility for the integrity of the data and accuracy of the data analysis. All authors participated in the interpretation of the data, the writing, reviewing, and editing of the manuscript, and had final responsibility for approving the published version.

CONFLICT OF INTEREST

GP, DS, SG, CP, and GN are all employees of Chiesi Farmaceutici S.p.A. KB has received honoraria and/or research funding from Alexion, Astellas, Bristol-Myers Squibb, Chiesi, Fresenius, Hansa, Hexal, Merck, Novartis, Otsuka, Pfizer, Roche, Sandoz, Siemens, and Veloxis Pharmaceuticals. UM received participation fees from Chiesi for scientific advisory boards. LR has been an advisor for Hansa and Biotest; has received speaker fees from Astellas, Novartis, Chiesi and Bristol-Myers Squibb; and has received grants from Chugai, Terumo and HemaT. GP, who is now an employee of Chiesi Farmaceutici S.p.A., worked at Dipartimento di Medicina e Chirurgia, UO Nefrologia, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy at the time of the study and received consulting fees from Chiesi for work as a medical research physician for the present study. OW has received research grants for clinical studies, speaker's fees, honoraria, and travel expenses from Amgen, Astellas, Bristol-Myers Squibb, Chiesi, Hexal, Janssen-Cilag, MSD, Novartis, Pfizer, Roche, and Sanofi. DS received consulting fees from Chiesi for work as a clinical study manager for the present study. NK has received speaker fees and participated in advisory boards for Abbvie, Amgen, Astellas, Chiesi, Fresnius Medical Care, Gilead, Merck Sharp and Dohme, Neovii, Novartis, Roche, Sanofi, and Shire. MB has received funds for travel from Astellas and Chiesi. JP has received consulting fees from Chiesi. DK has received honoraria from Chiesi and Astellas. TW has received honoraria from Chiesi and Therakos/Mallinckrodt, and research funding from Astellas, Chiesi, Neovii, Novartis, Sandoz, Sanofi, and Teva. AG-D has received travel grants, speaker fees, and/or advisory board honoraria from Alexion, Chiesi, MSD and Novartis, and has participated in clinical trials sponsored by Alexion, Astellas, Chiesi and Novartis. LW had received research funding from Chiesi.

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

The authors declare that this study (ClinicalTrials.gov, NCT02432833) was funded by Chiesi Farmaceutici SpA. The funder was involved in study design, collection, analysis and interpretation of data. All co-authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript. Fishawack Inc. has received funding by Chiesi Farmaceutici SpA to provide medical writing support.

ACKNOWLEDGMENTS

The authors received editorial/writing support in the preparation of this manuscript provided by Clemence Hindley, of Fishawack Communications Ltd., a Fishawack Health company, funded by Chiesi. OW is supported by an unrestricted grant of the Rudolf-Ackermann-Stifung (Stiftung für Klinische Infektiologie). The authors would like to thank all transplantation centers that enrolled patients into the study, as well as the patients and their families.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2021.10225/full#supplementary-material

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ORIGINAL RESEARCH
published: 21 March 2022
doi: 10.3389/ti.2022.10239

BNT162b2 Third Booster Dose Significantly Increases the Humoral Response Assessed by Both RBD IgG and Neutralizing Antibodies in Renal Transplant Recipients

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Background: An impaired humoral response to full dose of BNT162b2 vaccine was observed in renal transplant recipients (RTR).

Methods: To reveal predictors for humoral response to third vaccine, patients were stratified to positive (N = 85) and negative (N = 14) response groups based on receptor-binding domain (RBD) IgG ≥1.1 and neutralizing antibodies (NA) ≥ 16 dilution versus RBD IgG <1.1 or NA < 16, respectively. NA were detected using a SARS-CoV-2 pseudo-virus.

Results: Response rate increased from 32.3% (32/99) before the third dose to 85.9% (85/99) post-third vaccine with a significant rise in geometric mean titers (GMTs) for RBD IgG and NA [0.79 (95% CI 0.65–0.96) vs. 3.08 (95% CI 2.76–3.45), $p < 0.001$ and 17.46 (95% CI 12.38–24.62) vs. 362.2 (95% CI 220.7–594.6), $p < 0.001$ respective. 80.6% (54/67) seroconverted and 96.9% (31/32) remained positive following the vaccine with a significant increase in GMTs for RBD IgG and NA. Age, ESRD secondary to diabetic nephropathy (DN) and renal allograft function were independent predictors for antibody response in RTR. Mycophenolic acid (MPA) use and dose had no impact on humoral response following the third booster. AEs were recorded for 70.1% of RTR population. Systemic AEs were more common in recipients with a positive humoral response as opposed to non-responders (45.2% versus 15.4% respectively, $p = 0.04$).

OPEN ACCESS

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Received: 15 November 2021
Accepted: 18 February 2022
Published: 21 March 2022

Citation:
Hod T, Ben-David A, Olmer L, Scott N, Ghinea R, Mor E, Levy I, Indenbaum V, Lustig Y, Grossman E and Rahav G (2022) BNT162b2 Third Booster Dose Significantly Increases the Humoral Response Assessed by Both RBD IgG and Neutralizing Antibodies in Renal Transplant Recipients. Transpl Int 35:10239. doi: 10.3389/ti.2022.10239

Abbreviations: BMI, body mass index; CI, confidence interval; DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; ESRD, end stage renal disease; GMTs, geometric mean titers; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; NA, neutralizing antibodies; OR, odds ratio; RBD, receptor-binding domain; RTR, renal transplant recipients; SD, standard deviation; SOT, solid organ transplantation; IHD, ischemic heart disease; CHF, congestive heart failure; CVD, cardiovascular disease.

Conclusion: 85.9% of RTR develop NA to BNT162b2 third vaccine, found effective in both negative and positive responders prior to the vaccine. Antigenic re-exposure overcame the suppressive effect of MPA on antibody response in RTR.

Keywords: immunosuppression, humoral response, renal transplantation, COVID-19 vaccine, antibody response

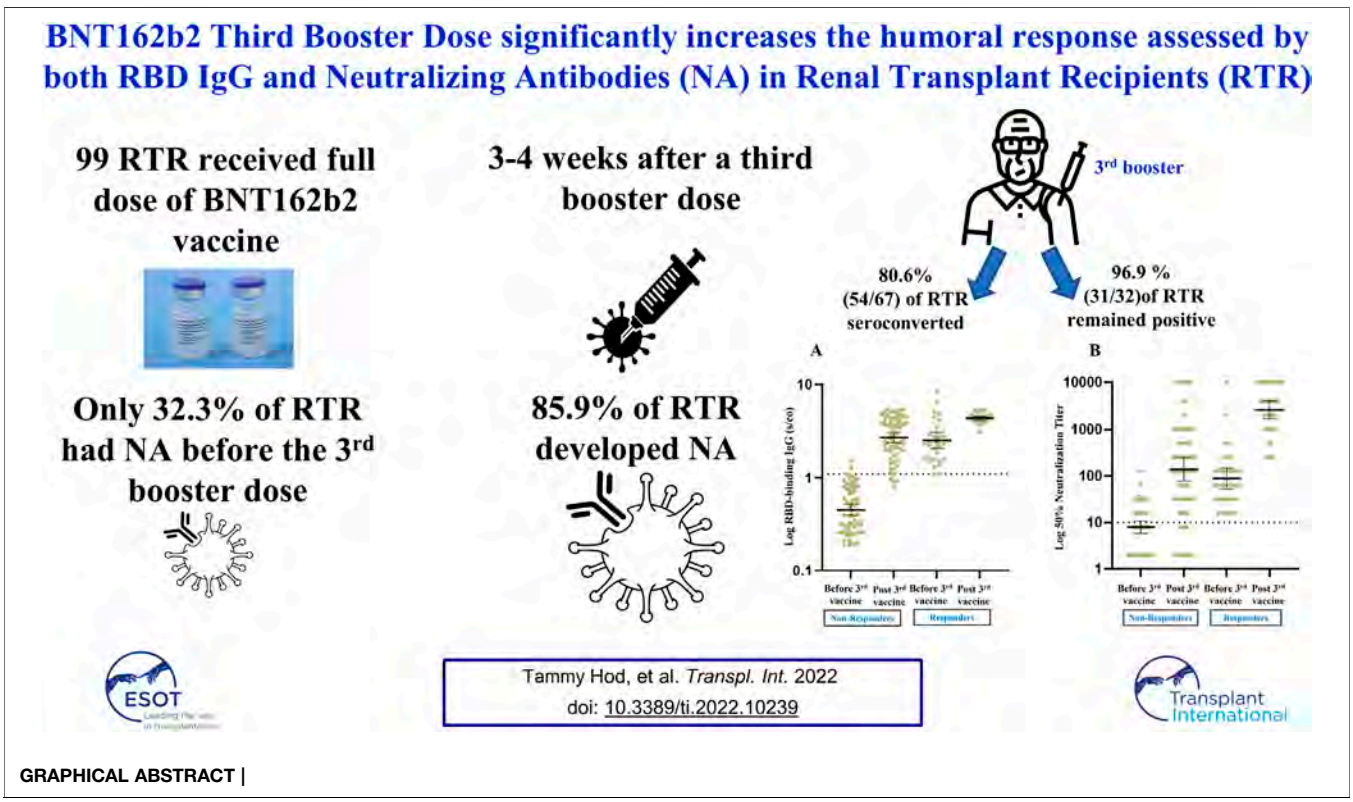
INTRODUCTION

Renal transplant recipients (RTR) among other solid organ transplant (SOT) recipients and immunosuppressed individuals are susceptible to significant morbidity and mortality from COVID-19 infection (1). A national campaign to vaccinate this vulnerable population took place with different studies reporting impaired response to the BNT162b2 mRNA vaccine (2–5). Over 60% of RTR did not develop an adequate humoral response, with seroconversion rates being as low as 5.7% in patients receiving belatacept (6). Studies of the vaccinated RTRs showed that the main factor impairing the ability to mount an antibody response to the vaccine was the administration of immunosuppressive drugs, particularly mycophenolic acid (MPA) (2, 3, 7).

In a recent study,(7) we showed that only 35% of 120 RTR developed neutralizing antibodies (NA) to the BNT162b2 mRNA vaccine, compared to 97.5% of 202 immunocompetent controls. In addition, NA geometric mean titers (GMTs) in RTR were significantly lower than those in the healthy population. Following the second BNT162b2 mRNA vaccine dose, the vast majority of RTR thus remained unprotected and susceptible to infection, leading to high rate of morbidity and mortality from COVID-19 infection in the vaccinees (8, 9). SOT recipients had an 82-fold higher risk of breakthrough infection and 485-fold higher risk of breakthrough infection with associated hospitalization and death compared to the general population.(10).

In July 2021, the Israel Government approved administration of a third booster dose of the BNT162b2 mRNA vaccine for all SOT recipients and other immunocompromised patients. The BNT162b2 mRNA vaccine, which has been found effective against the B.1.617.2 (delta) variant that has now been detected across the globe (11), was the only vaccine administered across the population in Israel.

Given the diminished antibody response observed following the two doses of the BNT162b2 mRNA vaccine in RTR, we sought to analyze the receptor-binding domain (RBD) IgG and NA responses to an homologous booster dose of the BNT162b2 vaccine in our population of RTR, with the aim to reveal predictors for serologic response, focusing specifically on the prior response detected following the second vaccine dose. Our working hypothesis was that the humoral response elicited in RTR to a third BNT162b2 dose would be higher than the reported response following the second dose. We also monitored the adverse events (AE) subsequent to the booster dose in our population.



GRAPHICAL ABSTRACT |

TABLE 1 | Demographic, clinical and biochemical characteristics of renal transplant recipients (RTR) stratified by antibody response.

Variable	Total cohort (N = 99)	Negative (N = 14)	Positive (N = 85)	p value
RTR characteristics				
Age, years, [median (IQR)]	66 (53–73)	71.5 (68–74)	63 (52–72)	**0.008**[b]
Female sex, n (%)	25 (25.3)	4 (28.6)	21 (24.7)	0.76
Transplant to 3rd vaccine, years [median (IQR)]	3.4 (1.4–9.2)	2.8 (1.0–6.2)	3.6 (1.4–10.0)	0.25
2nd to 3rd vaccine, days [median (IQR)]	175 (171–178)	177.5 (174–178)	175 (170–178)	0.34
3rd vaccine to antibody testing, days [median (IQR)]	21 (21–21)	21 (21–33)	21 (21–21)	0.28
ESRD etiology, n (%)				
APCKD	14 (14.1)	0 (0)	14 (16.5)	0.15
Diabetic nephropathy	20 (20.2)	6 (42.9)	14 (16.5)	
Glomerulonephritis	28 (28.3)	3 (21.4)	25 (29.4)	
Nephrosclerosis	14 (14.1)	2 (14.3)	12 (14.1)	
Other	16 (16.2)	3 (21.4)	13 (15.3)	
Unknown	7 (0.1)	0 (0)	7 (8.2)	
ESRD secondary to DN	20 (20.2)	6 (42.9)	14 (16.5)	**0.02**[a]
Time on dialysis, years [median (IQR)]	0.6 (0–1.5)	0.6 (0–3.0)	0.6 (0–1.5)	0.99
Transplant number, n (%)				
1	92 (92.9)	13 (92.9)	79 (92.9)	0.64
2	4 (4)	1 (7.1)	3 (3.5)	
3	3 (3)	0 (0)	3 (3.5)	
Donor type, n (%)				
Living	81 (81.8)	12 (85.7)	69 (81.2)	0.82
Deceased	16 (16.2)	2 (14.3)	14 (16.5)	
Unknown	2 (2)	0 (0)	2 (2.4)	
Medical history				
Hypertension	74 (74.7)	10 (71.4)	64 (74.1)	0.83
SBP 3-months average [median (IQR)]	131.8 (120.0–141.5)	139.5 (117.5–153.5)	131 (120.0–140.9)	0.25
DBP 3-months average [median (IQR)]	73.5 (68.0–79.5)	73 (66.8–79.5)	73.5 (68.0–79.5)	0.61
Ischemic heart disease	10 (10.1)	1 (7.1)	9 (10.6)	0.69
Congestive heart failure	10 (10.1)	2 (14.3)	8 (9.4)	0.58
Diabetes	37 (37.4)	7 (50)	30 (35.3)	0.29
HbA1C 6-months average (%) [median (IQR)]	6.4 (5.7–7.1)	6.4 (5.8–7.6)	6.4 (5.7–6.9)	0.59
Weight, (kg) [median (IQR)]	80 (70–92)	82.1 (70–89)	79.1 (70–92.2)	0.79
BMI, kg/m^2 [median (IQR)]	26.9 (23.2–31.1)	26.6 (23.2–31.8)	27 (23.6–30.9)	0.91
Average Laboratory results 1 month before antibody testing day [median (IQR)]				
White blood cell (K/μL)	7.3 (6.1–8.9)	7.1 (6.4–8.1)	7.4 (6.0–9.0)	0.73
Lymphocyte absolute (K/μL)	1.7 (1.3–2.1)	1.6 (1.2–1.7)	1.7 (1.4–2.2)	0.18
Neutrophils absolute (K/μL)	4.6 (3.6–5.7)	4.3 (3.9–5.2)	4.8 (3.6–5.7)	0.99
Neutrophil/lymphocyte ratio	2.7 (2.0–3.5)	2.9 (2.6–3.2)	2.7 (2.0–3.6)	0.48
Hemoglobin (g/dl)	13.2 (12.2–14.0)	12.7 (11.8–13.4)	13.2 (12.3–14.0)	0.36
Platelets (K/μL)	179 (149–223.5)	175 (168–196)	182 (147.5–225.3)	0.91
Creatinine (mg/dl)	1.1 (0.9–1.4)	1.4 (1.2–1.7)	1.1 (0.9–1.3)	**0.03**[a]
eGFR (CKD-EPI)**	64.7 (51.3–82.7)	46.6 (37.4–53.7)	67.9 (54.0–83.6)	**0.008**[b]
Glucose (mg/dl)	115.5 (101–145.2)	129 (123–170)	113 (100.9–141)	0.057
Albumin (g/dl)	4.1 (3.8–4.2)	4 (3.7–4.1)	4.1 (3.9–4.2)	0.36
Globulins (g/dl)	2.6 (2.4–2.9)	2.5 (2.3–2.7)	2.7 (2.4–2.9)	0.08
C-reactive protein (mg/L)	3.26 (1.17–8.79)	2.7 (1.52–6.72)	3.29 (1.08–8.88)	0.66

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; ESRD, end stage renal disease, SBP, systolic blood pressure.

[a] *<0.05.*

[b] *<0.01.*

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

When p value is significant, below 0.05 or below 0.01 the values are bolded.

TABLE 2 | RTR Immunosuppression Treatment on third vaccine Day Stratified by Antibody Response.

