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The molecular landscape of kidney rejection

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TRANSPLANT TRIAL WATCH
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Transplant Trial Watch

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Keywords: kidney transplantation, living donor liver transplantation, randomised controlled trial, acute kidney injury, tacrolimus

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

RANDOMISED CONTROLLED TRIAL 1

Fixed Low Dose Versus Concentration-Controlled Initial Tacrolimus Dosing With Reduced Target Levels in the Course After Kidney Transplantation: Results From A Prospective Randomized Controlled Non-Inferiority Trial (Slow and Low study).

by Stumpf, J., et al. EClinicalMedicine 2024; 67: 102381.

Aims

To assess if a slow and low tacrolimus regimen is non-inferior to classical dose of tacrolimus with regards biopsy proven acute rejection (BPAR) in an adult kidney transplant population.

Interventions

Participants were randomised to receive standard of care which was basiliximab induction, MMF, steroids and tacrolimus with trough levels 7–9 mg/mL or "slow and low" regimen of basiliximab induction, MMF, steroids and tacrolimus with 5 mg/day fixed for 7 days when to a trough level of 5–7 ng/mL.

Participants

432 adult kidney transplant recipients receiving ABO-compatible organs with low immunological risk scores, from living or deceased donors.

Outcomes

Primary efficacy outcome was the combined endpoint of BPAR, graft failure and death within 6 months. Secondary endpoints were renal function, delayed graft function. Chronic ABMR, DSAs, PTDM, infective incidence.

Follow-Up

6 months post-transplantation.

CET Conclusion

by John Fallon

This large multi-centre European open-label RCT demonstrated non-inferiority of a slow and low tacrolimus regimen with regards their composite end-point of BPAR, graft failure and death over a period of 6 months. However, one should be cautious in the interpretation. It is important to note that the study was conducted in immunologically low risk recipients, clearly recipients with a negative CDC cross-

Transplant Trial Watch

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match, but also no history of rejection in previous allografts, no DSAs, PRA <20% and no DCD organs, which in a wider context does limit the impact of the regimen's presented non-inferiority. When scrutinising the results more closely, the combined primary end-point occurred in 20.3% of slow and low and 18.8% of the standard care, risk difference and two-sided 90% confidence interval 1.5% (−6.0%; 9.0%; one-sided test of equivalence with a non-inferiority margin of 12.5% $p = 0.008$), but in this context a non-inferiority margin of 12.5% could be considered too large, but if reduced to margins closer to 5%, which one might consider more appropriate in this context, significance would likely not be reached. This combined with the finding that there was a statistically higher percentage of BANFF IA-III, i.e., above borderline, in the slow and low regimen compared with standard (11.6% vs. 5.2%, $p = 0.027$) could be a concern. The assessment on the impact of these is limited by the duration of follow-up being only 6 months, given these subtle event changes are impactful on the ultimate lifespan of a graft rather than necessarily acute losses. We must then consider conceptually the overall reason for interest in a slow and low regimen, which is the effects of early high trough levels. Slow and low avoided concerningly high trough levels within the first week, and by week 4 the levels in standard and slow and low are equilibrated, with acceptable therapeutic levels for nearly all patients throughout. However, despite this no difference was observed in secondary outcome parameter such as AE, SAE, kidney function, neurotoxicity, PTDM, or DGF (the study duration being too limited to consider implication to cardiovascular risk factors). While standardising early tacrolimus use is attractive for its clinical ease and its potential non-inferiority to standard care, the fact remains that variations in tacrolimus metabolism exist, and the present study is insufficient to confidently demonstrate the non-inferiority or reasoning behind a slow and low regimen.

Jadad Score
3.

Data Analysis
Modified intention-to-treat analysis.

Allocation Concealment
Yes.

Trial Registration
EudraCT—2013-001770-19.

Funding Source
Industry funded.

RANDOMISED CONTROLLED TRIAL 2

Effect of Dexmedetomidine on the Incidence of Postoperative Acute Kidney Injury in Living Donor Liver Transplantation Recipients: A Randomized Controlled Trial.

by Kwon, H. M., et al. International Journal of Surgery 2024 [record in progress].

Aims
The aim of this study was to investigate the role of intraoperative dexmedetomidine infusion on the incidence of acute kidney injury (AKI) in living donor liver transplant patients.

Interventions
Participants were randomised to receive either an infusion of dexmedetomidine or 0.9% saline.

Participants
214 living donor liver transplant patients.

Outcomes
The primary endpoint was the incidence of AKI. The secondary endpoints were levels of serial lactate during surgery, overall mortality, graft failure, early allograft dysfunction, major adverse cardiovascular events, chronic kidney disease, duration of mechanical ventilation, intensive care unit (ICU) and hospital length of stay.

Follow-Up
3 months posttransplantation.

CET Conclusion
by Simon Knight

This interesting paper from a single centre in South Korea investigated the use of dexmedetomidine (an alpha-2 agonist with anti-inflammatory and anti-oxidant properties) as a renoprotective agent during living-donor liver transplantation. 205 recipients were randomised to dexmedetomidine or control (saline) infusion during surgery. The authors report a significant reduction in risk of acute kidney injury in the dexmedetomidine group (35% vs. 50%), with lower postreperfusion lactate levels, although no difference in incidence of post-reperfusion syndrome. The study appears well designed, with adequate randomisation, allocation concealment and double-blinding. The exact method by which the clinical team were blinded to intervention is unclear—placebo was used, but how this was masked was not described. Given the evidence available from this study and others in cardiac surgery, it certainly warrants further investigation in more mixed multicentre cohorts.

Jadad Score
4.

Data Analysis
Per protocol analysis.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov—NCT03522688.

Funding Source
Non-industry funded.

CLINICAL IMPACT SUMMARY

by John O'Callaghan

This is an interesting study in living donor liver transplantation. The trial was conducted as a randomised, double-blind and placebo-controlled trial. The randomisation was computer generated and kept in sealed envelopes, opened prior to surgery by the anaesthetic nurse. AKI was defined using the KDIGO guidelines up to 7 days after surgery. There were very few dropouts and no group crossovers. The power calculation was based on the groups' previous work, where the risk of AKI was 59%. Altogether the setup, design and conduct of the trial is good.

The results showed a significant reduction in AKI when dexmedetomedine was used (35% versus 50%) and lower serum lactate levels until the end of surgery. There was no significant difference in CKD, MACE or EAD. There was no significant difference in ICU or hospital stay.

The majority of the reduction in AKI risk was seen in those with only stage 1 AKI (28% versus 38%). There was a moderate reduction in stage 2 AKI (6% versus 11%), but this was not statistically assessed, and no difference in the small risk of stage 3 AKI (1%). Therefore a far larger study would be required to demonstrate any difference in stage 2 or 3 AKI, and much longer follow up to establish if there are any consequences of the modest reduction in stage 1 AKI. Another option is to focus on patients with pre-existing CKD, who may benefit more from any protective effect.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

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NEWS AND VIEWS
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Cautious Optimism Warranted for Stem Cell-Derived Islet Transplantation in Type 2 Diabetes

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Keywords: type 2 diabetes, islet cells, induced pluripotent stem cells, islet transplantation, autologous

A 59 years old man in China has become the first patient to receive functional autologous islet tissue differentiated from inducible pluripotent stem cells (iPSC) [1]. In a previous case, injecting a poorly differentiated autologous iPSC product into the deltoid muscle led to a malignant teratoma [2] hampering proper evaluation due to insufficient information.

Wu et al reported on their proof-of-concept and safety study on 30th April 2024, in Cell Discovery [1]. The authors explore the use of endoderm stem cell lines [3] established from the patient's own peripheral blood mononuclear cells to generate islet tissues (E-islets) *in vitro* to treat a patient diagnosed with type 2 diabetes (T2D) 25 years ago. The patient had previously received a kidney transplant due to end-stage diabetic nephropathy, which required systemic immunosuppressive drugs to prevent rejection. A previously published protocol was used for *in vitro* differentiation of iPSCs into fully functional insulin producing islet cells [3]. A clinical dose defined as 1.2×10^6 IEQs was transplanted intraportally according to the established allogeneic islet transplant procedure [4] with a follow up of glycemic targets, reduction of exogenous insulin, and levels of fasting and meal-stimulated circulating C-peptide/insulin post transplantation. The present study suggests that stem cell-derived islet tissues could effectively restore islet function in a late-stage T2D patient. Additionally, the graft was well-tolerated with no instances of tumor formation or severe adverse events linked to the transplantation.

The novelty of this work lies in four key aspects: 1) the choice of iPSC to generate islets, as opposed to embryonic stem cells used in previously published clinical trials; 2) the utilization of an intermediate clinical grade cell line of endoderm stem cells (EnSCs) to facilitate non-tumorigenic, consistent, large-scale manufacture of the E-islets; 3) the use of autologous, patient-derived stem cells, and 4) the transplantation of a patient with type T2D. The use of human iPSCs as starting material is rapidly emerging and iPSCs have recently become a trusted autologous cell source that could be implemented for the treatment of Parkinson's disease [5], macular degeneration [6], and MSC-based therapy for steroid-resistant acute graft *versus* host disease [7]. We applaud the team from Shanghai Hospital, China, to conduct and report on this groundbreaking study but emphasize that there are several weaknesses that should be addressed.

To manufacture large-scale clinical-grade cell products of E-islet, multiple quality control (QC) release criteria must be met and reported throughout the process. This begins with generating of a GMP iPSCs clone, continuing with the development of a master cell bank of pancreatic endoderm stem cells (EnSCs), frozen as an intermediate product, and the final release of the drug product (E-islets) for transplantation. The references for the selected specific methods, justification of the timing, the batch size, and the rationale for the quality control platform are not clearly detailed by the authors [8].

Although, the efficiency of *in vitro* differentiation is good, it is not clear if multiple rounds of differentiation are needed to obtain enough yield of E-islets for a clinical batch. The single-cell transcriptomic (SCT) analysis data on E-islets highlights a significant presence of glucagon-positive

News & Views

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cells (almost half of the sequenced cells), which is not concordant with the characterization of E-islets by flow cytometry.

A real clinically relevant potency assay is one of the most challenging aspect to achieve for the new wave of biological drugs, also known as advanced therapy medicinal products (ATMPs) [9]. The authors selected a static stimulated insulin secretion assays that is a robust assay for primary human islets, but without any known sensitivity to distinguished quality control for stem cell derived islet like cells [10]. *In vivo* diabetic transplantation model was selected to determine the functionality and immunogenicity of the cells prior to transplantation. Although the authors created both a humanized mouse model using patient-specific blood mononuclear cells and non-human primates to study reversal of diabetes by E-islets, it is difficult to draw definitive conclusions about the function of these islets given the low number of mice used, pre-transplant blood sugar levels <400 mg/dL [11], lack of complete characterization of explanted grafts and short follow-up [12].

The advantage of using autologous iPSCs generated from a patient's somatic cell is to obtain patient-specific fully major histocompatibility complex (MHC) match. However, derivatives of autologous iPSCs have been shown to be rejected due to neoantigens that arise during *in vitro* cell manipulations and expansion, thereby questioning their immune-evasive potential [13]. The present study uses an autologous approach but since the recipient was already on immunosuppression due to the kidney transplant, this completely masks the immunological response to the autologous tissue, making this setting a wasted opportunity to answer an important question in the field.

Recent advances in differentiation protocols carefully developed over the past decades, represent one of the greatest achievements in the field. However most published protocols have been evaluated by a mixture of pluripotent stem cells generated from embryonic or reprogramming somatic cells into iPSC which makes it difficult to judge the impact on the differentiation. The field is still too young to declare the superiority of one strategy over the other [14].

Using the human islet equivalent number (IEQ) calculation for the dose selection of the stem-cell-derived islet cells is not accurate. The authors use the primary human islets volume-based method to estimate the number of E-islets but human islets are spherical structures ranging in size from 91 to 290 μm in diameter [15]. In contrast, the E-islets exhibit a uniform morphology consisting of smaller islet cells (one hundred μm), which impacts the tissue volume. Therefore, we advocate for reporting the clinical doses based on single cell counts using validated methods before allowing for 3D generation prior to transplantation.

One of the strengths of the paper is the thorough and comprehensive analysis of the safety of stem-derived cells, and it is hoped that this will serve as a reference for future studies.

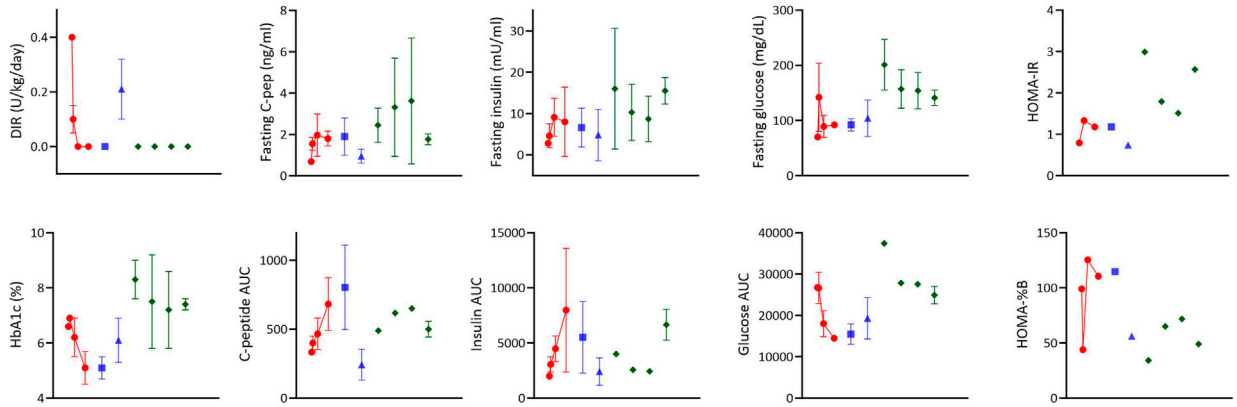
From the clinical point of view, the outcome of the endoderm stem cell-derived islet tissue transplantation is challenging to fully assess due to several key factors.