Immunosuppressive therapy	Total cohort (N = 99)	Negative (N = 14)	Positive (N = 85)	p value
Tacrolimus, n (%)	87 (87.9)	12 (85.7)	75 (88.2)	0.79
Tacrolimus daily dose (mg) on 3rd vaccine date [median (IQR)]	2 (1.5–3.0)	2.5 (1.5–3.0)	2 (2.0–3.0)	0.66
Tacrolimus daily dose (mg) per weight (kg) on 3rd vaccine day [median (IQR)]	0.03 (0.02–0.04)	0.03 (0.01–0.04)	0.03 (0.02–0.04)	0.74
Tacrolimus trough level 1M average before 3rd vaccine day (μg/L) [median (IQR)]	6.79 (5.6–7.7)	6.4 (5.8–7.6)	6.8 (5.4–7.9)	0.73
Mycophenolic acid (MPA), n (%)	79 (76.8)	13 (92.9)	63 (74.1)	0.12
MPA daily dose (mg) on 3rd vaccine date, [median (IQR)]	720 (360–720)	720 (360–720)	720 (0.0–720)	0.19
MPA daily dose (mg) per weight (kg) on 3rd vaccine date, [median (IQR)]	7.7 (3.6–9.5)	8.2 (5.1–10.3)	7.4 (0.0–9.2)	0.25
Prednisone, n (%)	74 (74.75)	10 (71.4)	64 (75.3)	0.76
Prednisone daily dose (mg) on 3rd vaccine date, [median (IQR)]	5.0 (0.0–5.0)	5.0 (0.0–5.0)	5.0 (3.0–5.0)	0.58
Prednisone daily dose (mg) per weight (kg) on 3rd vaccine date, [median (IQR)]	0.05 (0.00–0.07)	0.06 (0.00–0.06)	0.05 (0.03–0.07)	0.57
Immunosuppressive regimen				
Tacrolimus + MPA + prednisone, n (%)	45 (45.5)	7 (50)	38 (44.7)	0.71
Tacrolimus + MPA, n (%)	22 (22.2)	4 (28.6)	18 (21.2)	0.54
Tacrolimus + prednisone, n (%)	16 (16.2)	1 (7.1)	15 (17.6)	0.32
Cyclosporine + MPA + prednisone, n (%)	5 (5.1)	1 (7.1)	4 (4.7)	0.7
Tacrolimus + azathioprine, n (%)	2 (2.02)	0 (0)	2 (2.4)	0.56
Tacrolimus + azathioprine + prednisone, n (%)	2 (2.02)	0 (0)	2 (2.4)	0.56
mTORi (everolimus or sirolimus), n (%)	5 (5.1)	0 (0)	5 (5.9)	0.35

Abbreviations: MPA, mycophenolic acid; mTORi- mammalian target of rapamycin inhibitor.

METHODS

Study Population

This prospective study was conducted at the out-patient RTR clinic at Sheba Medical center. Ninety nine RTR who had previously received two doses of the BNT162b2 vaccine were vaccinated with an homologous third dose of the vaccine. Patients with a positive SARS-CoV-2 polymerase chain reaction test before or after the full two-dose vaccination were excluded from the study. Given the stronger response to the BNT162b2 vaccine in patients who received the vaccine prior to kidney transplant, patients vaccinated before transplant were also excluded. Vaccination was avoided during the first 3 months following transplantation and during active treatment for rejection. On the day of the third vaccination, blood was drawn, prior to administration of the booster dose, for baseline serology assessment of RBD IgG and NA. Three to 4 weeks following the booster dose, testing for RBD IgG and NA was repeated to assess the humoral response to the vaccine. For 76 of the 99 participants, we had RBD IgG levels 1 month post second vaccine. Written informed consent was obtained from all participants. The protocol and informed consent were approved by our Institutional Review Board (8314–21-SMC).

Immunosuppression

As described previously (7), the standard maintenance immunosuppression regimen for our RTR patients is a calcineurin inhibitor (usually tacrolimus), an anti-metabolite, usually a mycophenolate-based drug (mainly MPA), and prednisone. An early steroid withdrawal protocol is implemented between the fifth and eighth days post-transplant for RTR with a low immunological risk for rejection. The two-drug maintenance regimen for these patients is usually comprised of tacrolimus and MPA. Conversion to a mammalian target of rapamycin (mTOR) inhibitor (sirolimus or everolimus) is instituted according to the patient's risk of malignancy and intolerance to calcineurin inhibitors.

Primary Outcome

A positive response to the third booster dose of the BNT162b2 vaccine was defined as RBD IgG ≥1.1 and the presence of NA capable of reducing viral replication by at least 50% at a 16 fold dilution or above.

Data Extraction and Study Assessments

Patient information was obtained from the electronic patient records at the Sheba Medical Center, as described previously (7), and presented in **Table 1**. The MDClone data acquisition system at the Sheba Medical Center, which allows facile data retrieval, was used to retrieve average biochemical parameters that were recorded during 1 month prior to the third vaccine and any other relevant additional biochemical and clinical information (including average systolic and diastolic blood pressures in the 3 months prior to the booster dose, weight and BMI on the day of the third vaccine, average HbA1C level in the 6 months prior to the vaccine and total daily dose of the immunosuppressive medications on the day of the third vaccine, as described previously (7), and presented in the **Table 2**). In 15 patients, total daily mycophenolate dose was converted to the equivalent MPA dose by dividing the mycophenolate dose by 1.388. The use of cyclosporine, azathioprine, rapamycin and everolimus on the day of the third vaccine was also retrieved from the MDClone system.

Patients were instructed to report (using a specific questionnaire) any systemic (fever, fatigue, headache, myalgia, chills, nausea/vomiting, paresthesia) and local (pain, redness, or swelling at the injection site) reactions occurring within 30 days

TABLE 3 | RBD IgG and NA prior to third vaccine and post third vaccine stratified by Antibody Response to third vaccine.

Variable	Total cohort (N = 99)	Negative (N = 14)	Positive (N = 85)	*p* value
Baseline immune status on 3rd vaccine day				
Positive RBD IgG and NA on 3rd vaccine day, n (%)	32 (32.3)	1 (7.1)	31 (36.5)	**0.03***
Negative RBD IgG and NA on 3rd vaccine day, n (%)	67 (67.7)	13 (92.9)	54 (63.5)	**0.03***
IgG-RBD GMT on 3rd vaccine day (95% CI)	0.79 (0.65–0.96)	0.34 (0.23–0.51)	0.91 (0.74–1.12)	**0.0005****
NA GMT on 3rd vaccine day, (95% CI)	17.46 (12.38–24.62)	6.56 (3.12–13.80)	20.51 (14.1–29.85)	**0.02***
Response to 3rd vaccine				
IgG-RBD GMT post 3rd vaccine day (95% CI)	3.08 (2.76–3.45)	1.28 (0.87–1.86)	3.57 (3.28–3.88)	**<0.0001****
NA GMT post 3rd vaccine day (95% CI)	362.2 (220.7–594.6)	7.25 (2.42–21.71)	689.9 (456.3–1043)	**<0.0001****

Abbreviations: CI, confidence intervals; GMT, geometric mean titer; NA, neutralizing antibodies; RBD, receptor-binding domain.
**<0.05, **<0.001.*
When p value is significant, below 0.05 or below 0.01 the values are bolded.

after third vaccine dose and were actively screened for any other systemic and local complaints.

Antibody Detection Assays

Samples from vaccinated RTR were evaluated with an enzyme-linked immunosorbent assay (ELISA) that detects IgG antibodies against the RBD of SARS-CoV-2 as previously published (12, 13). A SARS-CoV-2 pseudo-virus (psSARS-2) neutralization assay (NA) was performed (14) using a propagation-competent vesicular stomatitis virus spike, which was kindly provided by Gert Zimmer, University of Bern, Switzerland.

Statistical Analysis

Descriptive statistics were expressed as frequencies and percentages for categorical data, and means ± standard deviation (SD) or median with interquartile range (IQR) for continuous variables. All continuous variables were assessed for normality by the Kolmogorov–Smirnov test and log-transformed as appropriate. Differences in baseline characteristics between the groups were tested using Chi-square for the categorical variables or t-test for the continuous variables. To compare the humoral response before and after the third vaccine dose, a paired t-test and McNemar's test were used.

Multivariable logistic regression analysis was used to identify factors associated with the vaccine-induced antibody response in the entire cohort. To analyze the association between antibody response and demographic, clinical and laboratory variables, a multivariable logistic regression analysis was constructed with a positive antibody response as the dependent variable, while adjusting for potential confounders. The variables used in the multivariate analysis were those with a p value <0.15 in the univariate analysis and those of clinical and biological relevance. Results are presented as odds ratio (OR), 95% confidence intervals (CI) and p-values. The correlation between IgG and log-transformed NA was analyzed using Spearman's correlation by two-tailed parametric t-test means with 95% CIs.

All data analyses were performed with the SAS 9.4 software (Cary, NC, United States). Scatter plots of log-transformed IgG and NA were obtained using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). A p-value of less than 0.05 was considered as the cut-off for statistical significance.

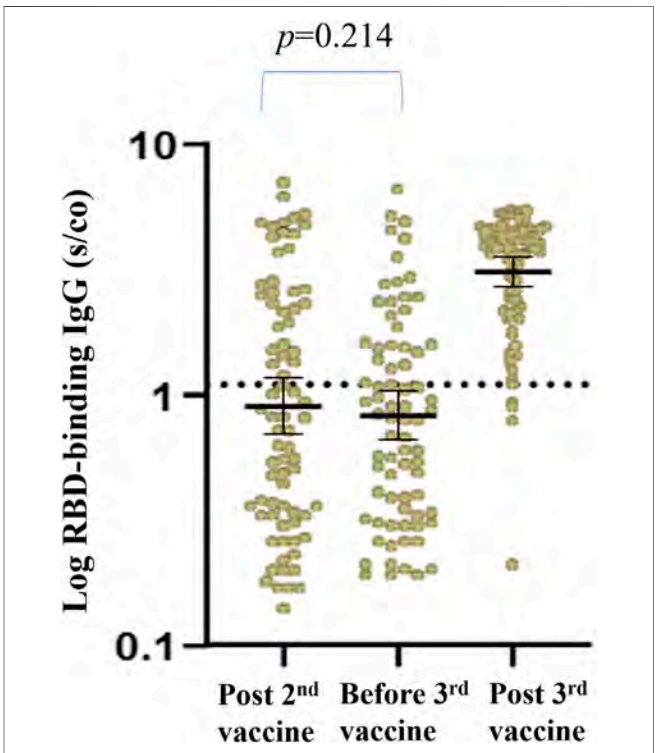


FIGURE 1 | Geometrical Mean (GM) of RBD IgG Antibody levels post second vaccine, on third vaccine date and post third vaccine.

RESULTS

Cohort Characteristics

Our study cohort comprised 99 RTR who received a third homologous booster dose of the BNT162b2 mRNA vaccine. Median age was 66 years (IQR, 53–73); 74 (74.7%) were males; and median body mass index (BMI) was 26.9 kg/m^2 (IQR,

TABLE 4 | Univariate Analysis for immune status before the third vaccine vs. post third vaccine in RTR.

	Before 3rd vaccine (N = 99)	Post 3rd vaccine (N = 99)	*p* value
All cohort			
IgG-RBD GMT (95% CI)	0.79 (0.65–0.96)	3.08 (2.76–3.45)	<**0.0001****
NA GMT (95% CI)	17.46 (12.38–24.62)	362.2 (220.7–594.6)	<**0.0001****
Positive responders			
n (%)	32 (32.3)	85 (85.9)	<**0.0001****
IgG-RBD GMT (95% CI)	2.53 (2.07–3.11)	3.57 (3.28–3.88)	<**0.0001****
NA GMT (95% CI)	89.12 (53.03–149.8)	689.9 (456.3–1043)	<**0.0001****
Negative responders			
n (%)	67 (67.7)	14 (14.14)	<**0.0001****
IgG-RBD GMT (95% CI)	0.45 (0.39–0.52)	1.28 (0.87–1.86)	**0.001***
NA GMT (95% CI)	8.01 (5.92–10.84)	7.25 (2.42–21.71)	0.85

Abbreviations: CI, confidence intervals; GMT, geometric mean titer; NA, neutralizing antibodies; RBD, receptor-binding domain.
** <0.05, ** <0.001.*
When p *value is significant, below 0.05 or below 0.01 the values are bolded.*

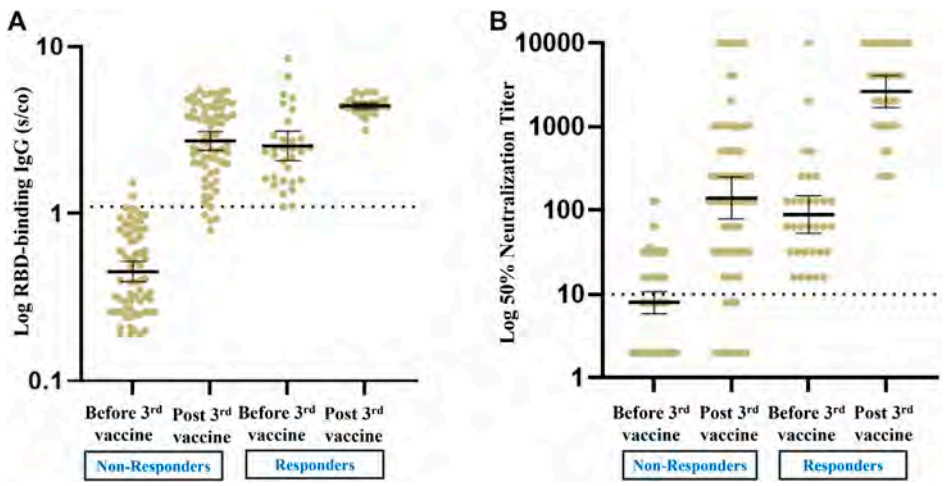


FIGURE 2 | Antibody response pre and post third vaccine in RTR with a positive versus negative humoral response before the third vaccine. **(A)** Geometrical Mean (GM) of RBD IgG Antibody levels. **(B)** Neutralizing Antibody levels.

23.2–31.1). Among the 99 RTR, for whom median time from transplant was 3.4 years, 81.1% had received a living donor transplant, and 69.7% had undergone pre-transplant dialysis, with median pre-transplant dialysis time being 0.6 years (IQR, 0–1.5). As shown in **Table 1**, 74.7%, 37.4%, 10.1% and 10.1% had a past medical history of hypertension, diabetes, ischemic heart disease, and congestive heart failure, respectively. 45.5% of the patients received the three-drug immunosuppression regimen of tacrolimus-MPA-prednisone, while 22.2% of the patients were treated only with tacrolimus and MPA (**Table 2**). Overall 93.4% of RTR were treated with a calcineurin inhibitor (87.9% with tacrolimus and 6.06% with cyclosporine), 76.8% with MPA, and 74.75% with prednisone.

Median time from the third vaccine to antibody testing was 21 days (IQR, 21–21). Ninety-four (94.95%) of the recipients had RBD IgG titers ≥1.1. Nine of the 94 recipients testing positive for RBD IgG nonetheless exhibited a low mean RBD IgG titer of 1.89 and did not develop NA; these patients were therefore considered as non-responders. Based on the two criteria—RBD IgG and NA—our RTR cohort included 85 patients (85.9%) in the positive response group (RBD IgG ≥1.1 and NA ≥ 16) and 14 (14.14%) in the negative response group (RBD IgG < 1.1 or NA < 16).

Univariate Comparison of Positive vs. Negative Response Groups

RTR who responded to the booster dose were younger, with a median age of 63 years (IQR, 52–75), as opposed to 71.5 years (IQR, 68–74) in non-responders ($p = 0.008$). The rate of end stage

TABLE 5 | Univariate and Multivariate Stepwise Logistic Regression Analysis for third vaccine Positive Antibody Response in RTR.

Effect	Univariate logistic regression		Stepwise logistic regression	
	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value
Age >65 vs. < 65	0.06 (0.01–0.47)	0.008	0.06 (0.00–0.88)	**0.04***
Gender F vs. M	0.82 (0.23–2.89)	0.76	0.56 (0.07–4.52)	0.59
Time from transplant to 3rd vaccine, years	1.08 (0.95–1.23)	0.22	1.16 (0.96–1.4)	0.13
Time from 2nd to 3rd vaccine, days	0.98 (0.93–1.03)	0.43	1.01 (0.97–1.06)	0.55
Time from 3rd vaccine to antibody testing, days	0.94 (0.85–1.04)	0.22	1.02 (0.87–1.20)	0.81
ESRD secondary to DN yes/no	0.26 (0.08–0.88)	0.03	0.11 (0.02–0.74)	**0.02***
eGFR (for every increase in 1 ml/min)	1.04 (1.01–1.08)	0.01	1.05 (1.00–1.09)	**0.04***
Glucose per 1 mg/dl increase	0.99 (0.98–1.00)	0.14	. . ..	. . ..
Globulins per 1 mg/dl increase	5.56 (0.99–31.2)	0.05	7.56 (0.77–74.5)	0.08
MPA use yes/no	0.22 (0.03–1.78)	0.16	0.09 (0.01–1.12)	0.06

AbbreviationsDN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; ESRD, end stage renal disease, MPA, mycophenolic acid.
[a]*<0.05.*
When p *value is significant, below 0.05 or below 0.01 the values are bolded.*

TABLE 6 | Local and Systemic Adverse Events (AEs) Reported after the third booster dose of BNT162b2 Vaccine Stratified by Antibody Response.

AEs	Total cohort (N = 97)	Negative (N = 13)	Positive (N = 84)	p value
Local AEs, n (%)				
Pain at injection site	50 (51.5)	5 (38.5)	45 (53.6)	0.31
Swelling	9 (9.3)	2 (15.4)	7 (8.3)	0.41
Redness	10 (10.3)	1 (7.7)	9 (10.7)	0.74
Systemic AEs, n (%)				
Fever	4 (4.1)	0 (0)	4 (4.8)	0.42
Fatigue	31 (31.96)	2 (15.4)	29 (34.5)	0.17
Headache	17 (17.5)	0 (0)	17 (20.2)	0.07
Myalgia	17 (17.5)	0 (0)	17 (20.2)	0.07
Chills	3 (3.1)	1 (7.7)	2 (2.4)	0.3
Nausea/vomiting	3 (3.1)	0 (0)	3 (3.6)	0.49
Paresthesia	2 (2.1)	0 (0)	2 (2.4)	0.57
Any local AE, n (%)	53 (54.6)	6 (46.2)	47 (56)	0.51
Any sytemic AE, n (%)	40 (41.2)	2 (15.4)	38 (45.2)	**0.04***
Any AE, n (%)	68 (70.1)	7 (53.6)	61 (72.6)	0.17

[a]*<0.05.*
When p *value is significant, below 0.05 or below 0.01 the values are bolded.*

renal disease (ESRD) secondary to diabetic nephropathy was significantly lower in the positive vs. the negative response groups (16.5% vs. 42.9%, respectively, $p = 0.02$). Average glucose blood levels in the month before the third vaccine was lower in the responders than in the non-responders, with a p value approaching significance ($p = 0.057$). Renal allograft function was significantly higher in the positive vs. the negative response group [median estimated glomerular filtration rate (eGFR) of 67.9 ml/min, IQR (54–83.6) and 46.6 ml/min, IQR (37.4–53.7), respectively, $p = 0.008$). For all other demographic, clinical and laboratory variables, the differences between the groups were not significant (**Table 1**).

A lower use of MPA was demonstrated for patients with a positive antibody response (74.1% for responders vs. 92.9% for non-responders, with a non-significant p value of 0.12). The total daily dose and daily dose per kg weight of tacrolimus, MPA and prednisone were not significantly different between the responders and the non-responders. The antibody responses were similar for the positive and negative response groups for the different immunosuppressive regimens administered, including the triple regimen containing MPA and double regimen of tacrolimus and prednisone (**Table 2**).

The differences in the humoral response between the positive and negative responders to the third vaccine dose is shown in **Table 3** (which also shows the humoral response to the second vaccine and prior to the third vaccine).

Response to the Second Vaccine Dose vs. the Third Booster Dose of the BNT162b2 mRNA Vaccine in RTR

Of the 76 patients for whom RBD-IgG was assessed 1 month after the second vaccine dose [median of 25 days, IQR (18–42.5)], 32 (42.1%) had RBD IgG titers ≥1.1 with a GMT of 2.82 (95% CI, 2.35–3.39). At a median time of 175 days (IQR, 171–178) from the second vaccine, a third booster dose was administered, and all 99 RTR were tested for RBD IgG and NA immediately before the third vaccine dose was given. Based on the above two criteria (RBD-IgG and NA) for a positive vs. a negative response, 32 (32.3%) of the RTR had a positive response before the third vaccine, with a GMT for RBD IgG of 2.53 (95% CI, 2.07–3.11) and a NA GMT of 89.12 (95% CI, 53.03–149.8). The GMT for RBD IgG after the second vaccine dose was not significantly different from that observed before the third vaccine (**Figure 1**). Therefore we compared between the humoral response before and after the third dose in our total cohort of 99 RTR. The humoral response was assessed 3 weeks after the third booster dose [median time of 21 days, IQR (21–21)]. The positive response rate based on RBD IgG and NA titers had increased from 32.3% before the vaccine to 85.9% (85/99) after the third vaccine dose, with RBD IgG and NA GMTs of 3.57 (95% CI, 3.28–3.88) and 689.9 (95% CI, 456.3–1043), respectively. Both the rate and the intensity of

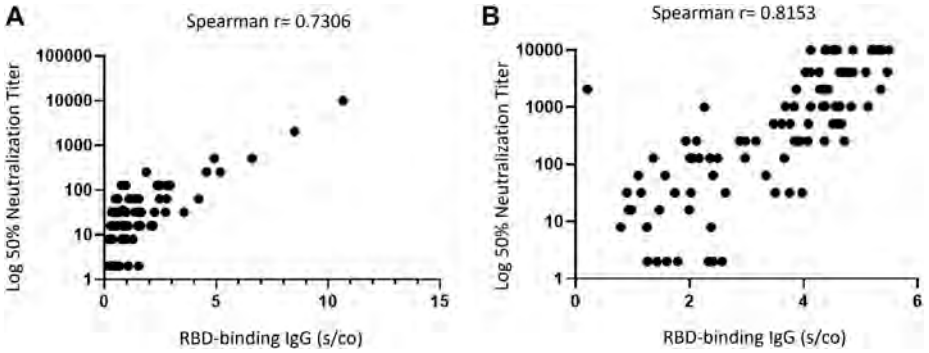


FIGURE 3 | Correlation between RBD IgG and Neutralizing Antibodies in RTR. **(A)** On third vaccine date. **(B)** Post third vaccine. Each Dot Represents a Combined IgG-RBD and Neutralizing Antibodies Result for One Participant.

response to the third booster dose were significantly higher than those observed before the booster dose (**Table 4**).

Of the 32 recipients with a positive humoral response prior to the third booster dose of the vaccine, 31 (96.9%) remained positive after the third vaccine, with a significant increase in GMTs for RBD IgG and NA. Sixty seven patients (67.7%) had a blunted antibody response before the third vaccine; among these, 54 (80.6%) exhibited a positive antibody response following the booster dose, with a significant increase in GMTs for RBD IgG NA (**Figure 2**).

Multivariable Logistic Regression for Positive Antibody Response

Multivariable logistic regression analysis found that the likelihood for a positive response decreased by 94% in RTR above 65 years of age vs. below that age (OR = 0.06, 95% CI 0.00–0.88, $p = 0.04$). For every 1 ml/min increase in eGFR the odds for a positive response increased by 5% (OR = 1.05, 95% CI 1.00–1.09, $p = 0.04$). ESRD secondary to diabetic nephropathy was also found to be an independent predictor for antibody response (OR = 0.11, 95% CI 0.02–0.74, $p = 0.02$) (**Table 5**).

Adverse Events

Adverse events were common, being recorded for 70.1% of the RTR cohort. Local and systemic adverse events were reported in 54.6% and 41.2% of the cohort, respectively. Pain at the injection site was the most frequent local adverse event, being experienced in 50 (51.5%) recipients following the third vaccine dose. Systemic adverse events, mainly fatigue, were reported for 31 (31.96%) of RTRs, with headache and myalgia being experienced only by the positive responders. Recipients with a positive humoral response following the third booster dose were more likely to experience systemic adverse events than non-responders (45.2% vs. 15.4% respectively, $p = 0.04$). No other differences in the prevalence of local or specific systemic adverse events were found between the responders and the non-responders (**Table 6**). No episodes of rejection were observed, and renal allograft function remained stable at a mean follow up of 60 days following the third vaccine dose. No allergic responses were documented.

DISCUSSION

The humoral response (both rate and intensity) to the third homologous booster dose of BNT162b2 vaccine was found to be significantly higher than that observed following the full two-dose vaccination and the baseline immune status prior to the third vaccine. RTR with a positive, as opposed to a negative antibody response were younger and were characterized by a lower prevalence of ESRD secondary to diabetic nephropathy, lower glucose in the 1 month prior to the vaccine, better renal allograft function, and a lower use of MPA. A multivariable model adjusted for age, sex, and times from transplant to the third vaccine dose, from second to third dose, and from third dose to serology assessment revealed that age, ESRD secondary to diabetic nephropathy, and renal allograft function are independent predictors for the humoral response to the third booster dose. The booster vaccination of RTR with the BNT162b2 vaccine was associated with a high rate of adverse events, with the most prevalent adverse event being pain at the injection site. The prevalence of systemic adverse events, mostly fatigue, but also fever, headache, myalgia, chills, nausea/vomiting, and paresthesia was higher in recipients with a positive (compared to a negative) antibody response.

The few studies on the humoral response to a booster vaccine dose in transplant recipients have reported conversion rates of 49–70%, as follows. Of 101 SOT recipients given three doses of the BNT162b2 vaccine, the response rate increased from 40% before the third dose to 68% 4 weeks after the third vaccine, but only 44% of seronegative patients seroconverted following the third dose (15). In 30 SOT recipients, antibody titers increased after the third dose in all the patients with low positive antibody titers after the first two doses but in only one quarter of patients (6/24) with negative antibody titers (16). A third dose of mRNA-1273 vaccine induced neutralizing antibody positivity in 60% of SOT recipients compared to only 25% of the placebo group (17).

A study of the humoral response to a third dose of the mRNA-1273 SARS-CoV-2 vaccine in 159 RTR with a minimal response to the full vaccine showed that the overall response rate to the booster dose was 49%, with a higher response rate in those with a weak compared to a negative response following the second vaccine (81.3% vs. 27.4% respectively) (18). In 71 RTR homologously vaccinated with the BNT162b2 there was an increase in the serological conversion rate from about 50% after the second dose to about 70% 1 month after the third dose (19). In a recent study, 10 RTR who had failed to respond to a second dose of the BNT162b2 vaccine received a third dose of the mRNA-1273 vaccine, which induced humoral and cellular responses in 60% and 90% of the patients, respectively (20). By analyzing both antibody and neutralizing levels, we observed a strong response to the third, booster dose, with an increase in the positive response rate from 32% before the third dose to 85.9% thereafter. In addition, in our cohort the booster dose elicited a strong and effective humoral response in RTR who were either seropositive or seronegative before the administration of the booster: 80.6% of the recipients who had not responded to two doses of the vaccine became seropositive following the third dose, and the intensity of the humoral response largely improved in those who were seropositive prior to the vaccine (**Figure 2**). The differences between studies observed in humoral response following a third dose in RTR could stem from different characteristics of the cohorts as well as differences in sensitivity of testing assays used. Nevertheless, the advantages of a third dose to RTR are clear.

The importance of assessing NA is that they show an antibody functionality that encompasses both the quantity and the affinity of the IgG antibodies. The NA assay is considered the gold standard antibody assay for antibodies, and for SARS-CoV-2, it appears to be the *in-vitro* assay most closely correlated with protection (21). Indeed, a correlation between the level of NA to the SARS-CoV-2 spike protein and symptomatic disease was observed (22). Presence of NA to SARS-CoV-2 post natural infection has been shown to provide protection from asymptomatic and symptomatic reinfection (23). In addition, and despite the high correlation observed between RBD IgG and NA before and after the third vaccine dose (**Figure 3**), a substantial number of the RTR in our cohort with positive RBD IgG did not exhibit adequate neutralization activity and were therefore considered as negative responders (2% and 9.1% recipients before and after the third dose, respectively). The use of NA is therefore crucial in the assessment of the humoral response to reduce false positive results, which could make patients wrongly believe they are protected from the infection.

The robust response observed in our cohort following the third booster dose is not surprising, given prior data linking vaccination strategies with higher, additional and booster doses to superior immunogenicity responses in immunocompromised populations (24–28). Of note, although some types of immunosuppressive therapy, especially the use of MPA, were found to be major suppressors of the antibody response following the first and second vaccine doses, in our cohort MPA treatment did not significantly impact the ability to mount a humoral response after the third booster dose. MPA specifically blocks the proliferation of B and T lymphocytes via the inhibition of inosine-5-monophosphate dehydrogenase, thereby suppressing cell-mediated and humoral immune responses (29, 30). Despite the reduced antibody titers in RTR, cellular immune responses have been documented at considerable rate, even in seronegative vaccinated patients (4, 31). It is possible that for patients receiving immunosuppressive therapy antigenic re-exposure with a higher total antigen load, as achieved in natural infection, is needed to trigger and expand the reduced immune response to previous antigenic exposures.

In prior publications, older recipient age and a lower eGFR were associated with a negative response to the third booster dose (15, 19). Interestingly, we found that ESRD secondary to diabetic nephropathy is predictive for a blunted immune response to the third dose. This finding may probably be attributed to the direct effects of hyperglycemia and insulin resistance, causing an immune-compromised state in this population, as is manifested by dysregulation of both the innate and adaptive immune responses in people with diabetes (32, 33).

We found the third booster dose of the BNT162b2 mRNA vaccine to be safe. Although the prevalence of adverse effects was higher than that observed in our RTR following the first and second BNT162b2 doses (7), no serious adverse effects were reported. The high rate of adverse effects in our cohort, with an increased prevalence of systemic adverse effects in the positive responders, reflects an immune system activation post vaccine exposure in RTR capable of mounting an effective humoral response to the vaccine.

Certain limitations should be taken into consideration in interpreting our results. The study is not an efficacy trial (there is no control group), but NA have been demonstrated to have a significant correlation with protection from SARS-CoV-2. The implications of our findings are limited by the small number of patients and the short follow-up period after vaccination. Antibodies may wane over time, and the half-life of the neutralizing response cannot be predicted. Furthermore, cellular immunity was not assessed.

The above notwithstanding, our results are encouraging, given the high rate of seroconversion and the impressive response in previously seropositive patients. Based on our data, we believe that a third booster dose is essential for transplant recipients, irrespective of seronegativity/seropositivity prior to the vaccine, to achieve neutralization antibody activity and a higher degree of protection from COVID-19 infection. Despite the high response rate, it is likely that the booster vaccine-induced immunity is lower in RTR and other immunocompromised patients than in immunocompetent individuals. In a significant number of RTR, antibody titers following the third vaccine may be low or not associated with neutralization and protection. Therefore, we should not get caught up in complacency and keep searching for other strategies to improve patient protection. It is crucial that we continue to promote social distancing and masking as well as full vaccination of all transplant recipients, household members, and caregivers to provide a ring of protection for our immunocompromised patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available upon reasonable request and not without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the protocol and informed consent were approved by our Institutional Review Board (8314-21-SMC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TH: conception and design, data acquisition, data interpretation, writing; AB-D: data acquisition; LO: data analysis; NS: data acquisition; RG: drafting; EM: revising; IL: conception and design; VI: data acquisition; YL: conception and design, data interpretation; EG: revising; GR: conception and design, data interpretation, revising.

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
published: 31 March 2022
doi: 10.3389/ti.2022.10109

Initial Experience With SARS-CoV-2-Neutralizing Monoclonal Antibodies in Kidney or Combined Kidney-Pancreas Transplant Recipients

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Background: Antiviral drugs have shown little impact in patient infected with acute respiratory coronavirus 2 (SARS-CoV-2). Especially for immunocompromised persons positive for SARS-CoV-2, novel treatments are warranted. Recently, the U.S. FDA has granted an emergency use authorization (EUA) to two monoclonal antibodies (mAb) targeting the viral spike protein: bamlanivimab and casivirimab and imdevimab. As per the EUA, all SARS-CoV-2 positive organ transplant recipients can receive mAb treatment.

Patients and methods: We queried our center's transplant registry to identify SARS-CoV-2 infected recipients treated with single doses of either Bamlanivimab or casivirimab/imdevimab up to May 31, 2021. We analyzed clinical outcomes, renal function and virus-specific antibodies. The co-primary endpoints were hospitalization due to COVID-19 and SARS-CoV-2 RT-PCR negativity.

Results: Thirteen patients at a median interval of 55 (IQR, 26-110) months from transplant were treated: 8 with bamlanivimab and 5 with casivirimab/imdevimab. In all, 4/13 (31%) patients were hospitalized at some time, while 11/13 (85%) achieved PCR negativity. 2/4 hospitalized patients received mAb as rescue treatment. Overall mortality was 23%, with one death attributable to transplant-associated lymphoma. All six patients infected with the B 1.1.7 variant were alive at last contact. *Conclusion*: mAb treatment appears effective when administered early to SARS-CoV-2-infected transplant recipients.