First, the definition of T2D in this context raises several questions. The early onset of the disease at 24 years of age is atypical for classic type 2 diabetes, which is more commonly diagnosed in older adults. Furthermore, the absence of precise information about the patient's previous medical history, including details such as family history, weight, insulin resistance, C-peptide levels, and autoimmunity at the time of disease onset, makes the classification of this case as T2D labile. This is especially concerning when considering the metabolic features of the patient in comparison to other cohorts of individuals with T2D (**Figure 1**) [16–18]. This diagnostic uncertainty is a common challenge encountered also in the context of pancreas transplantation for presumed T2D [19]. In such cases, the absence of comprehensive baseline data can pose challenges in accurately distinguishing T2D from other forms of the disease, including genetic subtypes like MODY or LADA.

Secondly, despite the patient being described as having "poor glycemic control," the reported data does not support this characterization and raises some ethical concerns. In fact, all the metabolic parameters of glucose control at baseline appear to be well within the target range, considering the current clinical goals [20]. This discrepancy raises doubts about the risk-benefit balance of the stem cell-derived islet tissue transplantation approach. Given the patient's stable glycemic control, alternative strategies could potentially achieve similar or even better outcomes without the risks and complications associated with a "first in man" experimental procedure. Such alternative approaches could include optimizing insulin dose titration or exploring the use of SGLT-2 inhibitors or GLP-1 agonists, which have demonstrated efficacy in the management of type 2 diabetes. Moreover, islet allotransplantation is a well-established procedure for patients having a kidney transplantation [21] and has been translated from an experimental procedure into a validated therapy during 25 years of research contribution [22]. In this context, pending the real need, the islet after kidney transplantation strategy should have been the first option considered as a potentially less risky alternative for this patient.

It is also difficult to distinguish the contribution of the transplanted tissue to the clinical outcome during the follow-up period. Residual function before the infusion was quite substantial with a 2 nmol/L C-peptide. Overall, there is a discrepancy in the early results with insulin independence achieved by 12 weeks, despite glucose levels remaining as high as before the transplant, and C-peptide levels at 12 weeks showing minimal difference compared to before the transplant. Significant clinical impact on glucose variability is also reported within 12 weeks, and even within 2 weeks after tissue infusion. However, this immediate improvement is not accompanied by a corresponding significant increase in C-peptide AUC or a decrease in glycated hemoglobin. This suggests that other factors may have influenced the outcomes, such as trial effects, changes in timing and carbohydrate intake [23] (as indicated by early-phase gastrointestinal disturbances and a 5 kg weight loss), or simply the reduction or suspension of insulin treatment. More congruent appears the improvement of insulin secretion and the improved glucose control in the following weeks, even if it is not possible to distinguish the endogenous contribution and any other potential confounding factors, such as the reported tapered drug administration of tacrolimus.

In conclusion, the field of stem-cell research are making substantial scientific advancements in developing a new generation of iPSCs as an unlimited source for generating cell types such as pancreatic beta cells and/or islet cells. Maintaining



	Case report	Healthy	T1D onset	T2D	T2D	T2D	T2D
	●	■	▲	●	●	●	●
n	Single case report	20	9	12	110	113	10
Reference	(1)	Unpublished		(2)	(3)	(3)	(4)
Ethnicity	Asian (China)	Caucasian (Italy)		Asian (China)			
Age	59	38.8±7.8	27.8±8.3	50.2±8.5	55.3±8.7	56.6±7.7	63.1±2.5
Gender M/F	1/0	12/8	5/4	6/6	not reported	not reported	8/2
Diabetes duration (yrs)	25	0	0.73±0.5	3.1 (2.4-5.7)	2.64±4	2.9±3.9	2.1±0.8
Insulin treatment	yes	no	ye	no	no	no	6/10
Oral hypoglycaemic	yes	No	no	7/12	110/110	113/113	4/10

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FIGURE 1 | Summary of metabolic features: stem-cell-derived islet cells transplantation vs T1D and T2D Cohorts. The figure summarizes the metabolic characteristics of the patient who received autologous islet tissue differentiated from induced pluripotent stem cells, in comparison with cohorts of individuals with type 1 diabetes (T1D) and type 2 diabetes (T2D). The data points for the case report patient are represented by the red circle, with four descriptive time points provided: 1) baseline; 2) mean value between baseline and 12 weeks (still on insulin treatment); 3) mean value between 12 and 52 weeks (still on antidiabetic treatment; 4) mean value after discontinuation of any diabetic treatment. For reference, data from healthy adults or T1D patients within the first year of onset followed at Ospedale San Raffaele in Milan are shown in blue symbols, and four cohorts of Chinese T2D patients reported in the literature are represented by green symbols. The table accompanying the figure provides the characteristics of the different cohorts.

optimism is encouraged. However, it remains unclear from the present study whether islet tissue generated from autologous stem cells is an efficient beta cell replacement therapy and if the immunogenic profile of autologous EnSCs used to generate E-islets triggers immune responses. Nevertheless, we believe that cell therapy has the potential to provide a markedly superior alternative to insulin therapy for patients with T1D. More data will be needed before expanded indications for T2D can be established.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

HS, VS, and LP drafted the article and revised it critically. All authors contributed to the article and approved the submitted version.

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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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Antibody Mediated Rejection and T-cell Mediated Rejection Molecular Signatures Using Next-Generation Sequencing in Kidney Transplant Biopsies

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Recently, interest in transcriptomic assessment of kidney biopsies has been growing. This study investigates the use of NGS to identify gene expression changes and analyse the pathways involved in rejection. An Illumina bulk RNA sequencing on the polyadenylated RNA of 770 kidney biopsies was conducted. Differentially-expressed genes (DEGs) were determined for AMR and TCMR using DESeq2. Genes were segregated according to their previous descriptions in known panels (microarray or the Banff Human Organ Transplant

Abbreviations: AMR, antibody-mediated rejection; B-HOT, Banff Human Organ Transplant; DEGs, differentially expressed genes; DSA, donor-specific antibodies; FC, fold change; FDR, false discovery rate; FPKM, fragments per kilobase of exons per million fragments; NGS, next-generation sequencing; TCMR, T-cell-mediated rejection.

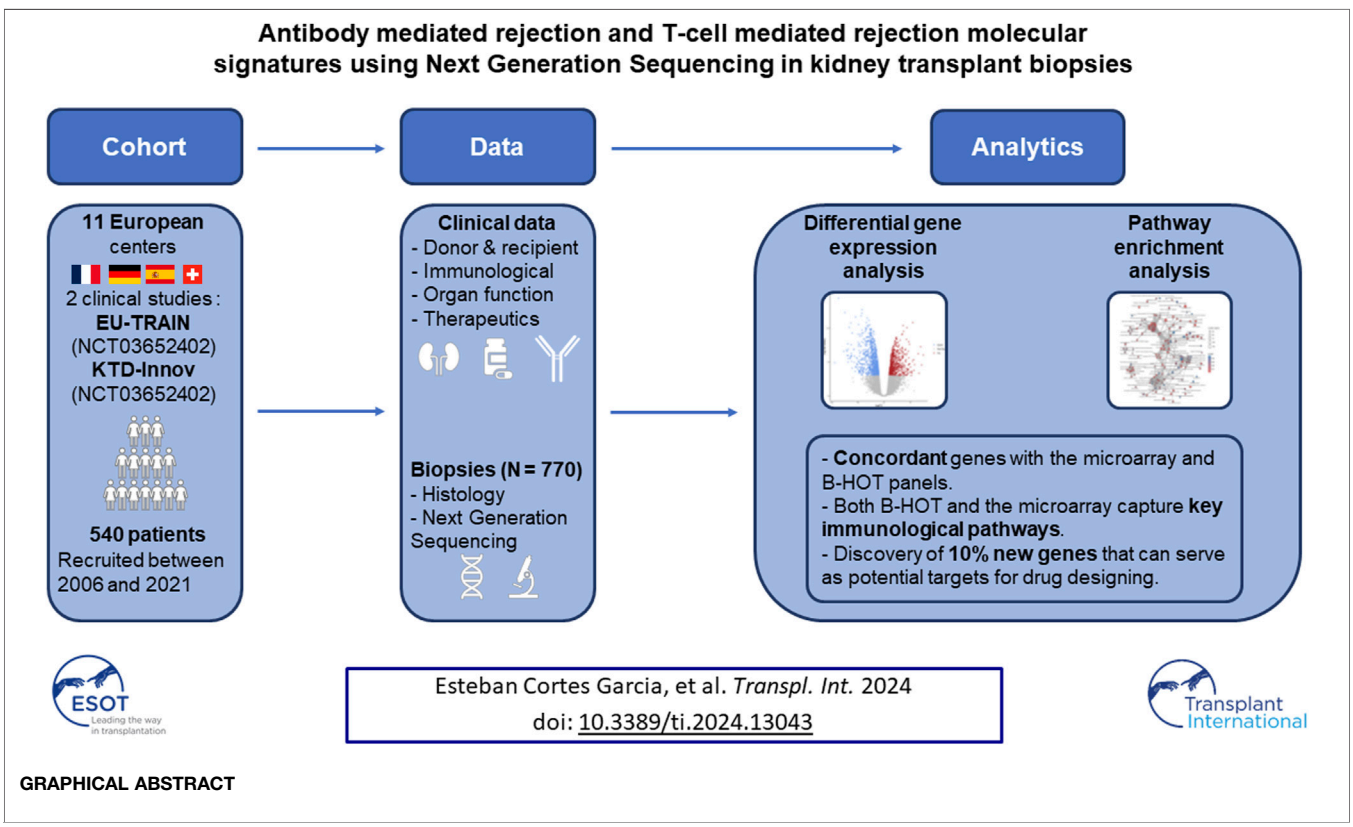
(B-HOT) panel) to obtain NGS-specific genes. Pathway enrichment analysis was performed using the Reactome and Kyoto Encyclopaedia of Genes and Genomes (KEGG) public repositories. The differential gene expression using NGS analysis identified 6,141 and 8,478 transcripts associated with AMR and TCMR. While most of the genes identified were included in the microarray and the B-HOT panels, NGS analysis identified 603 (9.8%) and 1,186 (14%) new specific genes. Pathways analysis showed that the B-HOT panel was associated with the main immunological processes involved during AMR and TCMR. The microarrays specifically integrated metabolic functions and cell cycle progression processes. Novel NGS-specific based transcripts associated with AMR and TCMR were discovered, which might represent a novel source of targets for drug designing and repurposing.

Keywords: next generation sequencing, RNA-seq experiment, kidney biopsies, molecular signature, allograft rejection, kidney transplantation

INTRODUCTION

Long-term kidney allograft survival is mainly limited by the occurrence of rejections [1, 2]. To improve kidney injury detection, the biennial revision of the Banff classification emerged as the gold standard for the diagnosis of rejection during the past 3 decades [3, 4]. From histology assessment of kidney biopsies, combined with clinical and immunological parameters, the classification is now encompassing molecular and digital biomarkers to improve its sensitivity and provide new diagnostic tools for the clinicians. Recently, transcriptome analysis has shown its capacity to accurately detect injuries and the degree of activity in solid organ transplant biopsies [5]. Previous studies focusing on the implementation of microarrays paved the way for the molecular understanding of rejection and allowed the development of gene expression-based classifiers [6]. However, this technology suffers from its necessity to design probes, limiting the past studies to the coding transcriptome only.

While lacking protein-coding ability, long noncoding RNAs (lncRNAs) act as functional RNA molecules, regulating protein-



GRAPHICAL ABSTRACT

coding gene expression through interactions with gene-regulatory proteins and microRNAs. Growing evidences in the literature showed the pivotal role played by lncRNAs in the establishment and maintenance of the immune response [7–9]. Therefore, they represent a complete novel source of biomarkers for the diagnosis of various cancers [10–12]. However, lncRNAs implication in the solid organ transplantation field remains poorly investigated. Combining the non-coding transcriptome on top of the coding might help our understanding of the pathophysiological mechanisms involved during kidney allograft rejection, could improve the molecular classifiers for its detection and prediction and provide new and unknown targets for drug designing and repurposing.

In the present study we investigated the discovery capability of Next-Generation Sequencing (NGS) to unravel both coding and non-coding transcriptome differentially expressed during rejection. For that purpose, we built a real-world, multicentric and extensively phenotyped cohort of 540 patients (770 biopsies) from two clinical studies: EU-TRAIN (NCT03652402) and KTD-Innov (NCT03582436). We performed an Illumina sequencing, analyzed the samples with differential gene expression analysis, identified known genes according to published gene panels (microarray or the Banff Human Organ Transplant) to identify new transcripts and implemented pathway enrichment analysis on the different subgroups.

MATERIAL AND METHODS

Study Population and Biopsy Cohort

EU-TRAIN (NCT03652402) and KTD-Innov (NCT03582436) studies are large, prospective multicenter cohorts that follow adult kidney transplant recipients for 1 year after transplantation. They involve collaboration between transplant centers, analytical platforms, and industrial partners across France and Europe.