Keywords: SARS-CoV-2, kidney transplantation, immunosuppression, bamlanivimab, monoclonal antibodies

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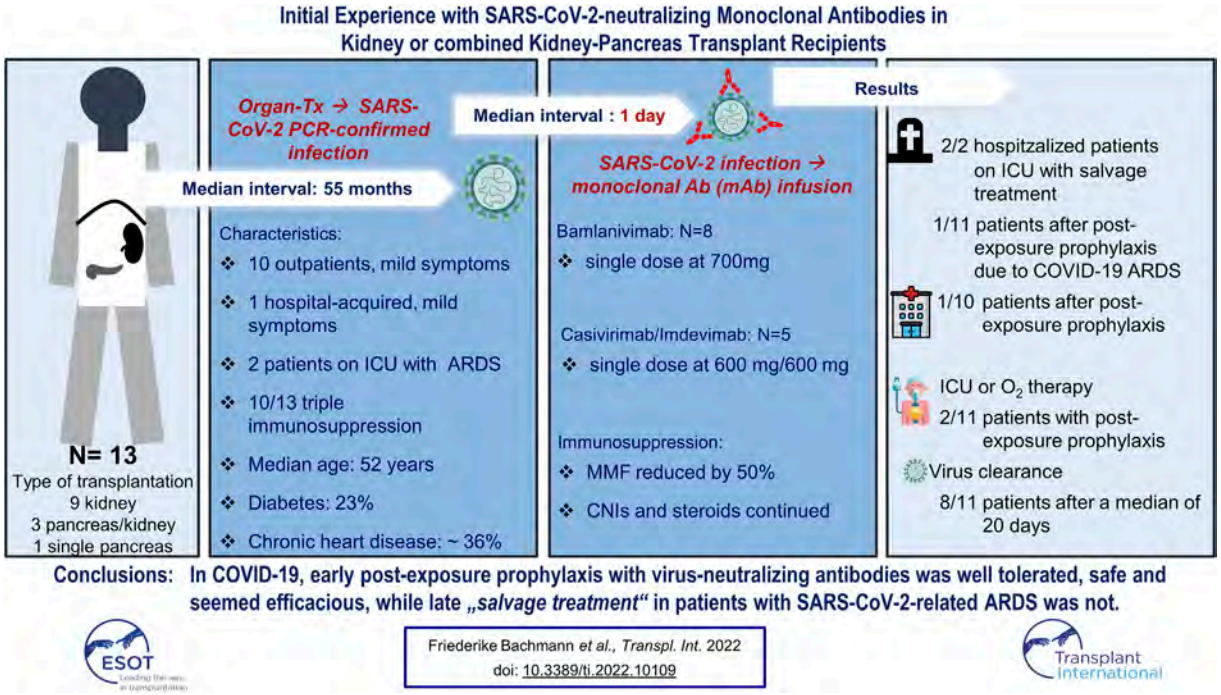
Received: *13 October 2021*
Accepted: *08 March 2022*
Published: *31 March 2022*

Citation:
Bachmann F, Budde K, Suttorp N, Lingscheid T, Stegemann MS, Osmanodja B, Schrezenmeier E, Duettmann W, Weber U, Naik M, Lehner LJ, Kahl A, Duerr M, Eckardt K-U, Waiser J, Choi M and Halleck F (2022) Initial Experience With SARS-CoV-2-Neutralizing Monoclonal Antibodies in Kidney or Combined Kidney-Pancreas Transplant Recipients. Transpl Int 35:10109. doi: 10.3389/ti.2022.10109



GRAPHICAL ABSTRACT |

BACKGROUND

Until now, antiviral drugs have largely failed to improve the natural course of coronavirus disease 19 (COVID-19) (1). In fact, mainly dexamethasone had positive effects on patients severely affected by SARS-CoV-2 (2,3). Therefore, antibody treatments (4,5) attracted strong interest prior to the availability of vaccines in order to ameliorate disease severity in subjects with proven infection. In addition to convalescent plasma (6), also monoclonal and "off-the-shelf" preparations of neutralizing intravenous immunoglobulins may be effective in fighting SARS-CoV-2 infection (7). Convalescent plasma might positively impact intensive care unit (ICU) admission and mortality rates (6). However, these preparations are difficult to standardize and a drug containing high amounts of a well-defined epitope-specific antibody might be superior and exert more reliable efficacy. In fact, two compounds have been granted an emergency use authorization (EUA) by the U.S. FDA for mild to moderate COVID-19 since November 2020 (8,9). In Germany both compounds are provided for unlicensed use since January 2021 due to pending approval in Europe.

Bamlanivimab, one of these two compounds, constitutes a neutralizing IgG1 monoclonal antibody (mAb) binding to the receptor binding domain (RBD) of the spike protein of SARS-CoV-2 (9). Bamlanivimab was shown to accelerate elimination of SARS-CoV-2 and, more importantly, reduced probability of emergency department visits by approximately 75% when given to outpatients (7). Casivirimab and imdevimab are two neutralizing mAbs targeting two different epitopes of the RBD (10). They are administered together as an "antibody cocktail" with a single dose in subjects who are at high risk for developing severe COVID-19 (5,11). The combined phase 1–3 trial met its key efficacy endpoints, in that viral load over time was significantly reduced and medically attended visits were less frequent in patients receiving any dose of casivirimab/imdevimab (11).

Chronic kidney disease (CKD) is associated with a higher risk of in-hospital mortality in COVID-19 patients than, for instance, malignancy or cardiac comorbidities, as demonstrated in several large studies (12,13). Overall mortality in hospitalized CKD patients was 26% (12). While many CKD patients are of advanced age with age being another major risk factor for poor outcomes, kidney transplantation constitutes a further risk factor for SARS-CoV-2 infected individuals (14,15). Besides immunosuppression, graft function was inversely correlated with COVID-19 disease severity (16). Because immunosuppression may suppress adequate production of protective antibodies, the use of external antibody preparations may be of higher effectivity in kidney transplant recipients, known to be at high risk for poor outcomes. Until now, experience with neutralizing mAbs in COVID-19 kidney transplant recipients under a defined immunosuppression is very limited. In a recent single-center, retrospective study, bamlanivimab reduced the hospitalization rate in a cohort where approximately 30% of patients were of immunosuppression. Of note, the authors estimated the number needed to treat (in order to prevent one hospitalization) to be 8 (17). To the best of our knowledge,

only one case series from outside the U.S. (18). has been reported so far.

PATIENTS AND METHODS

We queried our electronic patient database "TBase" (19) for the search terms "SARS-CoV-2," "COVID-19" and "bamlanivimab" or "casivirimab" or "imdevimab." Retrieved records were manually reviewed for all patients receiving treatment with mAbs preparation "bamlanivimab" or "casivirimab/imdevimab" until May 31, 2021. All patients had a minimum follow-up of 14 days after mAb infusion, the last of which was administered on May 22, 2021.

According to indication, solid organ transplant (SOT) recipients with a positive SARS-CoV-2 PCR were treated with 700 mg bamlanivimab or 1200 mg casivirimab/imdevimab. The decision to treat was made by a multidisciplinary team consisting of nephrologists, transplant physicians and infectious disease specialists. We collected clinical outcomes, medication history, laboratory results for infection parameters, renal function tests, blood count, and anti-SARS-CoV-2 serum antibodies (IgA and IgG using Euroimmun–ELISA, Lübeck, Germany). All patient samples from the current series were analyzed using NGS-based sequencing of viral genomes as described previously (20). Methods differed based on viral load in patients' nasal swab. Briefly, libraries were established using the KAPA RNA Hyper Prep kit (high viral load) or a PCR amplicon-based sequencing approach.

Imaging results were used to assess pulmonary infiltration patterns in patients with dyspnea. Telemedicine support, which has been in use for our KTR recipients for about 1 year, was offered to all patients following mAb treatment and comprised symptom-reporting, remote vital sign monitoring, medication intake and chat functioning (21). The co-primary endpoints were hospitalization due to COVID-19 by day 29 (11) and viral response, respectively. The latter was defined as a SARS-CoV-2 RT-PCR negative (swab) test on day 11 (+/− 2 days) after mAb administration. Sustained response was defined as an ongoing negative PCR negativity at further testing. In contrast to mAb pivotal studies, we used dichotomized PCR test results rather than log reduction of viral load. We additionally defined a composite secondary endpoint which was defined by admission to ICU, any form of ventilation, or death. Disease-specific survival was calculated from first positive SARS-CoV-2 PCR test until death from any cause or last follow-up. Cause of death for deceased patients was attributed to COVID-19 if their last PCR was positive and/or they met criteria for severe COVID-19 at last follow-up and/or they died due to pulmonary involvement of a previously documented SARS-CoV-2 infection.

RESULTS

In total, thirteen organ transplant recipients were identified and included in this report. The median age was 52 (IQR 42-60) years. Baseline demographics are shown in **Tables 1**, **2**. Patients were diagnosed with SARS-CoV-2 infection at a median of 55 months (IQR 26-110 months) after organ transplantation. None of the patients was fully vaccinated when testing positive. In six of all sequenced samples (46.1% of all patients), the B.1.1.7 lineage of SARS-CoV-2 virus was confirmed at baseline. At the time of diagnosis, 10 (76.9%) patients received triple immunosuppression consisting of a calcineurin inhibitor (CNI; tacrolimus or cyclosporine A), mycophenolic acid (MPA), and corticosteroids. Three (23.1%) patients were on a steroid-free regimen. Three patients were diabetic, and four patients had chronic cardiac disease. Four (30.7%) patients received telemedical care. Median interval from diagnosis confirmed by positive SARS-CoV-2 PCR test to administration of either mAb treatment was 1 day (range, 1–30) and median follow-up 40 days (IQR 23–50 days) (**Table 1**). Bamlanivimab was administered to the first eight patients, including all three hospitalized patients. When casivirimab/imdevimab became available at our center, we switched mAb treatment to this compound. Both antibody preparations were well tolerated and we did not observe any infusion-related reactions.

TABLE 1 | Patients' characteristics.

Variable	
Median age, years (IQR)	52 (42–60)
Male gender, N (%)	8 (61.5)
Kidney transplant, N (%)	9 (69.23.6)
Kidney-pancreas; single pancreas transplant, N (%)	3(23.1); 1(7.7)
Living donor, N (%)	5 (38.5)
BMI, median (range)	23 (16–32)
Diabetes, N (%)	3 (23.1)
Chronic heart disease, N (%)	4 (36.3)
Median interval SOT to SARS-CoV-2 positivity, months (range)	55 (26–125)
C-reactive protein (mg/L), median (range) at diagnosis	7 (2–106)
Lactate dehydrogenase (U/l), median (range) at diagnosis	246 (192–511)
Serum interleukin-6 (pg/ml), median (range) at diagnosis	14 (1.6–80.6)
Interval positive PCR—administration of MoAb, days (range)	1 (1–30)
Oxygen supplementation, N (%)	3 (23.1)
ICU admission, N (%)	3 (23.1)
COVID-19 symptoms at treatment: moderate/severe, N (%)	3 (23.1)
Median duration of follow-up, days (IQR)	40 (23–50)
Patients alive at last follow-up, N (%)	10 (76.9)
Renal replacement therapy, N (%)	3 (15.4)

For two of three patients (**Table 2**: patients 12 and 13) who were already hospitalized and had received other treatments, including dexamethasone and convalescent plasma, mAb administration was delivered as a rescue treatment. Therefore, MMF was discontinued and tacrolimus dosage was adjusted to trough levels of 4–6 ng/ml. One of these two patients had refractory PTLD/acute lymphoblastic leukemia with leukemic meningitis. Both patients had acute kidney injury requiring dialysis. They died on day 45 and day 60, respectively, after diagnosis of intractable COVID-19 with virus persistence until death.

Another hospitalized patient (**Table 2**: patient 11) developed nosocomial COVID-19 infection during treatment of peripheral vascular disease. MMF was discontinued. He was treated in-line with EUA on day 1 after diagnosis with moderate symptoms. RT PCR became negative on day 24 after diagnosis with his further clinical course being dominated by vascular disease-associated complications. Renal function was stable (on day 54 after diagnosis) and remained unaltered after discharge.

TABLE 2 | Outcomes.

Pat. No.	Gender	Donor Type	Immuno-suppression at COVID-19 dx	Interval transplant-SARS-CoV-2 positivity PCR (months)	Interval diagnosis to mAB treatment (days)	Variant of concern	Anti-SARS-CoV-2 S1 IgG/IgA antibodies baseline/course of the disease	Oxygen supplementation due to COVID-19	ICU admission/ ventilation type due to COVID-19	Response/ Sustained viral response	Survival status
1	M	Deceased donor	Tac, MMF, steroid	171	2 days	no	Positive on day 14 after diagnosis	yes	Yes; mechanical ventilation	y/n	Deceased on day 57 after Dx[b]
2	M	Deceased donor	Tac, MMF, steroid	45	1 day	no	Positive on day 83 after diagnosis	no	No	y/y	Alive, stable renal function
3	M	AB0i kidney	Tac, MMF	64	1 day	B.1.1.7	Positive on day 21 after diagnosis	no	no	y	Alive, stable renal function
4	F	Deceased donor	Tac, MMF	55	2 days	B.1.1.7	Positive on day 88	no	no	y	Alive, stable renal function
5	M	Kidney-pancreas	CyA, SRL, MMF	276	2 days	B.1.1.7	Positive on day 109 after diagnosis	no	no	y	Alive, stable renal function
6	F	Kidney-pancreas	CyA, MMF, Pred	239	1 day	no	Positive on day 105 after diagnosis	no	no	y	Alive, stable renal function
7	F	Kidney-pancreas	CyA, MMF, steroid	15	1 day	no	Positive on day 43	no	no	y/n.a.	Alive, stable renal function
8	F	Living donor	Tac, MMF, steroid	1	1 day	B.1.1.7	Positive on day 14 after Dx	no	no	y/y	Alive, stable renal function
9	M	AB0i kidney	Tac, MMF, steroid	18	1 day	B.1.1.7	Positive on day 88	no	no	y/n.a.	Alive, stable renal function
10	F	AB0i kidney	Tac, MMF, steroid	45	1 day	B.1.1.7	Pos on day 14 after Dx	no	No	y/y	Alive, stable renal function
11	M	Single pancreas	Tac, MMF, steroid	59	Nosocomial infection 1day	no	Negative on day 14, positive on day 78	no	no	y/y	Alive, discharged[a] on day 54 after Dx, stable renal/ pancreas function
12[d]	M	Deceased donor	Tac, MMF, steroid	26	15 days	no	Pos on day 13	yes	Yes; mechanical ventilation	no/no	Deceased on day 45
13[d]	M	Living donor	Tac, steroid	82	30 days	no	Pos on day 30	no	no	no/no	deceased on day 60 due to progressive PTLD[c]

Abbreviations: COVID, Coronavirusdisease 2019; mAb, monoclonal antibody; M, male; F, female; Tac, tacrolimus; CyA, cyclosporine A; MMF, mycophenolic acid; Dx, diagnosis; n.d., not done; IgA, Immunglobulin A; IgG, Immunglobulin G; S1, Spike antigen; ICU, intensive care uni; PCR, polymerase chain reaction.

[a]*Prolonged inpatient care due to arterial occlusion.*

[b]*Became negative on day 19 and turned positive on day 27.*

[c]*Duration positivity 60 days until death.*

[d]*mAB, were administered as rescue therapy.*

All remaining 10 patients received mABs during outpatient care with mild symptoms and according to the respective notice of the general ruling by the Federal Ministry of Health (Bundesgesundheitsministerium, BMG). Of note, none of the six carriers of the B.1.1.7 variant needed inpatient care or oxygen supplementation. In addition to standard PCR tests, six patients were tested for the presence of pre-infusion anti-SARS-CoV-2 antibodies. All six were seronegative. As patients were not treated within a prospective study, testing for serum antibodies was performed at discretion of the treating physician in charge.

Therefore, previous infection and preexisting immunity seems unlikely. MMF was reduced by 50% and steroids continued in patients on a steroid maintenance regimen. Despite continued immunosuppression following their organ transplants, 9/10 had an uneventful outpatient course and did not develop severe COVID-19 symptoms requiring hospitalization. SARS-CoV-2 PCR became negative in 6 patients after a median of 22 days (range 18–35). However, one patient with initial viral clearance had symptomatic disease recurrence, and multiple positive PCR tests later on. He was hospitalized on day 21 after diagnosis and died due to COVID-19 associated ARDS in the ICU on day 57 after initial disease onset.

In summary, two out of 11 patients (18%) with early antibody treatment reached the composite endpoint and were admitted to an ICU and required oxygen. With one patient dying due to Covid-19, mortality was still app. 10% in our series. This underscores an ongoing medical need in the severely immunocompromised. In this cohort, time to viral clearance occurred after a median of 20 days, and sustained viral clearance was achieved in 8 patients (73%). Testing for serum antibodies against one subunit of the spike protein (anti-S1 Ig A and IgG) revealed positive results for anti-S1 IgG in all patients after a median of 43 (range, 13–109) days.