The studies focus on adult patients (18 years or older) receiving a living or deceased kidney transplant. Participants must be willing to comply with study procedures and signed an informed consent. Patients with a history of multi-organ transplants, language barriers hindering participation, or vulnerability (minors, pregnant women, etc.) were excluded.

Both EU-TRAIN and KTD-Innov involve baseline visits at the time of transplant, followed by checkups at 3- and 12-months post-transplantation. Additional visits may be scheduled if a patient's kidney function deteriorated or protein levels raised. KTD-Innov recruited participants between July 2018 and December 2019 and focused on seven French transplant centers (Paris-Necker, Paris-Saint-Louis, Nantes, Bordeaux, Toulouse, Lyon, and Montpellier). The EU-TRAIN study, with a slightly broader enrollment window (November 2018–June 2020), encompasses nine centers across Europe (Paris-Saint-Louis, Paris-Necker, Nantes, Barcelona-Bellvitge, Barcelona-Vall d'Hebron, Berlin-Charité Mitte, Berlin-Charité Virchow, Geneva, Paris-Kremlin-Bicêtre). 770 renal biopsies were collected from 540 patients from the two prospective studies as well as two retrospective cohorts from Necker and St Louis hospitals (Paris, France) between 2006 and 2021. This study was approved by local institutional review boards and written informed consent was obtained from all patients.

Kidney Allograft Phenotypes

Lesions from biopsies were graded by local renal specialist from 0 to 3 according to the 2019 international Banff classification [13], and comprised: glomerulitis (g), peritubular capillary inflammation (ptc), interstitial inflammation (i), tubulitis (t), total inflammation (ti), endarteritis (v), transplant glomerulopathy (cg), interstitial fibrosis (ci), tubular atrophy (ct), vascular fibrous intimal thickening (cv), arteriolar hyalinosis (ah). C4d staining was performed by immunohistochemistry on paraffin sections using the human C4d polyclonal antibody. C4d staining was graded from 0 to 3 by the percentage of peritubular capillaries with linear staining. Earlier biopsies were reclassified to take into account the evolution of the classification.

Detection and Characterization of Circulating Donor-specific anti-HLA Antibodies

The presence of circulating donor-specific anti-HLA-A, -B, -Cw, -DR, -DQ and -DP antibodies was analyzed using single-antigen bead assays (One Lambda, Inc., Canoga Park, CA, United States) on a Luminex platform on serum samples collected at the time of transplantation and at the time of biopsy. For each patient, we recorded the number, class, specificities and mean fluorescence intensity (MFI) of all donor-specific HLA antibodies. Positiveness of a DSA was defined by a threshold of 500 for the mean fluorescence intensity. The maximum MFI for the immunodominant DSA (Anti-HLA iDSA MFI) was defined as the highest ranked donor-specific bead. HLA typing of donors and recipients was performed using DNA typing.

Experimental Procedures

After collection, all biopsies were stored in the RNAlater® solution at −20°C. They were then centralized at the Paris Cardiovascular Research Center (PARCC) in order to be processed by the Paris Transplant Group Precision Pathology Platform for total RNA extraction using the Promega® Maxwell® RSC miRNA Tissue Kit [14]. All samples were selected according to a minimal concentration of RNA of 20 ng/μL and an RNA integrity number superior or equal to 7. Purified RNAs were, then, stored in a −80°C fridge while waiting to be sent and sequenced by the GENOM'IC platform at Cochin hospital where the library was prepared according to the Illumina® Stranded mRNA Prep Ligation protocol [15] with a capture of the polyadenylated RNAs using oligo (dT) magnetic beads. Finally, an Illumina sequencing has been performed in order to obtain 2 × 30 millions paired-end reads on average.

RNA-Seq Data Processing and Quality Controls

After the sequencing, we used FastQC (v0.11.9) [16] to assess the pre-alignment quality controls. We performed the alignment

with the STAR algorithm (v2.7.4a) [17] and the Hg38.p13 reference genome. We finally verified its quality with STAR, Picard tools (v 2.22.9) [18] and RSeQC (v3.0.1) [19] metrics. Raw counts have been generated using the featureCounts program with the GC_000001405.39_GRCH38.p13 GTF annotation file and the BAM files resulting from the alignment. Quality controls results can be found in the **Supplementary Table S1**.

Differential Gene Expression Analysis

The identification of differentially expressed genes (DEGs) was performed using the DESeq2 method (v1.30.1) [20]. Gene expression count matrix has been pre-filtered by removing lowly-expressed genes using a threshold of at least 1 Fragment Per Kilobase Million (FPKM) in 20% of the total samples for each gene. The number of filtered genes reduced from 44,613 to 15,563. Fold changes (FC) and Wald statistics were computed for each comparison of interest with a correction for multiple hypothesis testing (Benjamini-Hochberg) and genes were ranked according to increasing adjusted *p*-values.

Two differential gene expression analysis were conducted including antibody-mediated rejection (AMR) and T-cell-mediated rejection (TCMR). Each diagnosis was tested against all histopathological diagnoses available in the cohort to obtain a molecular signature specific for the diagnosis of interest. This control group included all biopsies diagnosed with either TCMR or AMR (according to the design), isolated interstitial fibrosis and tubular atrophy, acute tubular necrosis, polyomavirus-associated nephropathy, thrombotic microangiopathy, recurrent or *de novo* glomerulonephritis, calcineurin-inhibitor toxicity or biopsies with no evidence of specific lesions (i.e., normal biopsies). Missing information, borderline (N = 69), mixed (N = 20) and suspicious rejection (N = 12) samples have been excluded from both designs. No threshold on the $\log_2$ fold change was applied and all significant (adjusted *p*-value <0.05) differentially-expressed genes were considered during the analysis.

The complete description of differentially expressed gene symbols, mean expressions, $\log_2$ fold changes, standard errors and Wald statistics as well as descriptions of the genes previously described in gene panels (B-HOT or microarrays) are shown in the **Supplementary Material**.

Pathways Enrichment Analysis

Pathways analysis was performed using both Reactome and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) repositories with ReactomePA (v1.34.0) [21] and clusterProfiler (v3.18.1) [22], respectively, by either choosing as an input the entire list of upregulated genes (Reactome and KEGG) or a subset consisting of the upregulated transcripts included in the B-HOT or the microarray gene panel (Reactome only). Raw *p*-values were corrected for multiple hypothesis testing with the Benjamini-Hochberg FDR controlling technique and two cut-offs were applied to filter non-significant results: threshold of 0.05 on the adjusted *p*-value and 0.2 on the q-value. Q-values correspond to the proportion of false positive results in a set of signaling pathways that are at least as significant (adjusted *p*-value) as the signaling pathway under consideration. While the adjusted *p*-value gives the expected false positive rate, the q-value gives the expected positive false discovery rate. Pathway names, annotations and statistics are reported in the **Supplementary Material**.

TABLE 1 | Baseline patient characteristics.

	NGS cohort (n = 540)	N
Recipient characteristics		
Age (years), Mean (SD)	51.1 (15.9)	540
Gender male, No. (%)	336 (63.2)	532
End stage renal disease causes		539
ADPKD, No. (%)	82 (15.2)	
Diabetes, No. (%)	48 (8.9)	
Glomerulonephritis, No. (%)	125 (23.2)	
Tubulo-interstitial, No. (%)	58 (10.8)	
Vascular, No. (%)	60 (11.1)	
Other, No. (%)	79 (14.7)	
Unknown, No. (%)	87 (16.1)	
Donor characteristics		
Age (years), Mean (SD)	54.4 (17.1)	534
Gender male, No. (%)	298 (55.7)	535
Hypertension, No. (%)	144 (28.6)	504
Diabetes, No. (%)	40 (7.8)	513
Creatinine (μmol/L), Mean (SD)	83.8 (51.0)	530
Donor type		
Living donor, No. (%)	100 (18.5)	539
Deceased donor, No. (%)	439 (81.5)	539
Expanded criteria donor, No. (%)	185 (42.1)	439
Transplant baseline characteristics		
Prior kidney transplant, No. (%)	103 (19.1)	538
Cold ischaemia time (hours), Mean (SD)	13.9 (8.4)	534
Delayed graft function, No. (%)	77 (14.5)	531
HLA-A/B/DR/DQ mismatch, Median (IQR)	5 (4–6)	465
Presence of D0 DSA, No. (%)	141 (27.9)	505

Delayed graft function was defined as the use of dialysis in the first postoperative week. Abbreviations: ADPKD: autosomal dominant polycystic kidney disease; DSA: donor specific antibody; HLA: human leucocyte antigen.

Statistical Analysis

Continuous variables were described by using means and standard deviations or medians and interquartile ranges. All analyses were performed using R (version 4.0.5, R Foundation for Statistical Computing). Values of $p < 0.05$ were considered significant, and all tests were 2-tailed.

RESULTS

Characteristics of the Population

The study cohort comprised a total of 770 kidney allograft biopsies from 540 patients collected between 2006 and 2021 from 11 international European centers (See **Supplementary Material**). Baseline characteristics including recipient and donor characteristics are summarized in **Table 1**. The population was mainly composed of men (n = 336, 63.2%) with a mean age of 51.1 ± 15.9 years at the time of transplantation, a history of glomerulonephritis as end stage renal disease (n = 125, 23.2%) and no history of a prior kidney transplant (n = 435,

TABLE 2 | Histological, immunological and functional characteristics at the time of biopsy.

	Included samples (n = 770)	N
Histological characteristics		
Time since transplantation (months), Median (IQR)	3.90 [2.97; 12.1]	770
Banff scores		
g score > 0, No. (%)	109 (14.8)	737
ptc score > 0, No. (%)	133 (18.2)	730
i score > 0, No. (%)	141 (19.1)	738
t score > 0, No. (%)	203 (27.5)	739
v score > 0, No. (%)	31 (3.8)	697
cg score > 0, No. (%)	47 (6.4)	734
cv score > 0, No. (%)	445 (66.4)	670
ci score > 0, No. (%)	420 (57.1)	736
ct score > 0, No. (%)	435 (59.2)	734
ti score > 0, No. (%)	206 (29.6)	696
i-IFTA score > 0, No. (%)	117 (18.7)	626
t-IFTA score > 0, No. (%)	13 (4.2)	312
ah score > 0, No. (%)	416 (57.8)	721
aah score > 0, No. (%)	40 (22.9)	175
mm score > 0, No. (%)	59 (8.3)	709
C4d score > 0, No. (%)	108 (15.2)	710
Diagnosis according to pathologist		
Normal, No. (%)	152 (20.6)	738
Borderline, No. (%)	69 (9.4)	737
T-cell mediated rejection, No. (%)	72 (9.9)	724
Antibody-mediated rejection, No. (%)	88 (12.0)	736
IFTA positive, No. (%)	365 (49.6)	736
Recurrent nephropathy, No. (%)	15 (2.0)	754
De novo glomerulonephritis, No. (%)	18 (2.4)	752
Acute tubular necrosis, No. (%)	68 (9.1)	749
Polyomavirus nephropathy, No. (%)	30 (4.0)	748
CNI toxicity, No. (%)	65 (8.7)	748
Thrombotic microangiopathy, No. (%)	34 (4.6)	748
Immunological characteristics		
Anti-HLA DSA, No. (%)	226 (31.3)	722
Anti-HLA DSA class		221
I, No. (%)	43 (19.5)	
II, No. (%)	114 (56.1)	
I and II, No. (%)	64 (29.0)	
Anti-HLA iDSA MFI, Mean (SD)	3,229 (4,060)	219
Functional characteristics		
Proteinuria (g/g), Median (IQR)	0.20 [0.10; 0.41]	736
eGFR (MDRD), Mean (SD)	42.8 (19.1)	727

eGFR, was calculated according to the MDRD, formula without the 1.21 ethnicity and 0.94 standardized creatinine factors. Abbreviations: (i) DSA: (immunodominant) donor specific antibody; eGFR: estimated glomerular filtration rate; HLA: human leucocyte antigen; IFTA: interstitial fibrosis and tubular atrophy; MFI: mean fluorescence intensity.

80.9%). The majority of the transplantations were performed from deceased donors (n = 439, 81.5%) with 185 (42.1%) exhibiting expanded criteria. In total, 141 (27.9%) patients had pre-existing anti-HLA DSA.

The median time from transplantation to the biopsy was 3.9 months (IQR: 3.0–12.1) (**Supplementary Figure S1**) with 460 (60%) protocol biopsies and 370 (40%) for cause biopsies. The mean number of biopsy per patient was 1.4 (median = 1), with a maximum of 5 biopsies per patient. The mean eGFR at the time of the biopsy was 42.8 ± 19.1 mL/min/11.73 m^2 with a mean proteinuria of 0.53 ± 1.58 g/g. One-third of the patients (n = 226, 31.3%) had positive anti-HLA DSA with the immunodominant DSA belonging mainly to the class II (n = 148, 67.6%) (**Table 2**).

Histological Phenotypes

Kidney allograft biopsies were either classified as normal (n = 152, 20.6%) or had histological evidence for one or multiple of the following diagnoses: T-cell mediated rejection (n = 72, 9.9%), antibody-mediated rejection (n = 88, 12.0%), mixed rejection 14 (10.6%), borderline rejection (n = 69, 9.4%), interstitial fibrosis and tubular atrophy (n = 365, 49.6%), recurrence of the initial nephropathy (n = 15, 2.0%), *de novo* or recurrent glomerulonephritis (n = 18, 2.4%), acute tubular necrosis (n = 68, 9.1%), polyomavirus-associated nephropathy (n = 30, 4.0%), calcineurin inhibitors-related toxicity (n = 65, 8.7%), and thrombotic microangiopathy (n = 34, 4.6%) (**Table 2**).