DISCUSSION

Immunocompromised subjects with SARS-CoV-2 infection are prone to an unfavorable course of the disease with a 10-fold increased mortality risk. Pre-emptive administration of monoclonal, virus-neutralizing antibodies that have constant, defined and reproducible characteristics has shown to benefit subjects with mild symptoms from confirmed SARS-CoV-2 infection (7, 10 and 12). We treated 13 consecutive SOT recipients from our center. All but one patient who had received pre-emptive treatment with the mAbs are alive after a follow-up of 40 days. Only one of these 11 patients experienced recurrence of viral infection and eventually died from intractable COVID-19. This patient's fate leads to the speculation that, in light of the half-life of the mAbs (13 days for bamlanivimab and 13–18 days for casivirimab/indevimab) (22), a single dose might not be appropriate in severely immunocompromised patients or viral immune escape took place due to mAb-monotherapy and insufficient immune-response by the patient. Two severely ill patients received bamlanivimab at a relatively long interval from SARS-CoV-2 infection and died due to complications from underlying disease and refractory COVID-19. Findings for anti-S1 IgG showed positive results for all patients after a median of 43 days from mAb administrations. Especially positive results after app. 80 days from therapeutical antibody infusion suggest "true seroconversion" rather than remaining concentrations of bamlanivimab or casivirimab/imdevimab, respectively. The overall good outcome is particularly remarkable, since in 6 of 13 cases the B.1.1.7 lineage was found to be the infectious agent, which is associated with higher reproducibility and case fatality. All 6 subjects carrying the B.1.1.7 lineage had an uneventful course without need for oxygen or other interventions, suggest efficacy against this variant of concern. Another strength of our study is the fact that prior infection was ruled out for six patients assuming none of these having "natural" immunity. Our study has some limitations: first, the sample size is small. Second, in this exploratory pilot study we did not attempt comparison with a control group not receiving a monoclonal antibody. Third, allocation to any one of the mAbs was by availability and individual decision rather than randomization making any comparison impossible. The dynamic situation of the pandemic is mirrored by incoming virus variants which may escape from treatments established in earlier phases of the pandemic. Of note, early data of a novel compound, sotrovimab, indicate its efficacy also for the Omicron variant (23). Quickly evolving virus variants may pose a novel threat to communities by questioning established strategies in intervals as short as weeks. For instance, the Omicron type features a magnitude of mutations clustering in the receptor binding motif (RBM) leading to an immune escape not only to the first class of mAbs, but also to covalescent plasma and certain types of vaccinations. Interestingly, the non-RBM targeting second class of mAbs is still effectively neutralizing the Omicron variant as very recently shown by a Swiss group (24).

CONCLUSION

Our initial experience with neutralizing mABs for SOT recipients with confirmed SARS-CoV-2 infection shows excellent tolerability and suggests high efficacy including infections with the B.1.1.7 variant. We conclude that in a setting of rescue therapy no clear benefit can be documented, a finding which is in accordance with FDA emergency use authorization while early administration appears efficacious in prevention of severe COVID-19 in heavily immunosuppressed patients with mild symptoms. However, rates of overall and sustained PCR responses were low, suggesting a potential discordance between viral replication and clinical course and the need for continued surveillance.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

FB designed the study, analyzed patient-level data, contributed patients and wrote the manuscript draft. MN conducted and is responsible for all statistical analyses. FB, KB, MC, and FH wrote the

manuscript. WD, UW, LL, AK, and MD contributed patients. K-UE contributed patients and critically revised the manuscript. NS, TL and MS organized antibody supply and collected and analyzed virological data. All authors approved the final version of the manuscript.

FUNDING

ES was funded by the Federal Ministry of Education and Research (BMBF) grants (BCOVIT and 01KI20161) and is enrolled in the Charité Clinician Scientist Program funded by the Charité–Universitätsmedizin Berlin and the Berlin Institute of Heal.

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
published: 21 March 2022
doi: 10.3389/ti.2022.10127

SARS-CoV-2 Infection Can Lead to an Increase in Tacrolimus Levels in Renal Transplant Patients: A Cohort Study

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The aim of this study is to evaluate the effect of SARS-CoV-2 infection on serum tacrolimus levels. Tacrolimus levels of 34 transplant patients diagnosed with SARS-CoV-2 in 2020 were compared with their pre-infection values and those of a control group with alternative infections. 20 out of 34 (59%) had high levels. At diagnosis, median tacrolimus level in the SARS-CoV-2 cohort was 9.6 µg/L (2.7–23) compared to 7.9 µg/L in the control group (p = 0.07, 95% CI for difference –0.3–5.8). The ratio of post-infection to pre-infection tacrolimus values was higher in the SARS-CoV-2 group (1.7) compared to the control group (1.25, p = 0.018, 95% CI for difference 0.08–0.89). The acute kidney injury rate was 65% (13 of 20) in SARS-CoV-2 patients with a level >8 µg/dl, compared to 29% (4 of 14) in those with lower levels (p = 0.037). Median length of stay was 10 days among SARS-CoV-2 infected patients with high tacrolimus levels compared to 0 days in the rest (p = 0.04). Four patients with high levels died compared to 2 in the control group. Clinicians should be aware of this potential effect on tacrolimus levels and take appropriate measures.

Keywords: SARS-CoV-2, kidney transplantation, immunosuppression, tacrolimus, AKI, tacrolimus levels

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***Received:** 20 October 2021*
***Accepted:** 16 February 2022*
***Published:** 21 March 2022*

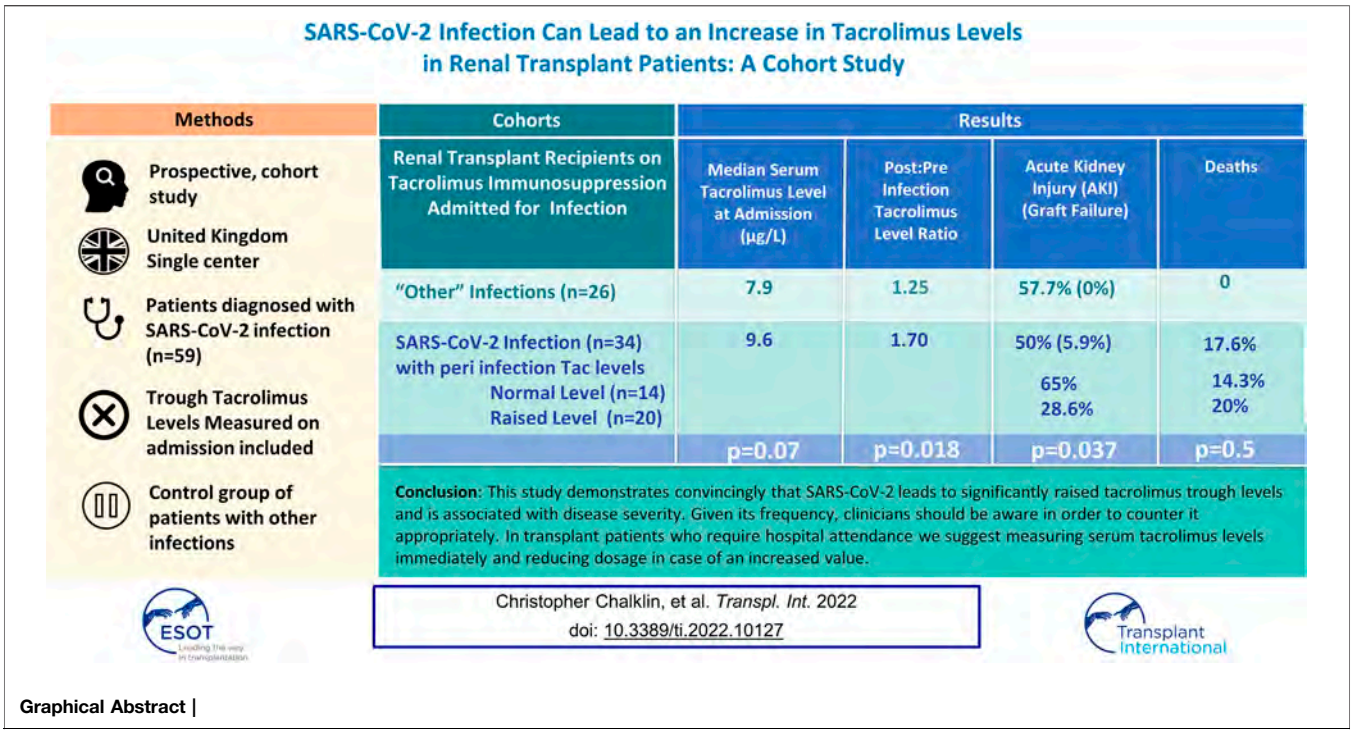
Citation:
Chalklin CG, Koimtzis G, Khalid U, Carrington-Windo E, Elker D and Asderakis A (2022) SARS-CoV-2 Infection Can Lead to an Increase in Tacrolimus Levels in Renal Transplant Patients: A Cohort Study. Transpl Int 35:10127. doi: 10.3389/ti.2022.10127

INTRODUCTION

Severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV-2) has emerged as worldwide pandemic (1). Risk factors for mortality include advanced age, chronic kidney disease, diabetes mellitus, obesity, cardiovascular disease, history of malignancy and chronic immunosuppression (1–5). As a result, transplant patients are more vulnerable with a higher mortality rate which differed amongst reports, prior to vaccination, between 11% and 50% (6–8).

British and American guidelines recommend immunosuppression modification during the treatment of SARS-CoV-2 infection in transplant patients (9, 10), mainly consisting of withdrawal of one or more immunosuppression drugs. Most commonly, antimetabolites such as mycophenolate derivatives are discontinued while other immunosuppressants such as calcineurin inhibitors are administered at a lower dose or occasionally stopped (6, 7). Some though, suggest that calcineurin inhibitors may also inhibit the replication of coronaviruses such as SARS-CoV-2 (11–13) although this is not the prevalent view currently.

Abbreviations: AKI, acute kidney injury; ARDS, acute respiratory distress syndrome; AUC, area under the curve; BMI, body mass index; COVID-19, Coronavirus disease 2019; MMF, mycophenolate mofetil; PCR, polymerase chain reaction; PgP, P-glycoprotein; RRT, renal replacement therapy; SARS-CoV-2, Severe acute respiratory distress syndrome coronavirus 2; SPK, simultaneous pancreas and kidney transplant.



Graphical Abstract |

Nevertheless, until now, there has been no data published on the effect that SARS-CoV-2 infection has on serum levels of immunosuppression medication.

During the "first wave" of the pandemic we observed a number of renal transplant patients with unusually high serum tacrolimus levels, therefore we set out to investigate if the presence of SARS-CoV-2 infection was associated with increased calcineurin inhibitor levels in the cohort of transplant patients affected by SARS-CoV-2 in Wales, and whether this had contributed to acute kidney injury (AKI) and patient outcome. We were also interested to see if this increase was more pronounced from the one seen in other acute inflammatory conditions or infections (14, 15).

MATERIALS AND METHODS

This is a cohort study performed by maintaining a prospective database of transplant patients cared for by the Cardiff Transplant Unit who were diagnosed with SARS-CoV-2 infection between 1st March 2020–31st December 2020 since, during this period, outcomes were not affected by vaccination. Transplant patients who presented to the emergency department in any of the South and Mid Wales hospitals or to the transplant telephone service with a presumed diagnosis of SARS-CoV-2 and had a positive result on real-time polymerase chain reaction (PCR) assay were included. One single transplant clinician collated the data prospectively and dedicated members of the team communicated with the treating team if patients were admitted to any of the surrounding hospitals or called them at home if not. Patients developing "classical" SARS-CoV-2 symptoms were initially directed to self-isolate, however, if unwell or upon deterioration to present to their local hospital. Data and outcomes of those more severely affected transplant recipients along with waiting list patients during the first wave of the disease from our region has been published (16).

Cases were recorded alongside demographics, symptoms at diagnosis, serum tacrolimus level at diagnosis and the previous visits, hospital admission, intensive care admission and 30-day outcomes including mortality. No patients were lost to follow-up.

Patients were on different immunosuppression regimes. The target range for tacrolimus levels in this unit is generally between 5 and 8 µg/L. Once patients were diagnosed with SARS-CoV-2 infection, their immunosuppression medication was reviewed. Patients who were on tacrolimus were identified, and those who had their trough serum level measured at the time or close (±2 days) to the diagnosis were included in the final analysis. Patients who did not have their level measured at the appropriate time or who were not taking tacrolimus were excluded from this analysis. The presence (or not) of diarrhoea was also recorded as a potential symptom of SARS-CoV-2 infection due to its effect on tacrolimus absorption from the gut.

Once the cohort of patients was identified, their serum tacrolimus levels were examined and the mean of the three most recent levels for each patient immediately prior to infection was calculated and represented their pre-infection level. Following admission, mycophenolate derivatives were withheld as a standard practice.

A "control" group of patients was identified by collecting the data of all sequential admissions with sepsis to our unit over a period of 1 year. Diarrheal illnesses were excluded as this is well known to affect serum tacrolimus concentrations (17).

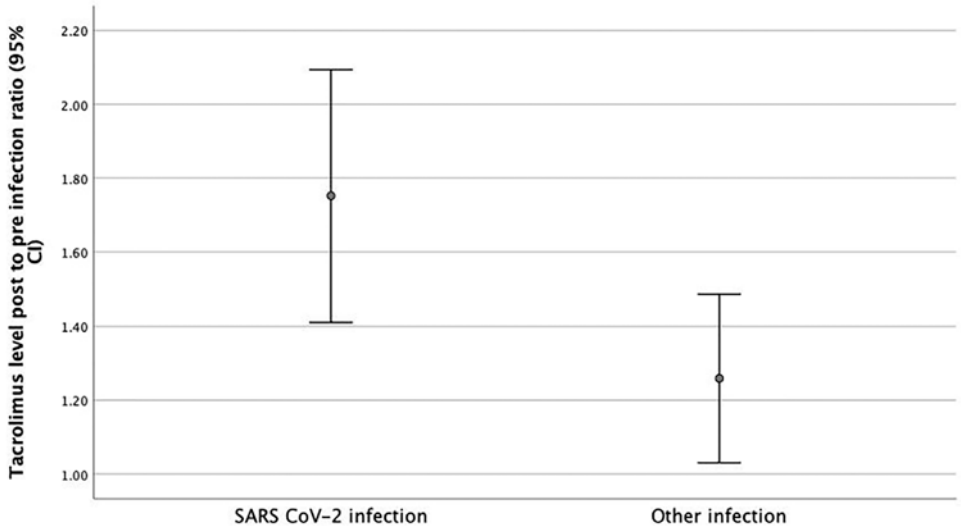


FIGURE 1 | Post to Pre-Infection Tacrolimus Level Ratio. Post to Pre infection Tacrolimus level ratios were higher in patients with SARS- CoV-2 infection (1.7 μg/L) at the time of diagnosis compared to transplant patients admitted with other infections (1.25 μg/L) (p = 0.018, mean ratio difference 0.49, 95% CI 0.08–0.89).

TABLE 1 | Characteristics of transplant recipients with available serum Tacrolimus levels at the time of diagnosis with SARS-CoV-2 compared to a control group of other infected patients who required hospital admission.

Group		SARS-CoV-2 (n = 34)	Control (n = 26)
Age (years)	Median (range)	54.5 (25–80)	55 (25–83)
Sex	Male	23 (67%)	14 (53.8%)
	Female	11	12
Ethnicity	White European	33 (97%)	24 (92.3%)
	East Asian	1	1
	South Asian	0	1
BMI (kg/m^2)	Median (range)	28.7 (22–41.5)	25.3 (18–39)
Type of transplant organ/donor	Kidney (living donor)	11 (32%)	10 (38.5%)
	Kidney (DBD)	14 (41%)	10 (38.5%)
	Kidney (DCD)	8 (23.5%)	5 (19%)
	Simultaneous pancreas and kidney	1 (3%)	1 (3.8%)
Transplant to infection diagnosis (months)	Median (range)	82.5 (1–317)	112 (22–328)
Hospital admission status and outcome	Outpatient	12 (35.3%)	—
	Inpatient	22 (64.7%)	26
	Intensive care unit	4 (11.8%)	0
	Graft dysfunction	17 (50%)	15 (57.7%)
	Graft failure	2 (5.9%)	0
	Death	4 (11.8%)	0

TABLE 2 | The serum tacrolimus levels where higher among SARS-CoV-2 infected patients compared to controls admitted due to other infections.

		SARS-CoV-2 (n = 34)	Control (n = 26)	
Serum tacrolimus level (μg/L)	Range	2.7–23	2–27.8	p = 0.07
	Median	9.6	7.9	95% CI (–0.3–5.8)

The same data was collected for these patients for comparison with patients presenting with SARS-CoV-2 infection.

Median values were compared with Mann Whitney test. In addition the ratio of tacrolimus level post- and pre-SARS-CoV-2 infection was generated for each patient and was compared with the respective ratio of patients who had other types of infection by performing a t-test without presumption of equal variances. Analysis was performed using IBM-SPSS version 25.0 software.

This article was prepared following the STROBE statement-checklist.

RESULTS

During this period, 59 transplant patients were diagnosed with SARS-CoV-2 infection. 52 of were taking tacrolimus and 34 had their trough serum tacrolimus level measured at the time of diagnosis (including all those admitted). Of these 34 patients, 20 (38.4% of infected patients on tacrolimus and 58.8% of those with available trough levels ±2 days to diagnosis) had a value above 8 μg/L and 14 had a value within the unit's 'normal range'. The 18 patients who had not had their tacrolimus level measured were patients with mild symptoms that did not attend any healthcare facility in person and were advised to self-isolate.