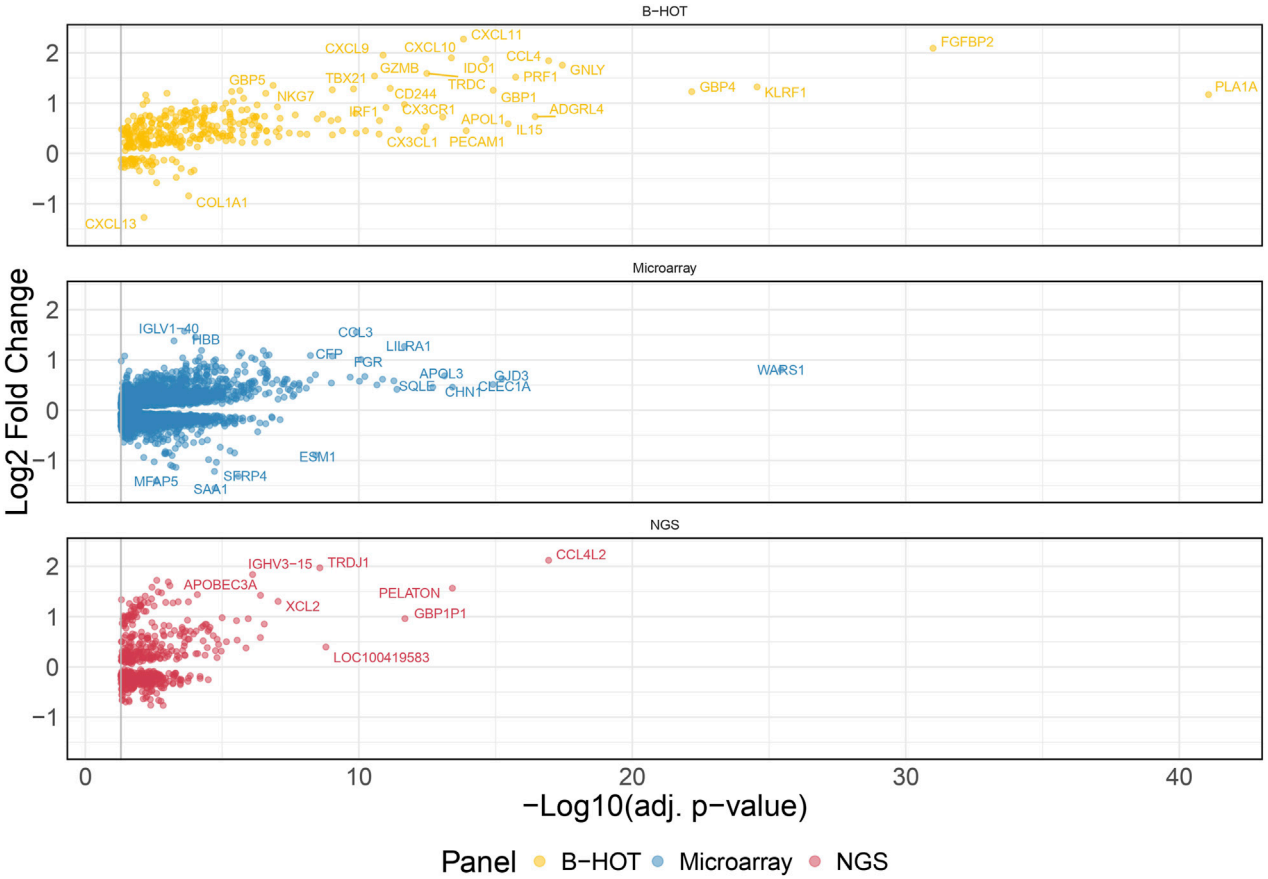


FIGURE 1 | Antibody-mediated rejection molecular signature. Volcano plot of the significant differentially expressed genes associated with antibody-mediated rejection. Dots are related to each gene. The significant transcripts are displayed according to a 0.05 threshold on the adjusted *p*-value (vertical grey line). NGS-specific transcripts are highlighted in red, B-HOT-related in yellow and microarray-related in blue. *X*-axis represents the -$\log_{10}$ of the adjusted *p*-value (the higher, the smaller is the *p*-value) and the *y*-axis represents the $\log_2$ fold change. Differences in gene expression between the AMR and non-AMR group are marked with positive (negative) values correspond to up- (down-)regulated transcripts in the AMR group. In total, 6,141 genes were differentially expressed showing the following distribution: 358 included in the B-HOT gene panel, 5,180 included in the microarray gene panel and 603 NGS-specific. Abbreviations: AMR: antibody-mediated rejection; B-HOT: Banff Human Organ Transplant; NGS: next-generation sequencing.

Molecular Landscape of Antibody-Mediated Rejection

60 AMR were compared to 576 non-AMR samples, resulting in 6,141 differentially expressed genes (DEGs). 358 (5.8%) were included in the Banff Human Organ Transplant (B-HOT) gene panel, 5,180 (84.4%) were included in the microarray gene panel, and 603 (9.8%) were new and defined as NGS-specific transcripts (**Figure 1** and **Supplementary Material**). Genes included in the microarrays were highly represented throughout the entire molecular signature (from 0.0% to 84.4% among the increasing top ranked genes and stabilizing at top 2,500 genes), while the B-HOT-related genes were mainly ranked in the top genes (from 100.0% to 5.8%%, reaching 47.0% among the top 100 genes, 25.4% among the top 500 genes and 17.0% among the top 1,000 genes), and the new NGS-specific genes were constantly comprised across the signature between 5.2% and 12.5% of the total (**Supplementary Figure S2**). Among the top 30 ranked genes, 20 genes (66.7%) were included in the B-HOT gene panel including *PLA1A*, *GBP1/4*, *GNLY*, *CCL4*, *IL15*, *IDO1*, *CXCL10/11*, 7 (23.3%) genes were included in the microarrays panel (*WARS1*, *GJD3*, *CLEC1A*, *CHN1*, *APOL3*, *SQLE*, *LILRA1*), and 3 genes (10%) were specific to the NGS gene panel with *CCL4L2*, *PELATON* (a long non-coding RNA) and *GBP1P1* (**Supplementary Table S2**).

The list of upregulated and differentially expressed genes was composed of 2,876 genes from which 2,299 (79.9%) were included in the microarrays, 313 (10.9%) were included in the B-HOT panel, and 264 (9.2%) were NGS-specific. Pathway analysis was performed using the Reactome repository. Top ranked (adj.*p*-value<0.05) known pathological categories were related to immune response: interferon signaling (q-value = $1.78e^{-11}$), neutrophil degranulation (q-value = $4.57e^{-11}$), signaling by interleukins (q-value = $4.11e^{-10}$), Toll-like receptors cascades (q-value = $6.80e^{-06}$), class I MHC mediated antigen processing and presentation (q-value = $1.37e^{-05}$), Fc Gamma/Fc Epsilon

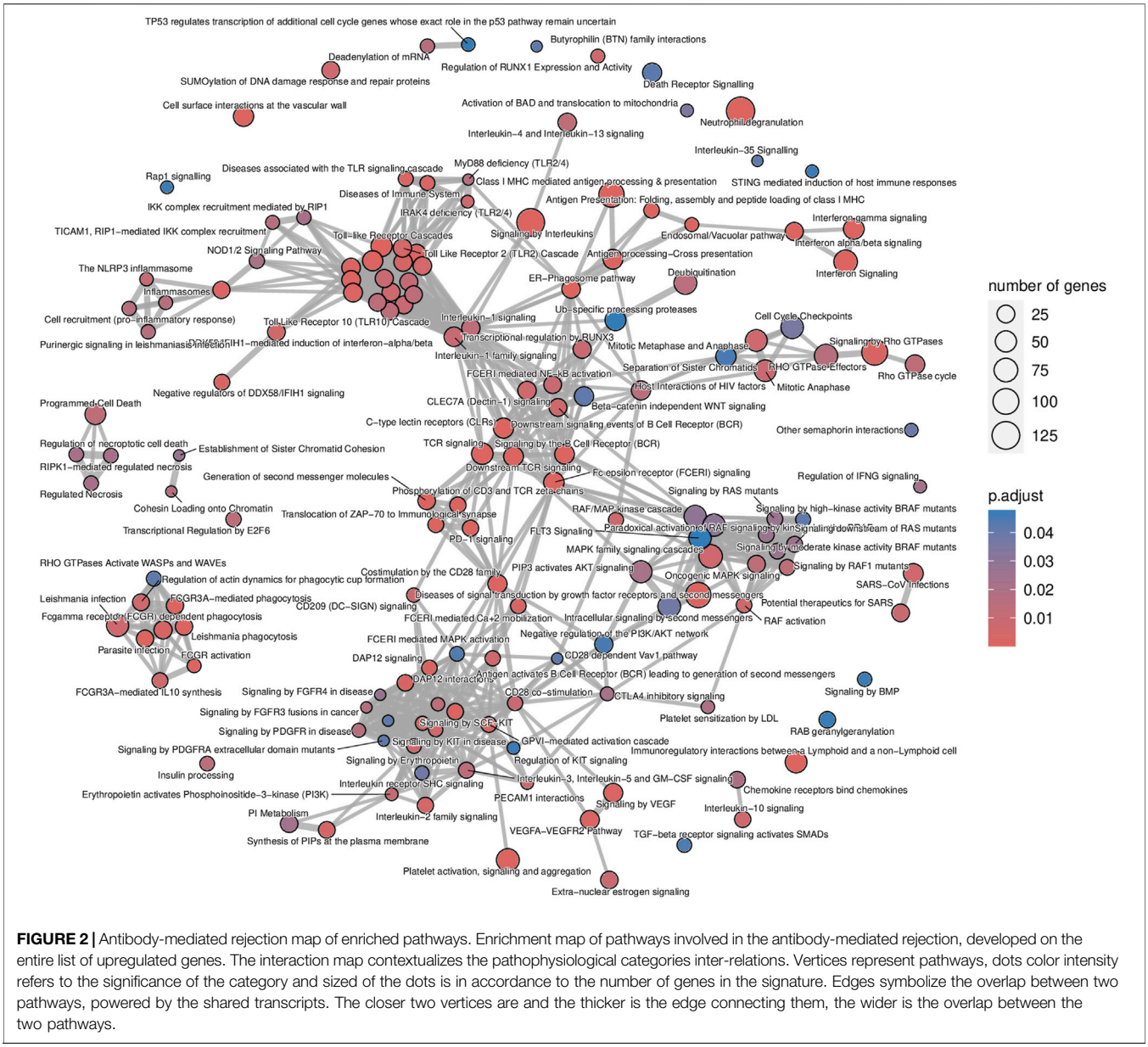


FIGURE 2 | Antibody-mediated rejection map of enriched pathways. Enrichment map of pathways involved in the antibody-mediated rejection, developed on the entire list of upregulated genes. The interaction map contextualizes the pathophysiological categories inter-relations. Vertices represent pathways, dots color intensity refers to the significance of the category and sized of the dots is in accordance to the number of genes in the signature. Edges symbolize the overlap between two pathways, powered by the shared transcripts. The closer two vertices are and the thicker is the edge connecting them, the wider is the overlap between the two pathways.

receptors (q-value = $2.18e^{-04}$ and q-value = $2.15e^{-05}$, respectively), signaling by the BCR (q-value = $5.40e^{-05}$), cell surface interactions at the vascular wall (q-value = $2.11e^{-04}$) and PECAM1 interactions (q-value = $8.14e^{-03}$). In addition, the TCR signaling (q-value = $1.78e^{-11}$), the PD-1 signaling (q-value = $3.50e^{-08}$), the CD28 co-stimulation (q-value = $7.43e^{-03}$) and the CTLA4 inhibitory signaling (q-value = $1.78e^{-02}$) pathways were found significantly enriched in the AMR signature (**Figure 2**; **Supplementary Figure S3** and **Supplementary Material**). Enrichment analysis derived from the KEGG database demonstrated additional significant pathways including the NK cell mediated cytotoxicity (q-value = $2.44e^{-10}$), Th17 cell differentiation (q-value = $2.98e^{-10}$) and Th1 and Th2 cell differentiation (q-value = $4.97e^{-07}$), and provided access to the entire set of cytokine and receptors (CCL4, CCL11, CXCL5/6/9/10/11, XCL2, IL15/16/27/34/35, TNF, TGFβ) and cell adhesion and endothelium-related molecules (CD58, MHCI/II, CD40, ITGA, CD2, CD4, PD-L1, CDH5, PECAM1) involved during antibody-mediated rejection (**Supplementary Figures S4 and S5** and **Supplementary Material**).

The analysis of enriched pathways restrained to the different panels highlighted specific functions. The B-HOT panel captured all the above-mentioned significant functions with a total of 191 entries in Reactome (**Supplementary Figure S6** and **Supplementary Material**) while the microarray panel was specifically enriched in SUMOylation processes, RHO/RAC GTPase cycle, cell cycle progression and FCGRIIIA-mediated phagocytosis with only 44 entries (**Supplementary Figure S7**

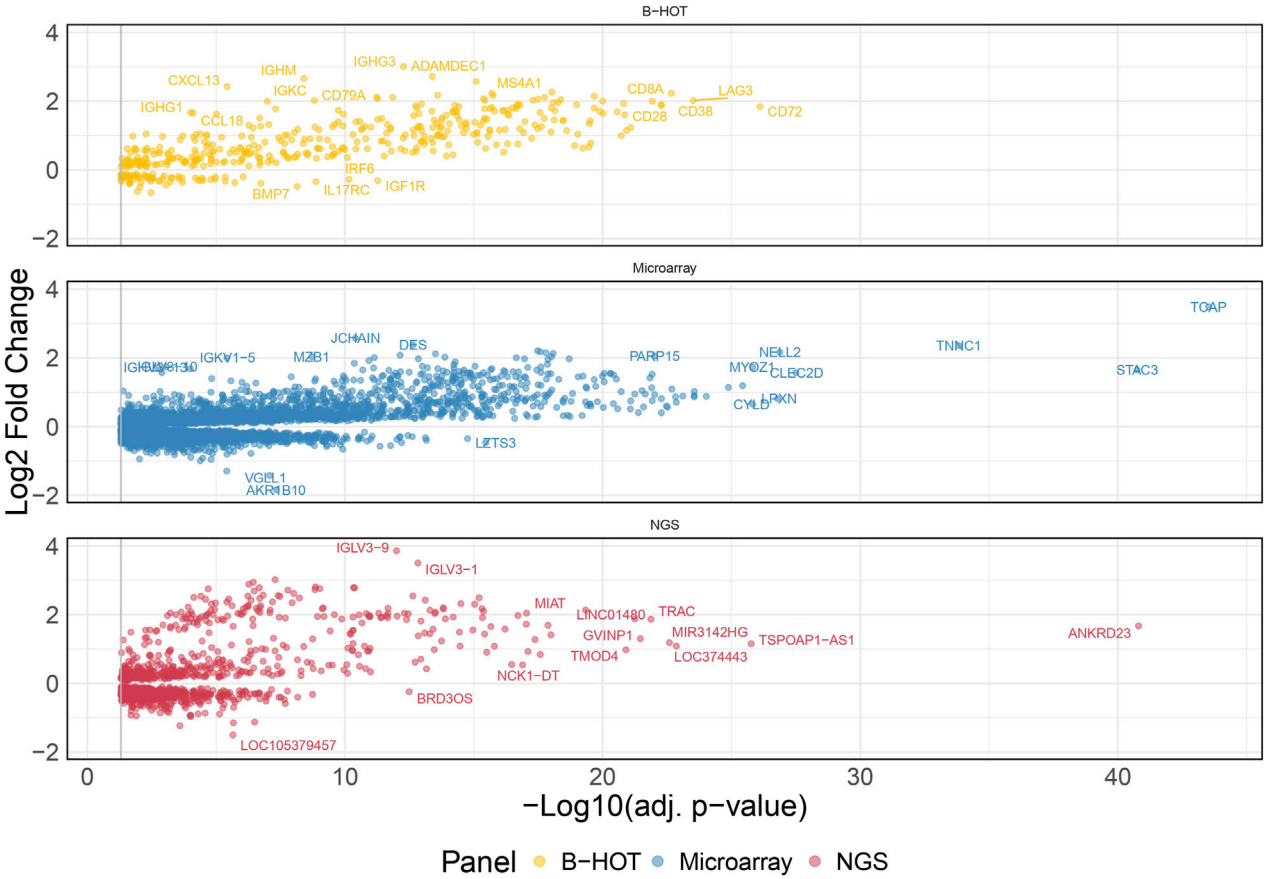


FIGURE 3 | T-cell mediated rejection molecular signature. Volcano plot of the significant differentially expressed genes associated with T-cell mediated rejection. Dots are related to each gene. The significant transcripts are displayed according to a 0.05 threshold on the adjusted *p*-value (vertical grey line). NGS-specific transcripts are highlighted in red, B-HOT-related in yellow and microarray-related in blue. *X*-axis represents the -$\log_{10}$ of the adjusted *p*-value (the higher, the smaller is the *p*-value) and the *y*-axis represents the $\log_2$ fold change. Differences in gene expression between the TCMR and non-TCMR group are marked with positive (negative) values correspond to up- (down-)regulated transcripts in the TCMR group. In total, 8,478 genes were differentially expressed showing the following distribution: 439 included in the B-HOT, 6,853 included in the microarray and 1,186 NGS-specific. Abbreviations: B-HOT: Banff Human Organ Transplant; NGS: next-generation sequencing; TCMR: T-cell mediated rejection.

and **Supplementary Material**). Finally, despite its 264 upregulated DEGs, the NGS-specific genes were only enriched in 4 non-specific metabolic functions (**Supplementary Figure S8**).

Molecular Landscape for T-cell Mediated Rejection

48 TCMR were compared to 589 non-TCMR samples and the molecular signature was defined by 8,478 genes. 439 (5.2%) were included in the B-HOT panel, 6,853 (80.8%) were included in the microarrays and 1,186 (14.0%) were NGS-specific (**Figure 3**). After ranking genes by their adjusted *p*-value, the proportions of transcripts included with each gene panel were mostly in favor of the microarray panel (from 100.0% to 80.8% with a local minimum of 60.8% among the top 265 genes). The proportion of genes included in the B-HOT first increased to reach a maximum of 30.5% among the top 118 genes, before decreasing to reach a minimum of 5.1%. Except among the top 5 genes, the newly discovered NGS genes were relatively stable (between 7.6% and 18.1%) (**Supplementary Figure S9**). Among the top 30 ranked genes, 22 (73.4%) were comprised in the microarray gene panel, 4 (13.3%) were comprised in the B-HOT panel (*CD72*, *LAG3*, *CD8A*, *CD28*) and 4 (13.3%) were NGS-specific (*ANKRD23*, *TSPOAP1-AS1*, *LOC374443*, *MIR3142HG*) (**Supplementary Table S3**).

The list of upregulated differentially expressed genes was composed of 4,482 genes from which 3,612 (80.6%) were included in the microarrays, 367 (8.2%) were included in the B-HOT panel and 503 (11.2%) were NGS-specific. Using the entire list of upregulated genes, significantly immunological Reactome enriched pathways comprised pathways related to: interferon signaling (q-value = $1.76e^{-22}$), signaling by ROBO receptors (q-value = $2.66e^{-22}$), TCR signaling (q-value = $2.40e^{-17}$), class I MHC mediated antigen processing and presentation (q-value = $1.88e^{-16}$), signaling by interleukins (q-value = $2.42e^{-16}$), signaling by the BCR (q-value = $8.04e^{-13}$),

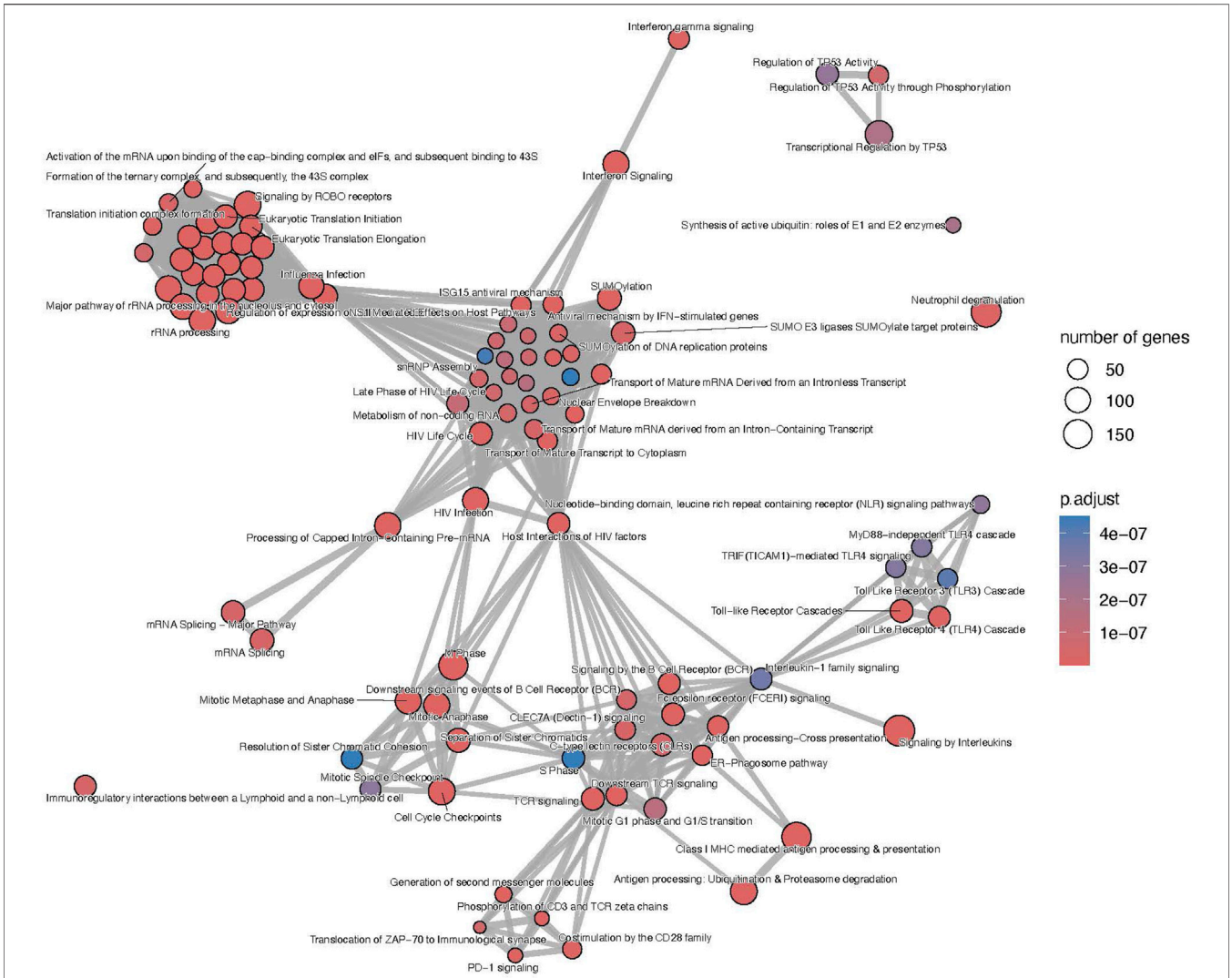


FIGURE 4 | T-cell mediated rejection map of enriched pathways. Enrichment map of pathways involved in the antibody-mediated rejection, developed on the entire list of upregulated genes. The interaction map contextualizes the pathophysiological categories inter-relations. Vertices represent pathways, dots color intensity refers to the significance of the category and sized of the dots is in accordance to the number of genes in the signature. Edges symbolize the overlap between two pathways, powered by the shared transcripts. The closer two vertices are and the thicker is the edge connecting them, the wider is the overlap between the two pathways. Only the top 100 pathways are displayed to improve readability.

Fc Epsilon receptor signaling (q-value = $1.79e^{-11}$) and Fc Gamma receptor dependent phagocytosis (q-value = $1.09e^{-04}$), TLR cascades (q-value = $9.18e^{-10}$), co-stimulation by the CD28 family (q-value = $3.88e^{-09}$), PD-1 signaling (q-value = $4.14e^{-09}$) and neutrophil degranulation (q-value = $6.69e^{-09}$). Out of the 466 Reactome entries, emphasis was also given on nonsense mediated decay and maturation of mRNA functions, SUMOylation processes, metabolism of non-coding RNA and cell cycle progression (**Figure 4** and **Supplementary Material**). The KEGG repository significantly presented enrichment of the Th17 cell differentiation (q-value = $4.72e^{-14}$), Th1 and Th2 cell differentiation (q-value = $8.13e^{-12}$), and NK cell mediated cytotoxicity (q-value = $1.10e^{-08}$). A wider range of activation/inhibition of cell adhesion molecules was also presented with lower/higher fold changes (min = −1.8, max = 3.9) compared to the AMR signature. The TCMR signature included the addition of CD22, PDCD1 and SELL and the inhibition of a multitude of molecules at the surface of the endothelial cells (**Supplementary Figure S10** and **Supplementary Material**).

Focusing on the different gene panels, the genes included in the B-HOT panel captured all the immunological functions described previously (**Supplementary Figure S11** and **Supplementary Material**) while the microarray genes specifically captured the nonsense mediated decay, SUMOylation, translation and mRNA maturation processes and the cell cycle progression (**Supplementary Figure S12** and **Supplementary Material**). Finally, with 503 upregulated genes, no enriched pathways were annotated for the new NGS-specific genes.

DISCUSSION

In this study we aimed at defining and describing the molecular profiles and biological functions associated with antibody-mediated and T-cell mediated rejection, combining a deeply phenotyped cohort of kidney allograft biopsies and next-generation sequencing analyses. For this purpose, we used the histological labels and the gene expressions as inputs for a differential expression analysis and ranked the significant genes according to the adjusted *p*-values. We, then, queried publicly available biological databases to understand the pathophysiological mechanisms derived from the upregulated DEGs. In this study, an emphasis was made to discriminate genes from known gene panels (B-HOT and microarray) already validated and used in clinical practice [23–26] and new genes discovered using the NGS technology.