Median age at diagnosis for these patients was 54.5 years (range 25–80), 23 patients (67%) were male and 33 (97%) were from a white European background. Median time between diagnosis and transplantation was 82.5 months (range 1–317). Five patients (14.7%) experienced diarrhoea (with another three developing it later). 22 patients (64.7%) required hospital admission with 4 of them (11.8%) requiring escalation to an intensive care setting. 17 patients (50%) suffered graft dysfunction (AKI) and 2 patients (5.9%) had graft failure requiring return to dialysis. Four patients (11.8%) died during hospital admission due to COVID-19 infection. All deaths occurred within the group of patients with graft dysfunction, but the patients who suffered graft loss survived. A summary of the characteristics of the patients in this cohort is provided in **Table 1**.

The control group consisted of 26 consecutive patients admitted with other infections to the transplant unit over 1 year period. Urinary tract infections were most common and affected 21 (80.8%) of the admitted patients. Other infections documented were non-SARS-CoV-2 respiratory infections (three cases, 11.5%), biliary (one case, 3.8%) and cryptococcal meningitis (one case, 3.8%). Median age at diagnosis was 55 years (range 25–83). Median time from transplantation to infection was 112 months (range 22–328). All patients were cared for on the transplant ward (level 1 and 2 care). No deaths or graft failure occurred in this group, 15 (57.7%) presented with an acute kidney injury.

The range of trough serum tacrolimus levels at the time of diagnosis for the patients in the SARS-CoV-2 cohort was 2.7–23 μg/L, mean 11.2, median 9.6. As mentioned, 20 of those patients had a level above 8 μg/L. This contrasted with a mean value of 8.5 μg/L and a median of 7.9 μg/L in the control group (Mann-Whitney $p = 0.07$, 95% CI for the difference between medians −0.3–5.8) (**Table 2**).

To ensure that the observed difference was real, a ratio was calculated of the post-infection tacrolimus trough level to the mean pre-infection value. This ratio was higher in the SARS-CoV-2 cohort (ratio 1.7) compared to the control group (ratio 1.25, $p = 0.018$, mean ratio difference 0.49, 95% CI 0.08–0.89).

(**Figure 1**) A higher incidence of graft dysfunction (AKI) was found in SARS-CoV-2 infected patients who had tacrolimus levels above 8 μg/dl following infection, 13 out of 20 compared to 4 out of 14 among those with lower levels ($p = 0.037$).

The median length of stay was higher among SARS-CoV-2 infected patients with higher tacrolimus levels (10 days, range 1–44) compared to those who did not have high levels (0 day, range 0–70, $p = 0.04$) although the incidence of ICU admissions was the same. Four of the 20 patients (25%) with high tacrolimus levels died, compared to 2 of the 14 patients with normal tacrolimus levels ($p = 0.5$).

DISCUSSION

This small study demonstrates convincingly that SARS-CoV-2 leads to significantly raised tacrolimus trough levels and is associated with disease severity.

Acute kidney injury has been recognized as a prominent complication of SARS-CoV-2 infection resulting from an immunological cascade leading to vascular, tubular, and glomerular injury (18). AKI complicates 4.3% of the cases of SARS-CoV-2 that require hospitalization and almost 20% of the critically ill patients require renal replacement therapy (RRT) (18–20). AKI has also been associated with higher mortality rate in critically ill patients with SARS-CoV-2 infection (21). The AKI is multifactorial but a specific proximal tubular injury has been described by Werion et al (22). A novel injury mechanism after SARS-CoV-2 entry, which is based on expression and functional network analysis between *ACE2* and solute channel genes has been considered.

Diarrhoea is a common symptom of SARS-CoV-2 in children (23) and it might affect up to 13.5% of adults (24). SARS-CoV-2 invades the gastrointestinal tract through binding with ACE2 receptors (for which it has 10–20 times higher affinity compared to SARS-CoV-1) (25) causing intestinal permeability changes. Mouse models have shown that ACE2 alterations might be associated with the uptake and imbalance of amino acids and colitis (26).

Five of the 20 patients with high tacrolimus levels had diarrhea, that is well recognized to lead to increased tacrolimus levels (17). The mechanism of increased levels in the rest is still unclear, but it is of unusually high frequency. Whether tacrolimus levels were raised in patients for whom a contemporaneous level was not available is difficult to say. It may be that high levels are associated with more severe disease that prompts hospital attendance or admission. We postulate that increased tacrolimus levels are either the result of decreased transit time in the gastrointestinal tract (small bowel) with increased enterohepatic circulation, being due to further reduction in Pgp levels compared to other infections or a direct effect of the increased permeability following binding of ACE2 receptors.

The current study brings to light another aspect of SARS-CoV-2 infection in transplant patients: tacrolimus-induced nephrotoxicity. In this cohort the serum tacrolimus level was significantly raised compared to previous values in the same patients as indicated by an 'infection to pre-infection' ratio of 1.7. Patients with those higher levels were at a higher risk for developing AKI and stayed longer in the hospital. In addition, the post-infection levels of tacrolimus among SARS-CoV-2 infected patients were increased compared to a control (non-SARS-CoV-2) infection group that required admission. It is well known that, in the setting of inflammation, ischemia and shock, P-glycoprotein (PgP) expression in the gut wall may be reduced leading to decreased PgP levels and increased blood tacrolimus trough concentrations up to two times, as it occurred in

the present study (14, 15, 27–30). The inclusion of a control group of serious infection that required admission confirms these particularly raised levels truly related to SARS-CoV-2 itself rather than a what is occurring with any severe infection.

SARS-CoV-2 infection could therefore impair renal function in transplant patients both by damaging the kidney directly and by causing drug-induced nephrotoxicity due to higher tacrolimus levels conveying a higher risk of overall morbidity.

As a conclusion, increased serum tacrolimus level is another effect of SARS-CoV-2 infection that has yet to be fully understood. Given its frequency, clinicians should be aware and be vigilant in order to counter it appropriately even in patients not experiencing diarrhoea. In transplant patients who require hospital attendance we suggest measuring serum tacrolimus levels immediately and reducing dosage appropriately in case of an increased value. This study is limited by the small sample size and the unmatched, heterogenous nature of the control group that could have also contributed to the difference in tacrolimus levels. Further research can determine the pathophysiological mechanism involved in this process, alongside careful control group design to gain more insight to it.

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. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CC, GK, and EC-W: Study design, data collection, data analysis, and writing of paper; UK and DE: Editorial input; AA: Supervising author.

FUNDING

This is from legacy research fund of the unit not directly associated with any products mentioned in this research.

CONFLICT OF INTEREST

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

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LETTER TO THE EDITOR
published: 16 March 2022
doi: 10.3389/ti.2022.10321

Antibody Response After a Second Dose of the BNT162b2 mRNA COVID-19 Vaccine in Liver Transplant Recipients

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Keywords: severe acute respiratory syndrome coronavirus 2, antibody responses, immunodeppressants, mycophenolate mofetil, coronavirus disease 2019

Dear Editors,

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread globally, and the World Health Organization declared a pandemic on March 11, 2020. A widespread health emergency with social and economic disruptions remains in effect worldwide. Vaccination against SARS-CoV-2 is therefore an essential tool to control the global COVID-19 pandemic.

Pioneering studies on vaccines against SARS-CoV-2 have verified their safety and efficacy in general populations (1). However, data remain scarce regarding fragile populations such as organ transplant recipients (2–4). Indeed, individuals on immunosuppressants have been specifically excluded from SARS-CoV-2 vaccine trials (1). Previous studies have described suppressed antibody titers in patients receiving mycophenolate mofetil (5). Thus, in consideration of the potential for blunted immune responses to vaccinations, quantification of the immunogenicity of SARS-CoV-2 vaccines in fragile populations represents an urgent issue.

We aimed to evaluate antibody response after the second dose of BNT162b2 mRNA vaccine (Pfizer/BioNTech). We measured IgG antibody titers to the S receptor-binding domain (RBD) in liver transplant recipients and healthy controls who had received two doses of BNT162b2 mRNA vaccine. We also analyzed how immunosuppressant regimens affected antibody responses.

We included individuals who had received the second dose of BNT162b2 mRNA COVID-19 vaccine between March and August 2021. This study included 56 liver transplant patients and 42 healthy controls at Matsunami General Hospital. Blood was collected at least 14 days after the second vaccination. RBD-IgG titers were measured using the SARS-CoV-2 S-IgG (IC) Assay Reagent assay kit (Fujirebio Inc. Tokyo, Japan). Titers greater than 1.0 arbitrary units (AU)/mL were considered positive (detection range, 0.1–20 AU/mL). This study conforms to the principles outlined in the 1975 Declaration of Helsinki and its later amendments. The study protocol was approved by the Ethics Committee of Matsunami General Hospital (approval no. 498, 2021).

RBD-IgG antibody titers were measured in 56 liver transplant recipients. The median age of liver transplant recipients was 65.0 years, comprising 76.8% males (n = 43) and 23.2% females (n = 13). None of participants had a prior polymerase chain reaction–confirmed diagnosis of

Abbreviations: COVID-19, coronavirus disease 2019; RBD, S receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

OPEN ACCESS

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Received: *23 December 2021*
Accepted: *18 January 2022*
Published: *16 March 2022*

Citation:
Sakai A, Morishita T and Matsunami H (2022) Antibody Response After a Second Dose of the BNT162b2 mRNA COVID-19 Vaccine in Liver Transplant Recipients. Transpl Int 35:10321. doi: 10.3389/ti.2022.10321

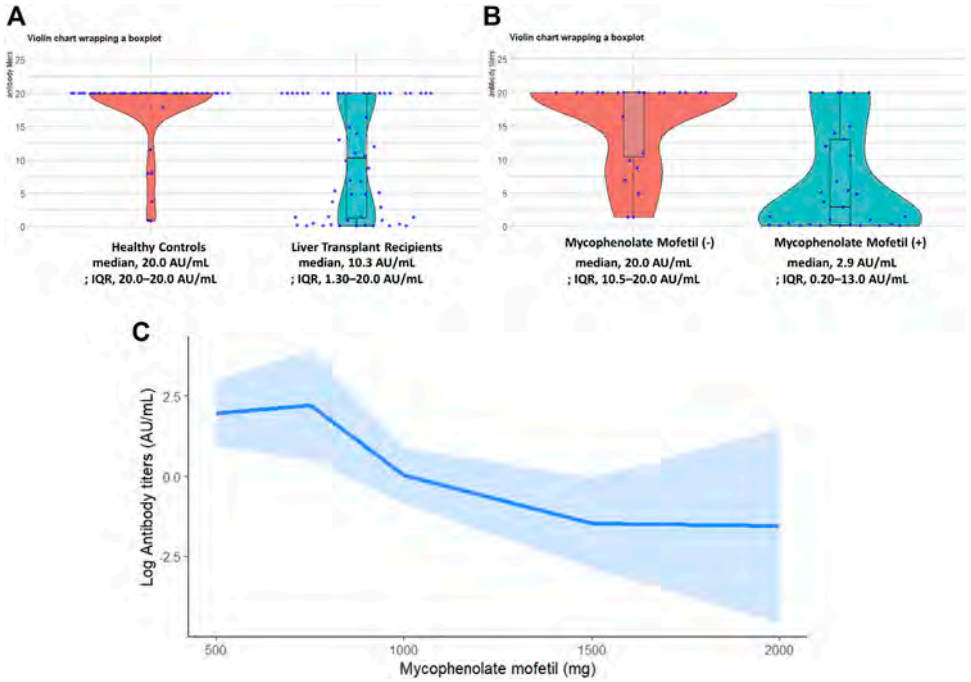

FIGURE 1 | (A) Violin charts wrapping a box plot show RBD-IgG antibody levels in the liver transplant recipient group ($n = 56$) and healthy donor group ($n = 42$) after the second dose. **(B)** Violin charts wrapping a box plot show RBD-IgG antibody levels in recipients taking mycophenolate mofetil ($n = 33$) and recipients not taking mycophenolate mofetil ($n = 23$) after the second dose of vaccine. Each point represents an individual patient, and horizontal lines indicate medians. Values above the detection limit are plotted as 20 AU/mL. Values below the detection limit are plotted as 0.1 AU/mL. **(C)** Association between RBD-IgG antibody titers and mycophenolate mofetil dose in liver transplant recipients by restricted cubic spline model with four knots. The shaded area represents the 95% confidence interval. IQR, interquartile range; RBD, S receptor-binding domain.

COVID-19, hospitalization and death between March, 2021 and September, 2021. Liver transplant recipients showed significantly decreased antibody titers as compared with healthy controls (**Figure 1A**). Liver transplant recipients developed significantly lower antibody titers when compared with healthy controls (adjusted mean difference −7.42; 95% confidence interval, −12.81 to −2.03; $p = 0.008$). Median time between liver transplantation and BNT162b2 vaccination was 15.5 years. Calcineurin inhibitor-based immunosuppressive therapy was used in 91.1% ($n = 51$), mycophenolate mofetil in 58.9% ($n = 33$), steroids in 1.8% ($n = 1$), and mTOR inhibitors in 1.8% ($n = 1$) among liver transplant recipients.

The overall seroconversion rate after the second vaccination was 86.7% in study participants. Liver transplant recipients showed a lower seroconversion rate (44/56; 78.6%) than healthy controls (41/42; 97.6%). The seroconversion rate was lower in recipients taking mycophenolate mofetil (21/33, 63.6%) than in those not taking mycophenolate mofetil (23/23, 100%; $p = 0.001$), whereas the seroconversion rate was higher in recipients taking calcineurin inhibitor (42/51, 82.4%) than in those not taking calcineurin inhibitor (2/5, 40.0%; $p = 0.06$).

Figure 1B compares antibody titers between liver transplant recipients with or without use of mycophenolate mofetil. Development of RBD IgG antibody titers was less likely in liver transplant recipients taking mycophenolate mofetil (median, 2.9 AU/mL; IQR, 0.20–13.0 AU/mL) than in those not taking mycophenolate mofetil (median, 20.0 AU/mL; IQR, 10.5–20.0 AU/mL; $p < 0.001$).

A restricted cubic spline plot (**Figure 1C**) shows the relationship between RBD IgG antibody titers and total mycophenolate mofetil dose in liver transplant recipients. An inverse linear relationship between RBD IgG antibody titers and mycophenolate mofetil dose was detected (p for effect = 0.008, p for non-linearity = 0.24).

Our findings in liver transplant recipients confirmed suboptimal immunogenicity after the second dose of BNT162b2 vaccine, supporting findings from other studies of kidney transplant recipients (3), allogeneic hematopoietic stem-cell transplant recipients (6) and lung transplant recipients (7). Concern remains about the occurrence of severe COVID-19 in some vaccinated immunocompromised transplant recipients.

The seroconversion rate was as low as 63.6% (21/33) in patients taking mycophenolate mofetil. Even in patients with confirmed seroconversion, antibody levels were low, suggesting that the threshold for protective immunity had not been reached. Restricted cubic spline modeling also verified a linear dose-response relationship between mycophenolate mofetil dose and reduced antibody titers in our study. Reduced antibody responses among organ transplant recipients, including liver transplant recipients, suggest that these recipients may remain at high risk of COVID-19 even after the second and third doses of mRNA vaccine.

Our findings have clinical implications for liver transplant recipients, emphasizing the need to consider a fourth dose of vaccination and the assessment of antibody titers even after the third dose of vaccination. Although a third vaccine dose was well tolerated by solid organ transplant recipients who had an insufficient antibody response after two dose of vaccination, the serological response was heterogeneous and a large proportion of recipients remain at risk for COVID-19 (8, 9). Approaches to improve antibody responses in transplant recipients may require temporary reduction or withdrawal of mycophenolate mofetil, or replacement to other immunosuppressants and additional measures such as subsequent a fourth dose of vaccination (10). The assessment of antibody titers even after the third dose of vaccination might be important to discriminate patients who should maintain their barrier measures.

Longevity of the antibody titer and the optimal period of monitoring antibody titers are still unveiled. Further longitudinal studies are warranted to investigate how cellular immune responses will be maintained or whether waning antibody titers still provide protection from breakthrough infections in transplant recipients with larger participants.

In conclusion, we have revealed mycophenolate mofetil contributed the attenuated antibody acquisition against SARS-CoV-2 among liver transplant recipients with dose-dependent manner. For populations that prove unlikely to acquire antibodies, active measurement of RBD-IgG antibodies may be warranted to assess protective immunity against COVID-19 and the need for additional vaccination.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the present study are not publicly available due to ethical/privacy reasons but are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Matsunami General Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AS, TM, and HM: study idea, design, manuscript AS, TM: study performance AS, TM: data analyses, manuscript. All authors: data ascertainment, manuscript discussion, manuscript revisions.

FUNDING

This study was supported by Sosaikouseikai Clinical Foundation.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

We are greatly indebted to the staff of Matsunami General Hospital, Matsunami Health Promotion Clinic and Matsunami Research Park for their generous cooperation in establishing the study.

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LETTER TO THE EDITOR
published: 22 March 2022
doi: 10.3389/ti.2022.10357

Enhanced SARS-CoV-2 Antibody Response After a Third Heterologous Vector Vaccine Ad26COVS1 Dose in mRNA Vaccine-Primed Kidney Transplant Recipients

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Keywords: Covid-19, Sars-CoV-2, kidney transplantation, Ad26COVS1, heterologous vaccination

Dear Editors,

We and others have shown that kidney transplant recipients (KTR) exhibit a reduced immune response with a seroconversion (SC) rate <50% after a regular 2-dose mRNA SARS-CoV-2 vaccination regimen (1, 2). Very limited data on a heterologous 3-dose vaccination with the vector vaccine Ad26COVS1 are available in this patient group. In the only published trial, a 3-dose homologous vaccination protocol was compared with a heterologous one in KTR without SC after a 2-dose mRNA vaccination (3). The third dose increased the antibody response and was well tolerated. However, still less than 50% of the initial non-responders developed SC 4 weeks after either a third mRNA vaccine (35%) or the vector vaccine Ad26COVS1 (42%) (3).

Herein, we provide additional data on the humoral response in 142 Austrian KTR (mean age 60.6 years, 59.9% male, median transplantation vintage 109 months) after double mRNA and triple heterologous vaccination (**Figure 1**). Patients provided written informed consent, and the study was conducted in compliance with the Helsinki Declaration of 1975, as revised in 2013. Out of 122 patients with follow-up, 76 patients being vaccinated with two doses of a mRNA vaccine (75% mRNA-1273, 25% BNT162b2) received a third dose of Ad26COVS1, administered on average 109 days (range 109.0–145.0 days) after the second dose. SC was determined on average 47 days (range 35.5–61.0 days) after the third vaccination by quantifying anti-SARS-CoV-2 spike IgG antibodies (LIAISON® SARS-CoV-2-TrimericS IgG chemiluminescent immunoassay, Diasorin S.p.A., Saluggia, Italy; cut-off value for seroconversion: ≥33.8 BAU/mL). After double mRNA vaccination the SC rate was 48%. Following heterologous triple vaccination an additional 54% of the initial non-responders achieved SC. Altogether, 97 out of 122 KTR (80%) achieved SC after either double mRNA vaccination or the heterologous triple vaccination. Forty-eight of the 142 KTR showed high-level SC after double mRNA vaccination. Twenty patients developed low antibody concentrations (arbitrary threshold <350 BAU/mL). After a third heterologous dose all these 20 patients significantly boosted their humoral response (1391.9 (SD 687.2) *vs.* 144.8 (SD 94.6) BAU/mL, $p < 0.001$). Non-responders after heterologous triple vaccination were significantly older (65.5 *vs.* 59.4 years; $p = 0.033$), were more often treated with prednisolone or belatacept (88% *vs.* 46.4%, 28% *vs.* 2.1%; $p < 0.001$ for both) and had a shorter median transplantation vintage (66.0 *vs.* 141.7 months; $p < 0.001$). They showed a trend of lower mean eGFR (48.1 *vs.* 55.5 ml/min/1.73 m^2; $p = 0.058$) and being treated more often with mycophenolic acid (84% *vs.* 64%; $p = 0.090$). Higher

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Received: *13 January 2022*
Accepted: *03 March 2022*
Published: *22 March 2022*

Citation:
Schimpf J, Davidovic T, Abbassi-Nik A, Sprenger-Mähr H, Lhotta K and Zitt E (2022) Enhanced SARS-CoV-2 Antibody Response After a Third Heterologous Vector Vaccine Ad26COVS1 Dose in mRNA Vaccine-Primed Kidney Transplant Recipients. Transpl Int 36:10357. doi: 10.3389/ti.2022.10357

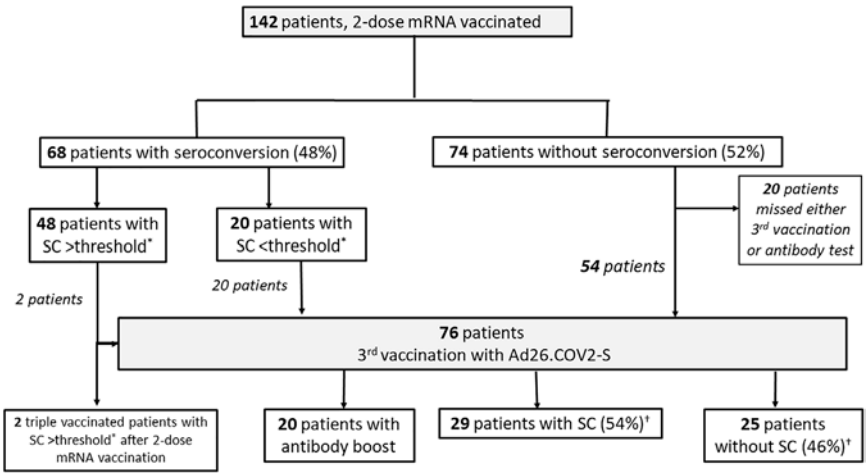

FIGURE 1 | Patient Flow Chart. *Seroconversion (SC) >threshold defined by anti-SARS-CoV-2 spike protein IgG antibodies >350 BAU/mL (LIASION© anti-SARS-CoV-2 TrimericS IgG chemiluminescent immunoassay, Diasorin S.p.A., Saluggia, Italy; cut off value for seroconversion: ≥33.8 BAU/mL). †% of patients (n = 54) without initial seroconversion after two mRNA doses.

mycophenolic acid doses did not correlate with inferior antibody response (p = 0.299). As a limitation, our study lacks cellular immune response and neutralizing antibody data. But anti-spike IgG antibodies are highly correlated with neutralizing antibodies, and a level >264 BAU/mL (95% CI: 108, 806) has been found to be associated with 80% vaccine efficacy against primary symptomatic Covid-19, although limited to the B.1.177 and B.1.1.7 SARS-CoV-2 variant (4). Fifty-three percent (40/76) of our patients with a third heterologous dose achieved this threshold, 62% of those with SC after initial non-response (18/29). Whether this threshold indicates the same vaccine efficacy against the now dominant SARS-CoV-2 Omicron variant is unknown. The longer transplantation vintage (9.0 *vs.* 4.6 years) and extended interval between second and third dose (109 *vs.* 80 days) in our cohort compared to the study by Reindl-Schwaighofer et al. (3) might be responsible for the higher seroconversion rate in our heterologous prime-boost vaccinees, as both factors significantly influence the vaccination response (1, 5). Nevertheless, due to our study design we cannot recommend one vaccine platform as superior over the other for booster vaccination in KTR, a clinically relevant question addressed by others (3). It remains to be proven whether a heterologous prime-boost regimen combining mRNA and vector vaccine improves the neutralizing humoral response against the now dominant SARS-CoV-2 Omicron variant in KTR as has been shown in the general population (6) or enhances the variant-specific cellular immune response (7) which might translate into better clinical outcomes.

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

Patients provided written informed consent, and the study was conducted in compliance with the Helsinki Declaration of 1975, as revised in 2013.

AUTHOR CONTRIBUTIONS

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

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LETTER TO THE EDITOR
published: 21 March 2022
doi: 10.3389/ti.2022.10319

First Experience With Extracorporeal Cytokine Adsorption Therapy After Lung Transplantation

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Keywords: lung transplantation, hemoadsorption, cytokine, Cytosorb, inflammation

Dear Editors,

Lung transplantation (LT) is accompanied by pro-inflammatory cytokine release, which correlates with the graft outcome (1–3). Extracorporeal cytokine adsorption therapy (ECAT) by Cytosorb® (CytoSorbents Corporation, Monmouth Junction, United States), a porous polymer beads adsorption cartridge, removes hydrophobic substances of molecular weight ≤60 kDa from the blood. ECAT is a promising therapy in hyperinflammatory situations (4–8), but has never been evaluated in LT. We evaluate for the first time ECAT on both circulating and membrane phagocyte-expressed inflammation biomarkers in the postoperative course of LT.

We conducted a prospective study at Bichat-Claude Bernard Hospital (Paris, France). Consecutive patients undergoing LT and admitted to the intensive care unit (ICU) postoperatively with extracorporeal membrane oxygenation (ECMO) were assessed. Cytosorb® cartridge was integrated into a bypass of the ECMO circuit at ICU admission. ECAT was performed during 24 h with the same cartridge. Blood samples were collected before cartridge placement (T0), after 24 h of ECAT (T1), and 24 h after cartridge removal (T2). We studied the evolution of membrane activation markers of neutrophils (CD66b and CD11b) and monocytes (CD14 and HLA-DR) by flow cytometry (Becton-Dickinson, FACS Lyric), the quantification of plasma levels of IL-6 and IL-8 by Luminex assay (Procartaplex®, Thermofisher) and L-lactate (Radiometer ABL90), and coagulation factors (factors II, V, VII, X, C protein, antithrombin III, and fibrinogen. Clinical data and outcomes are expressed in median (IQR). The study was approved by the French National Ethics Committee "Comité de Protection des Personnes Sud-Est II" (2017-A02625-48).

Six patients were transplanted for fibrosis ($n = 4$), chronic obstructive pulmonary disease (COPD) ($n = 1$) and silicosis ($n = 1$). At T2, neutrophil activation markers CD66b and CD11b expressions were significantly decreased as well as L-lactate levels (**Figure 1**). A downward trend was observed for

Abbreviations: COPD, chronic obstructive pulmonary disease; ECAT, extracorporeal cytokine adsorption therapy; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; LT, lung transplantation.

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Received: 21 December 2021
Accepted: 14 February 2022
Published: 21 March 2022

Citation:
Peyneau M, de Chaisemartin L, Faille D, Messika J, Mal H, Castier Y, Mordant P, Carrasco JL, Tanaka S, Lortat Jacob B, Ferrari P, Arrault X, Ajzenberg N, Chollet-Martin S, Montravers P and Tran-Dinh A (2022) First Experience With Extracorporeal Cytokine Adsorption Therapy After Lung Transplantation. Transpl Int 35:10319. doi: 10.3389/ti.2022.10319

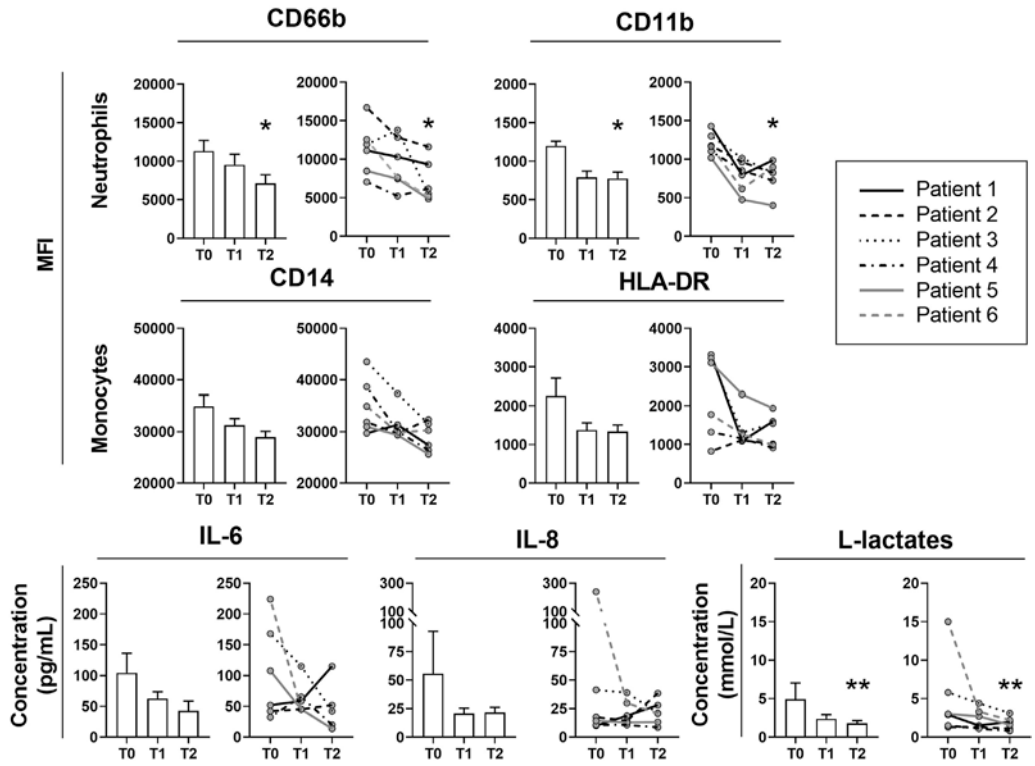

FIGURE 1 | Evolution of neutrophil and monocyte membrane activation markers, IL-6, IL-8 and L-lactate during extracorporal cytokine adsorption therapy by Cytosorb®. Activation membrane markers of neutrophils (CD66b and CD11b) and monocytes (CD14 and HLA-DR) were assessed by flow cytometry before cartridge placement (T0), after 24 h of ECAT (T1), and 24 h after cartridge removal (T2) and are expressed as mean fluorescence intensity (MFI). IL-6 and IL-8 were quantified by Luminex assay and L-lactates were assessed with Radiometer ABL90 Flex and concentrations are expressed in pg/ml and mmol/L respectively. Data are expressed in histograms (left) as the mean ± SEM or as individual values (right). Friedmann's test and Dunn's *post-hoc* tests are represented. *$p < 0.05$ **$p < 0.01$, as compared to T0.

monocyte activation markers (CD14 and HLA-DR), IL-6 and IL-8. No rebound effect was observed for any of these markers 24 h after cartridge removal. Coagulation markers were not altered. However, we observed one case of cartridge clotting after 12 h of treatment, without any consequences on the ECMO circuit. At T0, T1 and T2, norepinephrine doses were 0.75 (0.3–1.1), 0.25 (0.04–0.58) and 0.25 (0.03–1.15) µg/kg/min and PaO_2/FiO_2 ratio were 77 (74–118), 93 (88–107) and 79 (72–98) mmHg, respectively. Compared with a "control" cohort of 27 transplant patients over the same study period, the ICU length of stay and in hospital were longer for patients with ECAT, respectively of 64 (46–69) vs 41 (33–53) and 121 (82–146) vs 45 (38–63) days. However, at 1 year after LT, patients with ECAT were all alive, whereas the survival rate for patients in the "control" cohort without ECAT was 70.4%.

We present the first pilot study on the feasibility and efficacy of ECAT after LT. The decrease in neutrophil and monocyte activation markers has never been reported before and suggests a possible indirect immunomodulatory effect of ECAT on phagocyte activation. The decreased plasma IL-6 and IL-8 concentrations was not significant. However, the three patients with elevated IL-6 and/or IL-8 levels at T0 experienced a dramatic decrease at T1. Cytosorb® appears to be a safe and promising device to fight post-LT inflammation, and should be re-evaluated in a larger study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the French National Ethics Committee "Comité de Protection des Personnes Sud-Est II" (2017-A02625-48). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LC, AT-D, SC-M and PM designed the study. AT-D, ST, BL, JC, JM, HM, YC, PM included the patients and collected samples. PF

performed the placement of the Cytosorb® cartridge on the ECMO. MP and DF performed *in vitro* analysis. MP and LC analysed the results. AT-D and MP wrote the manuscript draft. AT-D, MP, LC, SC-M edited the manuscript, all authors approved the final manuscript.

FUNDING

This work was funding by a grant of the Cytosorb® Company.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

Service d'Anesthésie-Réanimation: Dan Longrois, Alexandre Mignon, Aurélie Snauwaert, Parvine Tashk, Maksud Assadi, Jules Stern, Sacha Rozencwajg, Adnan El Kalai, Aurélie Gouel, Fabien Lion, Laura Soldan, Adela Harpan, Marie-Pierre Dilly, Yassine Rkik, Atanas Sabahov, Claire Depont, Elie Kantor, Laetitia Desplanque, Nils Carrara, Sonia Yung, Morgan Roue, Alexandra Younes, Charles Moulin, Lea Copelovici, Iulia Balcan, Emmanuelle Busch. Service de Pneumologie B et Transplantation Pulmonaire: Gaëlle Weisenburger, Vincent Bunel, Cendrine Godet, Mathilde Salpin, Tiphaine Goletto, Chahine Medraoui, Domitille Mouren, Charlotte Thibaut de Menonville, Armelle Marceau, Gilles Jebrak, Lise Morer, Sabrina Trigueiros, Lucie Genet, Alice Savary, Zohra Brouk, Gwenn Frere, Agnès Abadie, Diego Ferreira, Sandrine Tissot. Service de Chirurgie Vasculaire, Thoracique et Transplantation Pulmonaire: Arnaud Roussel, Quentin Pellenc, Jean Senemaud, Iannis Ben Abdallah, Pierre Cerceau, Regis Renard.

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TRANSPLANT QUIZ
published: 17 March 2022
doi: 10.3389/ti.2022.10148

Anasarca, and Lymphadenopathy in a Kidney Transplant Patient: A Diagnostic and Therapeutic Challenge

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Keywords: kidney transplant, immunosuppressants, kaposi sarcoma, anasarca, bloody pleural effusion, axillary lymphadenopathy

CASE REPORT

A 57-year-old male kidney transplant recipient, originating from Congo and living in Switzerland for 10 years, was referred to our emergency department on the 26th of March 2021 for dyspnea. The clinical examination revealed anasarca progressing over 2 months, bilateral lower limbs edema and hydrocele. There were no skin or mucosal lesions at presentation. Symptoms started shortly after the patient returned from a 2-week trip to Kinshasa, Congo. His past medical history was relevant for a living-donor kidney transplantation in March 2019, in the context of end stage renal disease due to diabetic and hypertensive nephropathy. The patient had a history of subclinical C4d positive acute antibody mediated rejection (ABMR), treated with 1 dose of Rituximab in October 2019. Comorbid conditions included insulin-dependent type 2 diabetes mellitus, hypertension, treated obstructive sleep apnea, and stable monoclonal gammapathy (MGUS) with IgG lambda (62.3 mg/L). Immunosuppression at admission consisted of Ciclosporin, Mycophenolate mofetil, and Prednisone 5 mg/day.