In the present study, active antibody-mediated rejection was found in 9.4% of the analyzed samples. This incidence aligns well with the most recently reported incidence of AMR ranging from 3% to 12% in a recent systematic review including 28 studies [27]. Its molecular signature included features of macrophages activation (*CD40*, *CD58*, *IDO1*), NK cells activation (*GNLY*, *FGFBP2*, *CD16a*), cytotoxic T cells activation (*CD8*), helper T cells activation (*CD4*), endothelial cells activation (*ICAM1*, *PECAM1*, *VCAM1*, *CDH5*), and B cells activation (*CD22*, *CD40*, *CD86*), which showed great consistency with the microarray studies [28, 29]. From both innate and adaptive immune systems, the enrichment analysis confirmed the ability of the B-HOT gene panel to capture both components occurring during rejection but presented a lack of metabolic functions, such as SUMOylation processes and cell cycle progression and checkpoint that are specifically present in the microarrays. Regarding the NGS-specific gene panel, 603 new genes (comprising 264 upregulation) were found associated with AMR but no annotation was available in the public repositories. They were mainly composed of long non-coding RNAs that are poorly described in the literature. *PELATON*, for instance, was part of the new NGS-specific top ranked genes and was found to be a regulator specifically located in macrophages and monocytes nucleus, for which the downregulation is associated to decreased phagocytosis functions [30]. In this study, we found that *PELATON* was upregulated during AMR ($\log_2$FC = 1.56), in line with a probable increased phagocytosis function occurring in the microcirculation inflammation and, consequently, potentially leading to increased differentiation into antigen-presenting cells, T-cell recruitment and activation and, ultimately, B-cell proliferation and transformation into plasma cells.

Compared to the AMR signature, the TCMR signal presented a similar profile compared to the published studies in terms of genes (*CD72*, *LAG3*, *CD8A*, *CD28*, *ANKRD* family) and activated cell types and functions [31]. However, a key difference existed in the repertoire of inhibited cell adhesion molecules, showing strong inhibition of the endothelial and epithelial cells receptors, emphasizing the cell infiltration observed at the histological level. Regarding the different gene panels, the microarray was specific of mRNA maturation processes and nonsense mediated decay, which could be due to a lack of annotation of the different repositories. In our study, the B-HOT panel was enriched by the main immunological functions but did not include the top adjusted *p*-value ranked genes, potentially limiting its ability to accurately diagnose TCMR. The addition of new genes, for example, from the microarray or discovered with the NGS technology, could potentially help the molecular classifiers. Finally, for the NGS-specific markers, they were composed of lncRNA which lacked annotation in the current repositories. Few of them are described in the literature such as MIR3142HG which was shown to be a positive regulator of IL-8 and CCL2 [32].

The main advantage of the present study is that the cohort's diverse phenotypes encompass most of the clinical scenarios encountered in routine practice. It also gathered samples and patients representing a real-life setting in terms of population demographics, rejection prevalence and immunosuppression therapies. Lastly, this is, to our knowledge, the first RNA-seq experiment applied in such cohort characteristics (size, heterogeneity, description) to study the molecular signature of rejection in kidney allograft biopsies. A literature review on PubMed comprising the key words "NGS," "transplantation," "kidney" and "rejection" resulted in 46 articles published over the last 5 years: 11 (23.4%) were related to cell-free DNA, 9 (19.6%) were related to infections (comprising also BK virus), 4 (8.7%) were focusing on cell subpopulations, 5 (10.9%) were related to response to treatment and 5 (10.9%) were related to HLA matching. Five references mentioned either the use of NGS or the B-HOT gene panels but showed limitations in the number of patients/biopsies, number of genes under study, in their design (sick vs. well, single centre), or in the representativity of the different diagnoses [33–37].

Regarding the study limitations, one of the main issues is the sampling bias regarding the technique requiring an extra core. The sequenced core might be different from the one analyzed by the pathologist both in terms of quality (i.e., number of glomeruli and arteries) and severity of the disease, which is not the case for the Nanostring technology and the B-HOT gene panel where an extra core is not needed. Second, while NGS might help to discover new genes and physio-pathological pathways, its use in clinical practice is limited in terms of access to the technology and its cost. In our study, most of the genes associated with AMR and TCMR were included in the microarray and the B-HOT-gene panels, validating the relevance and the accuracy of the genes included. Finally, from a clinical aspect, our cohort was mainly treated with corticosteroids, mycophenolic acid and tacrolimus, which might have an impact on the observed molecular expressions. The presented results should be validated on patients treated with different types of immunosuppressive therapies including mTOR inhibitors or Belatacept.

CONCLUSION

We discovered 9.8% and 14.0% novel transcripts associated with antibody-mediated rejection and T-cell mediated rejection, respectively. The main immunological functions were positively captured by both the microarray and B-HOT gene panels. Those new NGS specific transcripts might represent a novel source of targets for drug designing and repurposing.

DATA AVAILABILITY STATEMENT

The complete list of activated pathways and differentially expressed genes are available in the synapse public Synapse database (https://www.synapse.org/Synapse:syn60959798/files/).

ETHICS STATEMENT

The Protocol, informed consent form, and other required documents have been approved by the Ethics Committees of all participating countries before enrolment of subjects in the study. AP-HP, as sponsor, obtained prior approval from the CPP (French Ethic committee) for its Minimal Risk and Restriction research studies, within the scope of the Board's authority and in accordance with statutory and regulatory requirements, under the IDRCB number 2018-A00733-52 (EU-TRAIN) and 2018-A00090-55 (KTD-Innov).

AUTHOR CONTRIBUTIONS

AL, SB, CL, and OA designed the study. OA, ECG, AG, CU-D, AL performed the data analysis and wrote the manuscript. ECG, AG, CU-D, PD, LA, MRac, CC, VG, FMe, JD, OB, FH, FMo, JV, KB, MG, SB, RD, P-AG, MRab, LC, MQ, NK, EM, FV, J-LT, RS, CLeg, DA, CLef, AL, and OA contributed to data acquisition and interpretation. OA, ECG, AG, CU-D and AL interpreted the data. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

Author PG was employed by GenoSplice.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13043/full#supplementary-material

Supplementary Data Sheet S1 | AMR signature.

Supplementary Data Sheet S2 | TCMR signature.

Supplementary Data Sheet S3 | AMR (B-HOT panel) Reactome pathways.

Supplementary Data Sheet S4 | AMR (microarray panel) Reactome pathways.

Supplementary Data Sheet S5 | AMR Reactome pathways.

Supplementary Data Sheet S6 | TCMR (B-HOT panel) Reactome pathways.

Supplementary Data Sheet S7 | TCMR (microarray panel) Reactome pathways.

Supplementary Data Sheet S8 | TCMR Reactome Pathways.

Supplementary Data Sheet S9 | AMR KEGG pathways.

Supplementary Data Sheet S10 | TCMR KEGG pathways.

Supplementary Data Sheet S11 | Supplementary Appendix.

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ORIGINAL RESEARCH
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Clinical Impacts of Allograft Biopsy in Renal Transplant Recipients 10 Years or Longer After Transplantation

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We aimed to investigate the clinical value of allograft biopsy performed long after renal transplantation. We retrospectively evaluated 99 allograft biopsies in recipients with transplantation vintages of 10 years or longer. Mixed-effects model showed that 1-year estimated glomerular filtration rate (eGFR) slopes after biopsy were significantly greater than those before biopsy [–3.13, –4.42 mL/min/1.73 m^2/year, p = 0.01]. Renal biopsy changed the treatment strategies in more than half of the patients. Improvement in eGFR slopes was pronounced in 51 patients with treatment modification based on the biopsy results [2.27 (95% confidence interval (CI): 0.66, 3.89) mL/min/1.73 m^2/year], whereas no improvement was observed in those without [0.33 (95% CI: –1.05, 1.71) mL/min/1.73 m^2/year, $P_{interaction}$ = 0.001]. Among the treatment modifications, enhancement of immunosuppression (IS) led to the most remarkable improvement in eGFR slope. Patients with g scores ≥2 were more likely to receive IS enhancement than those with g scores = 0 [odds ratio; 15.0 (95% CI: 1.65, 136)]. Patients with active glomerulitis (g ≥ 1) without chronicity (cg ≤ 1) showed the most significant improvement in eGFR slope. Given the prevalence of active glomerulitis (g ≥ 1, 21%), which is responsive to treatment even long after transplantation, and the observed magnitude of eGFR slope improvement, renal biopsy can indeed improve allograft prognosis.

Keywords: allograft biopsy, Banff score, eGFR slope, graft function, pathology, kidney transplantation

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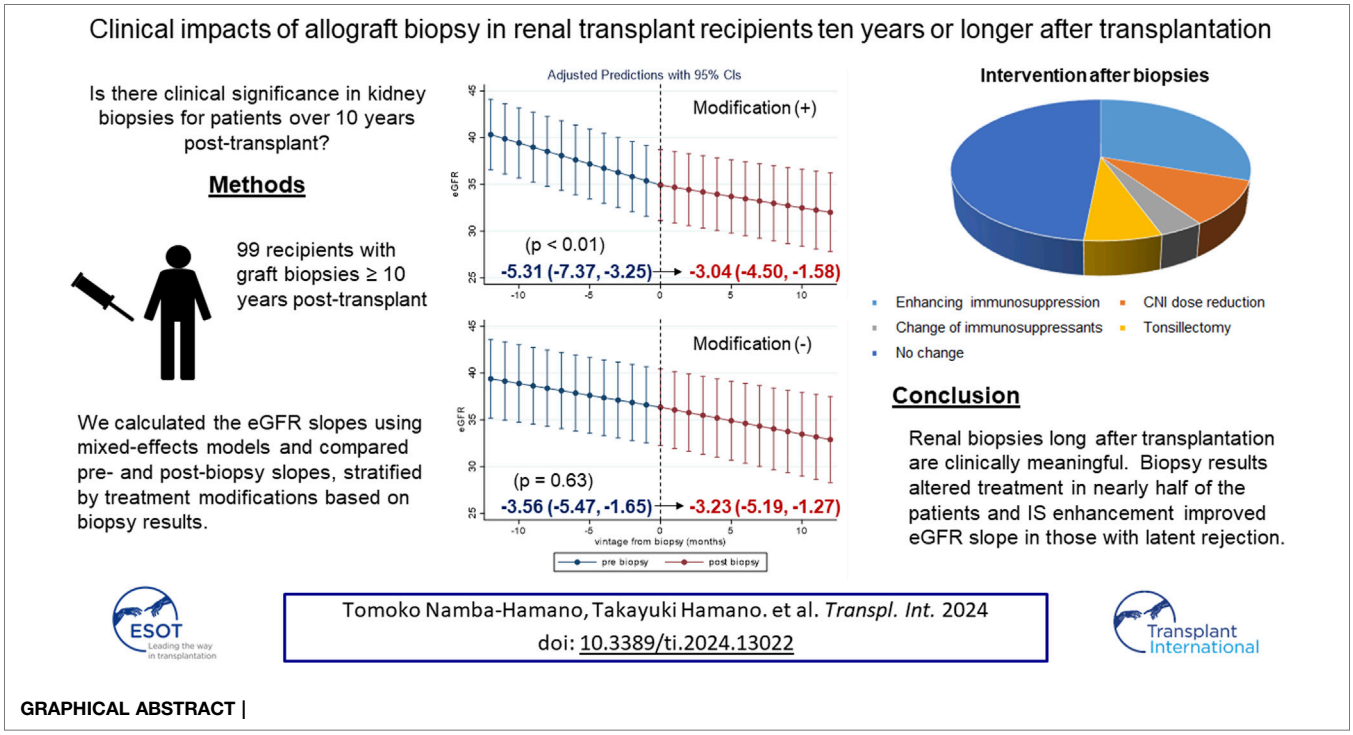
Abbreviations: ABMR, antibody-related rejection; ANOVA, analysis of variance; CI, confidence interval; CNI, calcineurin inhibitor; CsA, cyclosporine A; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen; IgAN, immunoglobulin A nephropathy; IQR, interquartile range; IS, immunosuppression; IFTA, interstitial fibrosis and tubular atrophy; KDIGO, Kidney Disease: Improving Global Outcomes; KTR, kidney transplant recipient; MMF, mycophenolate mofetil; PSL, prednisolone; TAC, tacrolimus; TG, transplant glomerulopathy; UACR, urine albumin-to-creatinine ratio.



GRAPHICAL ABSTRACT |

INTRODUCTION

Introduction of novel immunosuppressive agents has significantly improved graft outcomes in kidney transplant recipients (KTRs) since the 1980s. While the 1-year graft survival rate is over 90%, more than 10% of KTRs in the US and European countries lose their graft function by 5 years post-transplantation. Therefore, improving the long-term graft survival rate remains challenging [1, 2]. Furthermore, allograft kidney dysfunction is caused by immunological factors, such as rejection, and non-immunological factors, such as hypertension, glucose intolerance, and donor age [3]. In addition, allograft kidney dysfunction can be driven by conditions unique to transplantation, such as drug toxicity, infection, and recurrent nephritis [4].

Allograft renal biopsy plays an important role in differentiating between these conditions. In particular, indication biopsy plays an essential role in diagnosing rejection in an early phase. Immunosuppressive therapy for early rejection diagnosed by biopsy led to better graft outcomes subsequently, especially in patients in whom early intervention recovered the renal function to near baseline [5]. However, it remains unknown whether allograft biopsy long after transplantation can positively affect renal allografts through treatment modification. Although the risk of T cell-mediated rejection was reported to be relatively low in long-term transplanted grafts, the causes of graft loss were revealed to be multifactorial and more complex than expected previously [6]. Allograft biopsy performed long after transplantation might contribute to diagnosing its etiology, thus improving graft outcomes. However, there have been few reports focusing on renal biopsies performed at 10 years or longer after transplantation. Therefore, we aimed to examine the clinical impacts of renal biopsies in those transplant recipients. Specifically, we addressed the following three issues: 1) whether indication biopsy, performed more than 10 years after transplantation, improves graft prognosis, 2) whether the treatment changes based on renal biopsy results could lead to improvement in renal function after renal biopsy, and 3) the pathological findings associated with an eGFR improvement. In this study, since most patients were proteinuria-negative, we used eGFR slope [7] instead of urine albumin-to-creatinine ratio (UACR) reduction as a surrogate outcome.