Diagnostic work-up prior to hospital admission included an ultrasound of the lower limbs excluding thrombosis, normal transthoracic echocardiography as well as blank urinalysis without proteinuria. A CT scan was performed on 10 March 2021 (**Figures 1A,B**) and showed bilateral pleural effusion, predominantly on the right side with passive contact atelectasis. There were no ground glass opacities.

In the emergency department, initial blood work-up showed normal renal function, with normal electrolytes. Serum albumin was normal. The blood count showed mild thrombocytosis and mild hypochromic microcytic anemia with a Hb of 122 g/L. Leucocyte count was 5.1 g/L, with mild eosinophilia (1.16 g/L), and lymphopenia (0.3 g/L). CRP was mildly elevated at 17 mg/L. EBV and CMV viremias were negative. Quantiferon tuberculosis (TB) test was negative.

A right thoracentesis of 3 L was performed, relieving the dyspnea. Pleural fluid was bloody (1.45 × 10⁷/L erythrocytes) and filled criteria for an exudate. Pleural culture, PCR for TB, adenosine deaminase as well as cytology were all negative in the pleural fluid analysis.

Bronchoscopy with broncho-alveolar lavage was obtained and showed a cell count of 10⁷/L, with 83% macrophages, 16% lymphocytes, and 1% neutrophiles.

A whole-body 18-FDG PET-CT was obtained (**Figures 2A,B**), and showed pathological diffuse peritoneal hypermetabolism, as well as hypermetabolic right inguinal and left axillary lymph nodes.

OPEN ACCESS

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Received: *25 October 2021*
Accepted: *11 January 2022*
Published: *17 March 2022*

Citation:
Huegli S, Jaques DA, De Seigneux S and Haidar F (2022) Anasarca, and Lymphadenopathy in a Kidney Transplant Patient: A Diagnostic and Therapeutic Challenge. Transpl Int 35:10148. doi: 10.3389/ti.2022.10148

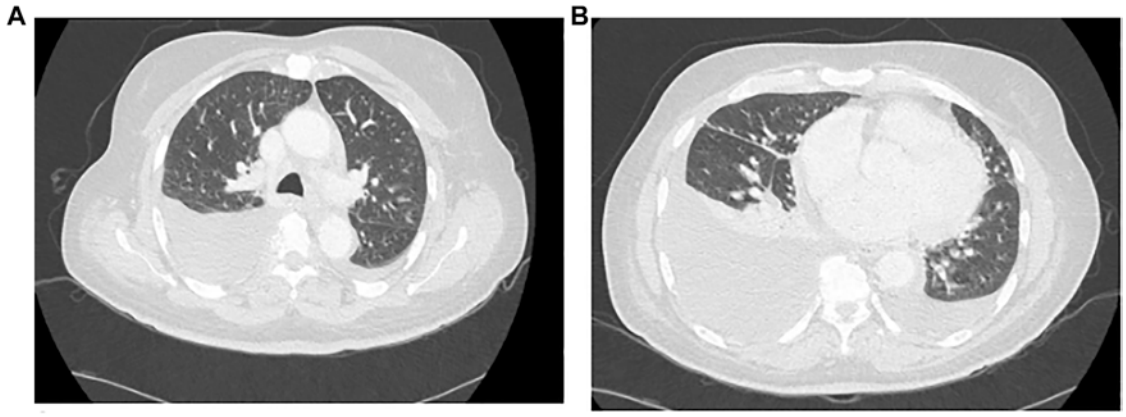

FIGURE 1 | Chest CT Scan, **(A)** Thoracic high, **(B)** Thoracic low.

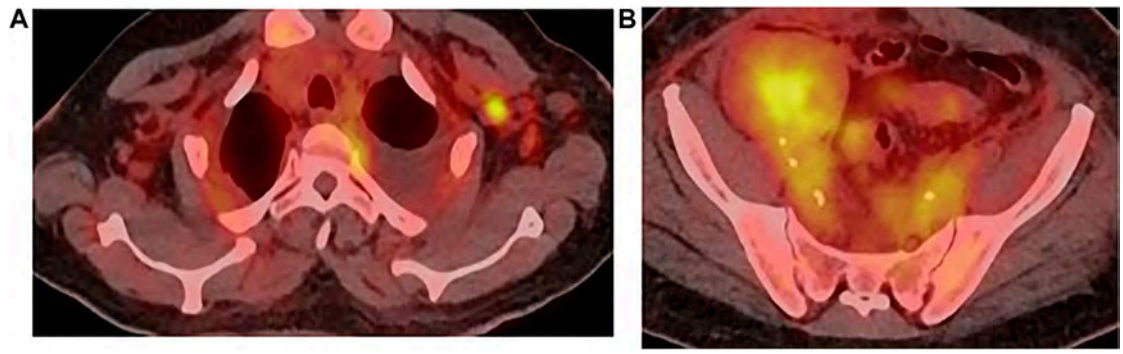

FIGURE 2 | PET-CT, **(A)** axillary, **(B)** inguinal.

TEST QUESTIONS

(1) The blood lymphocyte subsets show: $CD3^+$ = 1,050/ml; $CD4^+$ = 550/ml; $CD8^+$ = 500/ml; $CD19^+$ = 4/ml; $CD56^+$ $CD16^+$ (NK cells) = 124/ml. These results are compatible with:
   (a) $CD8^+$ cells depletion
   (b) CD4+ cells depletion
   (c) Severe B lymphocyte (CD19+) cells depletion
   (d) Abnormal NK cells level
   (e) Post transplant lymphoproliferative disease (PTLD)

(2) The broncho-alveolar lavage showed a cell count of 107/L, with 83% macrophages, 16% lymphocytes, and 1% polyneutrophiles. These results:
   (a) Are compatible with community acquired pneumonia
   (b) Are compatible with Mycobacterium tuberculosis infection
   (c) Are compatible with SARS-CoV-2 infection
   (d) Are compatible with intra-alveolar hemorrhage
   (e) Are normal

(3) What procedure would you recommend as the next step towards diagnosis?
   (a) Kidney graft biopsy
   (b) Left axillary lymph node biopsy
   (c) Abdominal surgical exploratory laparotomy
   (d) Bone marrow biopsy
   (e) Presumptive antituberculous treatment

(4) In terms of diagnosis, which answer is correct in this case?
   (a) Because of his African origin, the patient is at increased risk for Kaposi sarcoma (KS)
   (b) KS is secondary to HPV infection
   (c) PTLD can be excluded because EBV viremia is negative.
   (d) TB is excluded because of the negative TB Quantiferon test
   (e) CMV infection is a possible diagnosis

(5) How would you manage immunosuppression in the case of suspicion of malignancy or disseminated infection?
   (a) Stop Mycophenolate mofetil, keep Prednisone and ciclosporin
   (b) Increase immunosuppression by increasing the ciclosporin trough level
   (c) Stop all immunosuppression
   (d) Increase immunosuppression by doubling the dose of MMF
   (e) Increase immunosuppression by switching from ciclosporin to tacrolimus

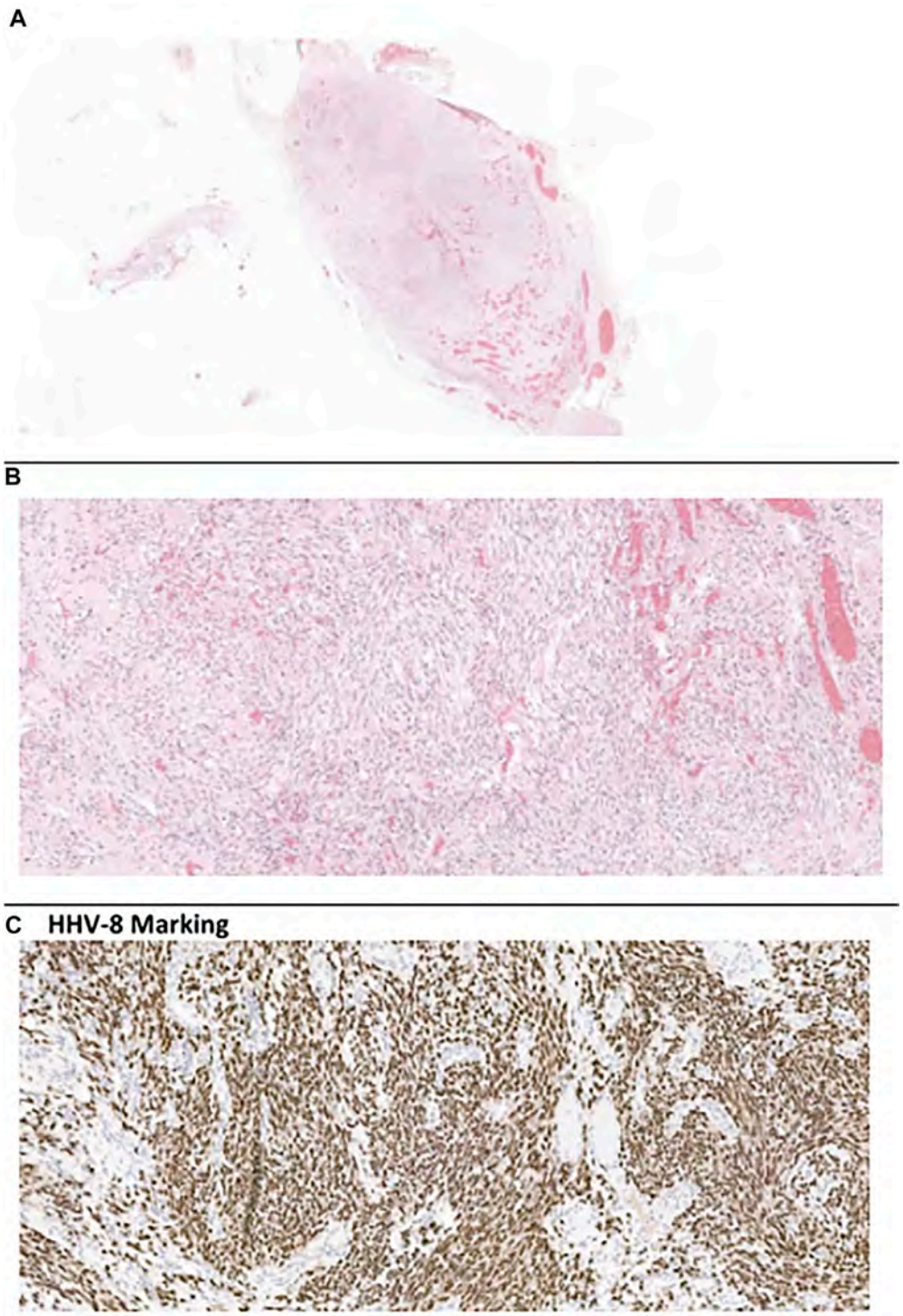

FIGURE 3 | Left axillary lymph node biopsy. **(A, B)** H-E coloration, **(C)** HHV-8 marking.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

SH wrote the manuscript and was in contact with the clinical case. DJ and SD reread the manuscript and provided significant alterations. FH participated in the first draft of the manuscript and all the rereads, and oversaw the clinical case.

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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APPENDIX

Answers and Discussion

Question 1

The correct answer is c.

The blood lymphocyte subsets show severe depletion in $CD19^+$ cells (B lymphocytes) with a level below 90/ml. This is secondary to the Rituximab injection that the patient received in October 2019 to treat subclinical ABMR.

PTLD Lymphoma cannot be diagnosed on these blood lymphocyte subsets alone.

Question 2

The correct answer is e.

The bronchoalveolar lavage (BAL) results are normal. However, the cell count in the BAL is of limited diagnostic value in clinical practice, in particular for infectious etiologies.

BAL cellularity in COVID-19 is predominantly neutrophilic (70%) and to a lesser extent macrophagic (27%) (1). In TB, BAL is characterized by increased neutrophil frequencies and decreased proportions of lymphocytes and macrophages (2).

The results for community acquired pneumonia would also be predominantly neutrophilic. There is no evidence of intra-alveolar hemorrhage, given the absence of red blood cells.

In the bronchoscopy, bacterial culture, PCR for tuberculosis, PCR for *Pneumocystis jirovecii*, and PCR for *Legionella pneumophilia* were all negative. Viral PCRs for HSV-1 and 2, SARS-CoV-2, CMV, adenovirus, and various respiratory viruses were also negative.

Question 3

The correct answer is b.

Given the results of the PET-CT, the next step towards diagnosis is a lymph node biopsy. We chose the left axillary lymph node as it was readily accessible. The right inguinal lymph node was not clinically observable. This strategy was overall less invasive than abdominal surgical exploratory laparotomy.

Given the normal complete blood count, we would advise against a bone marrow biopsy as a next step.

Presumptive antituberculous treatment could be discussed. In our patient, the results of the pleural effusion and bronchoscopy were negative for TB, and a pleural biopsy showed no sign of TB. Furthermore, this strategy would not rule out other causes of lymphadenopathy and pleural effusion, so a lymph node biopsy was necessary.

As none of the findings indicate renal dysfunction, kidney graft biopsy would be of low yield. In our patient, a left axillary lymph node biopsy was obtained (**Figures 3A,B**), and showed infiltration of the whole lymph node by fusocellular cells, with strong nuclear staining for HHV-8 (**Figure 3C**), establishing the diagnosis for KS. HHV-8 viremia was positive (12,000 GEq/ml).

Because of the bloody pleural effusion and the peritoneal hypermetabolism on the PET-CT, we concluded on a KS with visceral involvement. Biopsy of the pleura also showed KS, and so we did not do any gastro-intestinal investigations, but peritoneal KS was suspected.

Question 4

The correct answer is a.

KS is associated with HHV-8 infection. The endemic form is common in sub-Saharan Africa (3). KS occurring in solid organ transplant recipients is uncommon, but the risk is 100–200 times greater than that of the general population. It is more frequent in developing countries, with rates mirroring the HHV-8 seroprevalence (<5% in North America and Northern Europe, 30% in countries in the Mediterranean and the Middle East, and 50–60% in Sub-Saharan Africa). It usually appears in the first year post-kidney transplant but has been reported up to 18 years after the transplant (3). The classical presentation is cutaneous, with angiomatous lesions predominating on the legs and lymphedema. Visceral disease without cutaneous involvement occurs in about 10% of patients, mostly in the lymph nodes, intestines, and lungs (4).

Our patient had axillary and inguinal lymphadenopathy and anasarca. This situation evokes several differential diagnoses. CMV infection could explain diffuse lymphadenopathy but does not usually cause anasarca. Furthermore, CMV viremia was negative.

PTLD would be a reasonable differential diagnosis. Most cases post-transplant are associated with EBV infection, however, 20–40% are EBV negative. EBV negative cases occur more frequently after the first year of transplantation. In our patient, EBV viremia was negative, but this does not exclude PTLD. The gold standard for diagnosis of PTLD is the lymph node biopsy.

TB is also a probable differential diagnosis, given the bloody pleural effusion, lymphadenopathy, and the African origin of our patient. It cannot be ruled out only by a negative Quantiferon test. In our case, pleural fluid culture and PCR for TB were negative, but these tests have very low sensitivity and cannot exclude a pleural TB. Adenosine deaminase in the pleural fluid, which was also negative in our case, has a high negative predictive value if the effusion is lymphocyte dominant, but would be difficult to interpret in this case, given the bloody pleural effusion. Bronchoscopy and pleural biopsy are warranted to exclude TB. They were negative for TB in our case.

Question 5

The correct answer is a.

In the case of a suspicion of malignancy or diffuse infection in a kidney transplant recipient, there is a strong indication that to reduce immunosuppression, which we did initially by interrupting mycophenolate mofetil, and keeping ciclosporin with prednisone 10 mg/day. To stop all immunosuppression would not be appropriate, as it would greatly increase the risk of graft loss.

After the diagnosis of KS was made, ciclosporin was switched to everolimus. mTor inhibitors were found to have an anti-tumoral effect on cutaneous KS in a small case study of 15 kidney-transplant recipients (5).

Doxorubicine is considered a first line treatment for KS and is usually associated with a slow response. After a multidisciplinary

discussion, chemotherapy was started, with liposomal doxorubicin 20 mg/m^2 every 2 weeks.

Unfortunately, the patient did not respond well to chemotherapy, with the persistence of lymph node involvement and recurrence of symptomatic pleural effusion. His general condition deteriorated and did not allow for second line treatment. As he deteriorated, the immunosuppression was completely withdrawn, without any rejection episodes. He died approximately 6 months after the KS diagnosis was made of a sudden cardiac arrest after bronchoaspiration.

Transplant International

Official journal of the European Society for Organ Transplantation

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