PATIENTS AND METHODS

Study Design and Population

We conducted a multicenter retrospective observational study at Osaka University Hospital, Inoue Hospital Attached Clinic, and Takatsuki General Hospital in Osaka, Japan. We enrolled KTRs, who had undergone indication biopsies of allografts between March 2002 and January 2019 and had transplantation vintages of 10 years or longer at the biopsy.

In patients who underwent multiple biopsies more than 10 years post-transplant, the first biopsy conducted after 10 years was used for analysis. We excluded the patients who received additional immunosuppressive therapy immediately before renal biopsy. The study was conducted in accordance

with the principles of the Declaration of Helsinki. The Ethics Committee of Osaka University Hospital approved the study and waived the need for informed consent because of the retrospective study design (approval number: 17334-3). In addition, we provided the patients with the option to opt out of participation whenever during the study.

Data Collection

Patient characteristics at the time of graft biopsy were collected as baseline data. In addition, information regarding transplantation, including transplantation vintage, donor characteristics (age at transplantation, sex, and living or cadaveric donor), simultaneous pancreas-kidney transplantation, immunological factors (ABO compatibility and human leukocyte antigen (HLA)-matching status), and dialysis vintage before transplantation, was collected. According to the Banff 2017 classification [8], except for i-IFTA, biopsy samples were re-scored by a renal pathologist (T.N.). The i-IFTA score, a score of the chronic active T-cell-mediated rejection component, was evaluated retrospectively according to Banff 2019 [9]. A microvascular inflammation (mvi) score was defined as a combination of the glomerulitis score (g) and peritubular capillaritis score (ptc) according to the Banff 2013 classification [10]. The eGFRs were calculated using the following Japanese standard formula: $194 \times \text{creatinine}^{-1.094} \times \text{age}^{-0.287}$ (if female, ×0.739) [11].

Statistical Analyses

Data are presented as numbers (percentages) for categorical variables, means (standard deviations) for normally distributed variables, or medians (interquartile ranges [IQR]) for skewed variables. We compared the baseline characteristics between KTRs with and without treatment modification based on the biopsy results. The means of normally distributed variables and the proportion of each category were compared using the Student's t-test and Fisher's exact test, respectively. The Wilcoxon rank-sum test was used to compare the distributions of categorical variables. Differences in continuous variables across groups were tested using a one-way analysis of variance (ANOVA).

The study outcome was the improvement in 1-year eGFR slope after allograft biopsy, defined as (post-biopsy eGFR slope–pre-biopsy eGFR slope). For pre-biopsy and post-biopsy eGFR slope calculations, we used the eGFR data of the preceding 1 year before the index biopsy and the 1-year follow-up data after biopsy. We separately estimated the eGFR slopes for each individual in the pre- and post-biopsy periods using linear mixed-effects models. Random intercepts and time slopes were included to determine the eGFR trajectory in these models. As a primary analysis, we compared the eGFR slopes for the two periods using paired t-tests in all patients and in those stratified by treatment modification after biopsy. For sensitivity analysis, we created a linear mixed-effects model with time-dependent eGFR as the dependent variable to investigate whether eGFR improvement after biopsy differed between patients with and without treatment modifications. In this model, we included a 3-way interaction term among continuous-time, categories (pre-and post-biopsy), and treatment modification in addition to continuous-time, categories, and treatment modifications as fixed effects. The two-way interaction between time and the pre- or post-biopsy period would explain whether the effect of time on eGFR (eGFR slope) was different across biopsy periods. The addition of the 3-way interaction term to the model allowed us to explore whether treatment modification modifies eGFR slope difference across the biopsy period.

Logistic regression models were employed to analyze the Banff scores associated with IS enhancement. Covariates in the multivariate analysis included sex, recipient age, and eGFR at the time of biopsy.

Statistical significance was set at $p < 0.05$. All the statistical analyses were performed using Stata version 16 (StataCorp, College Station, TX, United States).

RESULTS

Clinical Characteristics of Participants

Figure 1 shows a flowchart of the participant selection process. Between March 2002 and January 2019, 1,638 patients underwent allograft biopsy. Among them, 106 had a transplantation vintage of 10 years or longer. In addition, seven patients received additional IS for probable rejection just before the biopsy. Therefore, 99 patients were eligible for this study based on all the exclusion criteria.

Table 1 shows the baseline characteristics of all the 99 patients. The median age, eGFR, and proteinuria at biopsy were 50 years, 34.8 mL/min/1.73 m^2, and 0.49 g/day, respectively. The median transplantation vintage at biopsy was 13.7 years. Of the 99 patients, 78 (79%) underwent living-donor kidney transplantation. More than half of the living donors were recipients' mothers (52.6%), followed by their fathers (14.1%). The remainder, except for one case, were kidney transplants from deceased donors, five of which were simultaneous pancreas-kidney transplants. There were five (5.1%) ABO-incompatible transplants. Ninety percent of the patients received steroid-based IS supplemented with calcineurin inhibitors (CNI), mostly cyclosporine A (CsA). The mean trough levels of cyclosporin users were 62.3 ± 17.8 ng/mL, and that of tacrolimus users were 3.04 ± 1.20 ng/mL. Mycophenolate mofetil (MMF) was used most commonly as an antimetabolic agent. Sixteen patients had been diagnosed with acute rejection prior to this study. All cases were of acute cellular rejection, and no patients had acute humoral rejection. No cases had been diagnosed with BK virus-associated nephropathy among the subjects included in the analysis.

In nearly half of the patients (51%), the doctors in charge changed their treatment strategies based on the biopsy diagnosis. Patients were divided into two groups according to treatment modification. Baseline characteristics of both groups were similar, except for age (**Table 1**). Details of the treatment modifications are described as follows. Enhancing immunosuppressive therapy was the most common treatment modification (30 patients; 58.8%), followed by a reduction in CNI doses in 9 patients (19.6%) and a change in immunosuppressive agents in 4 patients (7.8%). Enhancing immunosuppressive therapy

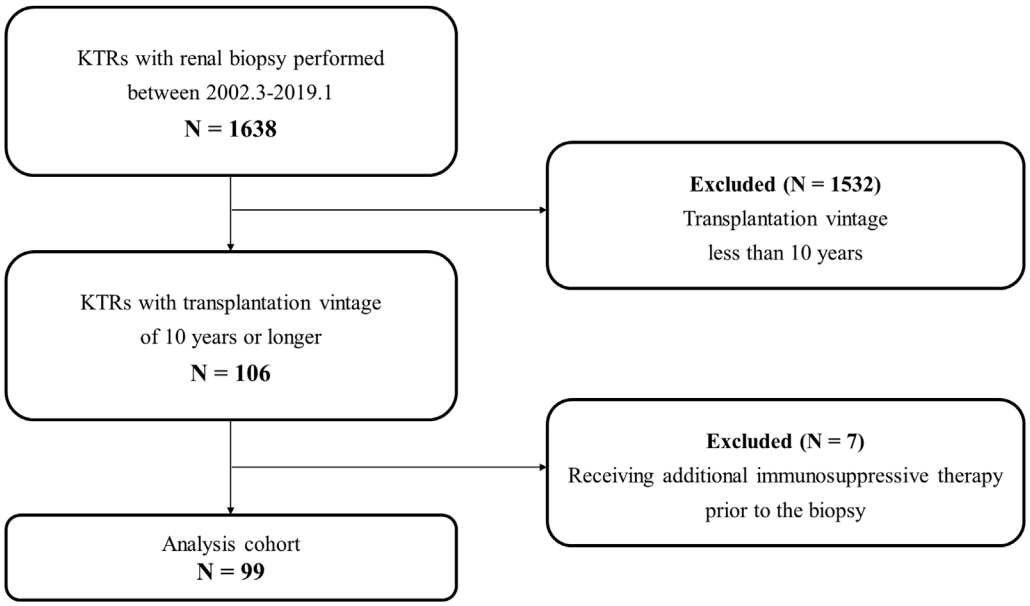


FIGURE 1 | Flow diagram of the study population. Abbreviations: KTR, kidney transplant recipient.

included methylprednisolone pulse therapy, 15-deoxyspergualin, and increased doses of immunosuppressive agents. As a methylprednisolone pulse therapy, we administered methylprednisolone sodium succinate for 3 consecutive days in 12 patients. 15-deoxyspergualin was administered for 7 successive days per course. The usual number of treatment cycles was 5 or 6 courses, with an average dosage of 4.9 mg/kg per administration in 9 patients. As an immunosuppression enhancement, increased doses of tacrolimus (2 patients), prednisone (2 patients), cyclosporine (1 patient), azathioprine (1 patient), and mycophenolate mofetil (1 patient) were administered (**Supplementary Table S1**). These patients had their immunosuppressive medication increased by 2.25 times compared to before the biopsy. Everolimus (1.5 mg/day) was administered to two patients. Seven (13.8%) patients were diagnosed with active IgA nephropathy, and subsequently underwent tonsillectomy.

Histological Findings of Biopsy Samples

Among the acute Banff scores, few patients had positive i- and t-scores. All the patients had v scores of zero (**Table 2**). Regarding chronic Banff scores, the ci, ct, and cg scores were positive in 55%, 65%, and 39% of patients, respectively. Only few patients had positive cv scores. Notably, the ah scores, indicating hyalinosis of the small arteries, and the aah scores, implying CNI toxicity, were positive in most cases (92% and 80%, respectively). In univariate analysis, the treatment modification group had significantly higher ti scores than the no-modification group (**Table 2**). We found no difference in other scores, including the i-IFTA score, between the two groups. The breakdown of glomerular lesions (IgA nephropathy, diabetic nephropathy, and membranous glomerulopathy) is shown in **Supplementary Figure S1**; there was no significant difference between the two groups (p = 0.64). A substantial percentage of enrolled patients did not undergo C4d staining.

One-Year eGFR Slopes Stratified by the Presence of Treatment Modification

To evaluate the clinical impact of graft biopsy, we compared the eGFR slope 1 year before and after the biopsy (**Figure 2**). The median number of serum creatinine measurements recorded per patient was 31 during the 2 years [interquartile range (IQR): 26–35]. The 1-year eGFR slope before biopsy for all the 99 patients was −4.42 mL/min/1.73 m^2/year [95% confidence interval (CI): −5.77, −3.06], and the eGFR slope after the biopsy was −3.13 mL/min/1.73 m^2/year (95% CI: −4.33, −1.93). A significant improvement was observed in eGFR slope after the biopsy [1.28 mL/min/1.73 m^2/year (95% CI: 0.29, 2.27), p = 0.01]. Stratified analyses were performed based on the treatment modifications. Among the patients with no treatment modification, we did not observe significant improvement in the 1-year eGFR slope [−3.56 mL/min/1.73 m^2/year (95% CI: −5.47, −1.65) before biopsy, −3.23 mL/min/1.73 m^2/year (95% CI: −5.19, −1.27) after biopsy]. On the other hand, eGFR slope significantly improved in patients with treatment modification [−5.31 mL/min/1.73 m^2/year (95% CI: −7.37, −3.25) before biopsy, −3.04 mL/min/1.73 m^2/year (95% CI: −4.50, −1.58) after biopsy, $p < 0.01$] (**Figure 3**). Even after censoring at eGFR less than 15, a significant improvement in the eGFR slope was observed among patients with treatment modification [−5.01 mL/min/1.73 m^2/year (95% CI: −6.64, −3.38) before biopsy, −3.17 mL/min/1.73 m^2/year (95% CI: −3.49, −0.20) after biopsy, p = 0.03]. In contrast, the patients without treatment modification showed no significant change in eGFR slope

TABLE 1 | Clinical characteristics of patients.

	Total	Without treatment modification*	With treatment modification**	*p*-value
Recipient information				
Number of patients	99	48	51	
Biopsy time from TPL (year)	13.7 (11.4, 19.3)	14.8 (11.8, 19.3)	12.9 (10.7, 19.3)	0.16
Dialysis vintage (year)	1.7 (0.8, 5.6)	2.3 (0.7, 6.4)	1.3 (0.9, 3.9)	0.33
Male gender (%) (n)	64 (63)	67 (32)	61 (31)	0.54
Age at biopsy (year)	50 ± 11	53 ± 12	47 ± 11	<0.01
Prior biopsy-proven acute rejection (%) (n)	6.2 (16)	12.5 (6)	19.6 (10)	0.42
Indication for biopsy (%) (n)				
Decline of eGFR	47 (48)	21 (44)	26 (51)	0.60
Increase in urinary protein	33 (33)	15 (31)	18 (35)	
Both	8 (8)	5 (10)	3 (6)	
Others	11 (11)	7 (15)	4 (8)	
eGFR at biopsy (mL/min/1.73 m^2)	34.8 ± 15.0	35.2 ± 14.5	34.4 ± 15.5	0.79
Urinary protein excretion at biopsy (g/day)	0.49 (0.18, 0.89)	0.45 (0.16, 0.8)	0.49 (0.20, 1.02)	0.38
Systolic blood pressure at biopsy (mmHg)	125 ± 16	126 ± 17	125 ± 16	0.8
Diastolic blood pressure at biopsy (mmHg)	77 ± 10	77 ± 10	78 ± 11	0.54
HbA1c at biopsy (%)	5.47 ± 0.78	5.48 ± 0.75	5.47 ± 0.80	0.95
Donor information				
Donor age at TPL (year)	50 ± 13	51 ± 15	49 ± 11	0.45
Gender of donors (male), n (%)	23 (28)	13 (33)	10 (23)	0.31
Deceased donation, n (%)	20 (20)	11 (24)	9 (18)	0.46
Relationship of living donors, n (%)				
Mother	41 (53)	16 (44)	25 (60)	0.60
Father	11 (14)	5 (14)	6 (14)	
Sister	8 (10)	6 (17)	2 (5)	
Spouse	8 (10)	4 (11)	4 (10)	
Brother	6 (8)	3 (8)	3 (7)	
Others	4 (5)	2 (6)	2 (5)	
Simultaneous pancreas transplantation, n (%)	5 (5.1)	4 (8.3)	1 (2.0)	0.20
ABO incompatibility, n (%)	5 (5.1)	2 (5.9)	3 (7.1)	1.00
HLA mismatch				
A (0, 1, 2)	27, 33, 2	9, 20, 0	18, 13, 2	0.04
B (0, 1, 2)	15, 44, 3	8, 20, 1	7, 24, 2	0.91
DR (0, 1, 2)	13, 46, 1	7, 21, 0	6, 25, 1	0.87
Average HLA mismatch	2.1 ± 1.1	2.2 ± 1.0	2.1 ± 1.1	0.59
Immunosuppressants, n (%)				
Corticosteroid	90 (91)	45 (94)	45 (88)	0.49
Cyclosporine A	55 (56)	25 (52)	30 (59)	0.55
Tacrolimus	40 (40)	20 (42)	20 (39)	0.84
Mycophenolate mofetil	53 (54)	24 (50)	29 (57)	0.55
Azathioprine	21 (21)	9 (19)	12 (24)	0.63
Mizoribine	15 (12)	10 (21)	5 (10)	0.16
Antihypertensives, n (%)				
Angiotensin-converting-enzyme inhibitor	28 (28)	11 (23)	17 (33)	0.27
Angiotensin II receptor blocker	62 (63)	26 (54)	36 (71)	0.10
Calcium channel blocker	47 (47)	25 (52)	22 (43)	0.42
Diuretics	20 (20)	9 (19)	11 (22)	0.81
β blocker	21 (21)	9 (19)	12 (24)	0.43
Mineral corticoid receptor antagonist	7 (7)	3 (6)	4 (8)	1.00

*Abbreviations: TPL, transplantation; eGFR, estimated glomerular filtration ratio; HLA, human leukocyte antigen. * denotes patients without treatment modification, while ** denotes patients with treatment modification.* p-*value for the difference between patients with treatment modification and those without.*

[−3.57 mL/min/1.73 m^2/year (95% CI: −4.84, −2.32) before biopsy, −3.07 mL/min/1.73 m^2/year (95% CI: −4.47, −1.67) after biopsy]. In other words, the magnitude of eGFR slope improvement was significantly pronounced in patients with treatment modification [2.27 mL/min/1.73 m^2/year (95% CI: 0.66, 3.89)] than in patients without [0.33 mL/min/1.73 m^2/year (95% CI: −1.05, 1.71)]. For sensitivity analysis, we employed a mixed-effects model using all data collected during the 2 years. A 3-way interaction term among the continuous-time, categories (pre- and post-biopsy), and treatment modification was significant in this model (p = 0.001). This indicated that eGFR slope changes were affected by treatment modification.

TABLE 2 | Distribution of each Banff score stratified by treatment modification based on biopsy results.

A. Active lesions				B. Chronic lesions				C. Acute and chronic Banff scores and arterial scores			
	Without treatment modification*	With treatment modification**	*p*-value		Without treatment modification*	With treatment modification**	*p*-value		Without treatment modification*	With treatment modification**	*p*-value
***i* score**	**n (%)**	**n (%)**		***ci* score**	**n (%)**	**n (%)**		***ti* score**	**n (%)**	**n (%)**	
0	43 (90)	46 (90)	1.00	0	23 (48)	22 (43)	0.85	0	39 (81)	32 (63)	0.04
1	5 (10)	5 (10)		1	18 (38)	22 (43)		1	7 (15)	18 (35)	
2	0 (0)	0 (0)		2	7 (14)	6 (11)		2	2 (4)	1 (2)	
3	0 (0)	0 (0)		3	0 (0)	1 (2)		3	0 (0)	0 (0)	
***t* score**				***ct* score**				***i-IFTA* score**			
0	46 (96)	49 (96)	1.00	0	19 (40)	16 (31)	0.57	0	24 (50)	23 (45)	0.36
1	2 (4)	2 (4)		1	25 (52)	32 (63)		1	14 (29)	14 (27)	
2	0 (0)	0 (0)		2	4 (8)	3 (6)		2	7 (15)	5 (10)	
3	0 (0)	0 (0)		3	0 (0)	0 (0)		3	3 (6)	9 (18)	
***g* score**				***cg* score**				***ah* score**			
0	42 (89)	36 (72)	0.15	0	32 (70)	28 (56)	0.11	0	3 (6)	5 (10)	0.80
1	4 (9)	9 (18)		1	8 (17)	5 (10)		1	12 (26)	11 (22)	
2	1 (2)	4 (8)		2	1 (2)	3 (6)		2	21 (45)	25 (50)	
3	0 (0)	1 (2)		3	5 (11)	14 (28)		3	11 (23)	9 (18)	
***v* score**				***cv* score**				***aah* score**			
0	47 (100)	49 (100)		0	46 (98)	47 (96)	1.00	0	12 (26)	9 (18)	0.77
1	0 (0)	0 (0)		1	1 (2)	2 (4)		1	7 (14)	6 (12)	
2	0 (0)	0 (0)		2	0 (0)	0 (0)		2	16 (34)	20 (40)	
3	0 (0)	0 (0)		3	0 (0)	0 (0)		3	12 (26)	15 (30)	
***ptc* score**				***mm* score**							
0	37 (77)	33 (65)	0.19	0	39 (85)	36 (72)	0.29				
1	8 (17)	7 (14)		1	6 (13)	10 (20)					
2	1 (2)	5 (10)		2	1 (2)	1 (2)					
3	2 (4)	6 (11)		3	0 (0)	3 (6)					

** denotes patients without treatment modification, while ** denotes patients with treatment modification.* p-*value for the difference between patients with treatment modification and those without.*

Difference in eGFR Slope Improvement Based on the Types of Treatment Modifications and Indications of Biopsy

The differences in eGFR slope change, before and after biopsy, were compared across the types of treatment modifications. The most remarkable improvement in eGFR slope after the biopsy was observed in patients whose IS was enhanced based on the biopsy results (including methylprednisolone pulse therapy, 15-deoxyspergualin administration, and an increase in IS dosage). An improvement in eGFR slope was observed in some, but not all, patients who underwent tonsillectomy or had their CNI doses reduced (**Figure 4A**). **Supplementary Figure S2** shows the eGFR trajectories in representative cases of eGFR improvement after IS enhancement (nine cases). Among them, eight patients underwent methylprednisolone pulse therapy. In three patients with increased CNI doses after biopsy, post-biopsy trough levels of CNI were 1.6 times higher than their pre-biopsy levels. Their eGFR slope ameliorated from −3.66 mL/min/1.73 m^2/year (95% CI: −13.6, 6.31) before biopsy to −0.24 mL/min/1.73 m^2/year (95% CI: −9.99, 9.52) after biopsy. Unfortunately, the sample size was too small to detect a significant difference.

In contrast, there was no significant difference in eGFR slope changes among the various indications for biopsy (**Figure 4B**).

Pathological Findings Related to Antibody-Mediated Rejection Prompted IS Enhancement

IS enhancement was found to be more effective for improving eGFR slope than the other interventions. We compared the Banff scores between patients with IS enhancement and those without. In

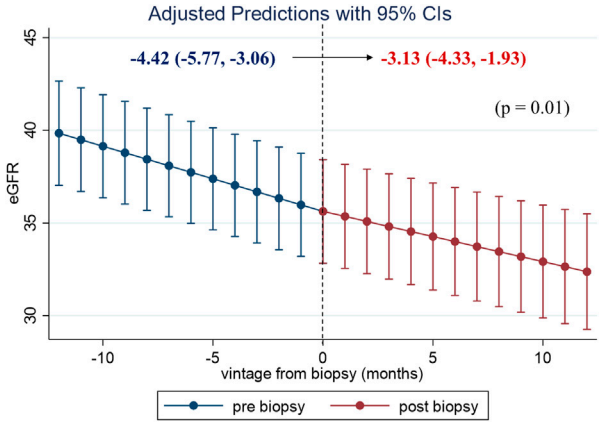


FIGURE 2 | One-year eGFR trajectory, pre- and post-biopsy, with slopes for all the patients. The eGFR level with 95% confidence interval (CI) at each time point in the 99 patients was estimated using a mixed-effects model. A significant improvement in eGFR slope was observed after the index biopsy. Abbreviations: eGFR, estimated glomerular filtration rate; CI, confidence interval.

univariate analysis, patients with IS enhancement had significantly higher g, ptc, and cg scores than those without (**Supplementary Figure S3**). The three scores were correlated; higher g-scores were associated with a higher percentage of patients with positive ptc and cg scores (**Supplementary Figures 4A, B**). Patients with higher cg scores were more likely to have positive ptc and mvi (g + ptc) scores ≥ 2 (**Supplementary Figures S4C, D**). Half of the patients (48.3%) with the enhancement of immunosuppressants had mvi score of 2 or higher. Among the four parameters, only the g-score showed a significant positive trend, with eGFR slope improvement after biopsy (**Figure 5A**). With an increase in the g-score, the proportion of patients with IS enhancement increased, whereas the proportion of patients without treatment modifications decreased (**Figure 6A**). The group with g scores ≥2 showed an odds ratio as high as 15.0 (95% CI: 1.65, 136), compared to the group with a g score of zero (**Figure 6B**). Regarding biopsy indications, the proportion of creatinine elevation did not show any difference (approximately 50%) among the three groups stratified by g-scores (**Figure 6C**).

To explore the impact of activity and chronicity of glomerular changes on eGFR slope improvement, we divided the patients into three groups according to the combination of g and cg scores; 1) g = 0 and any cg score (no activity), 2) g ≥ 1 and cg ≥ 2 (active and chronic), and 3) g ≥ 1 and cg ≤ 1 (active without chronicity). Patients with active glomerulitis showed better improvement in eGFR slope than those without (g score = 0). In particular, patients with active glomerular lesions, but without chronicity, showed the greatest improvement in eGFR slope; a significant trend was observed (p = 0.029) (**Figure 5B**).

DISCUSSION

The following novel findings were obtained in this study: First, the eGFR slope showed significant improvement after renal biopsy in patients with a transplantation vintage of 10 years or longer. This indicated the clinical importance of indication biopsy even during the late phase post renal transplantation. Second, treatment modifications based on the biopsy results, especially IS enhancement, had the greatest impact on improving the eGFR slope. This implied that patients exhibit a state of alloimmunity, for which immunosuppressive agents are effective, even long after transplantation. Third, physicians are likely to increase IS in cases with allograft glomerulopathy or with active inflammation in the glomerulus and peritubular capillaries. Among the findings, only glomerulitis was associated with improved eGFR slope after biopsy. However, no association was observed between the indications for biopsy and the severity of glomerulitis. This indicated that the diagnosis of transplant glomerulitis could not be inferred from the clinical information before biopsy. We observed little differences in baseline characteristics between patients with and without treatment modifications. Moreover, no significant difference was found in eGFR slope changes among the various indications for biopsy. These results underscore the importance of renal biopsy in clinical decision-making, even in long-term transplant recipients.

Overall, this study showed that allograft biopsy plays an important role in patients with a transplantation vintage of 10 years or longer.

This is the first study demonstrating clinical consequences after treatment modifications based on long-term pathological findings after transplantation. The rate of eGFR decline, along with the change in albuminuria [12, 13], have been reported to be an excellent surrogate marker for assessing renal outcomes [14, 15]. To evaluate the effect of treatment modification on hard outcomes, such as kidney failure with replacement therapy, we selected the eGFR slope among various surrogate endpoints, instead of albuminuria change, since urinary protein was negative in most KTRs, except for those with recurrent nephritis. Moreover, the association between graft outcome and eGFR slope for at least 12 months after biopsy has been confirmed recently in KTRs [16]. This result supports the validity of eGFR slope as a surrogate endpoint for graft survival. In our study, the improvement in eGFR slope after biopsy was as noticeable as 2.27 mL/min/1.73 m^2/year in the group with treatment modification. The value was clinically substantial, since a meta-analysis of clinical trials reported that a change in eGFR slope by 0.75 mL/min/1.73 m^2/year corresponds to an average 27% lower hazard of kidney failure with replacement therapy [7, 15]. In Japan, the mean eGFR at re-initiation of dialysis after transplantation was reported to be 5.45 mL/min/1.73 m^2 [17]. In this study, the mean eGFR level at biopsy, and the 1-year eGFR slope decline after biopsy, in patients with treatment modification, was 34.4 mL/min/1.73 m^2 and 3.04 mL/min/1.73 m^2/year, respectively; therefore, it was 9.5 years from the time of biopsy to the initiation of renal replacement therapy. On the other hand, if the patients had not received treatment modification based on biopsy results, it would have been 5.5 years after biopsy that they reached kidney failure with replacement therapy, based on the pre-biopsy eGFR slope (−5.31 mL/min/1.73 m^2/year). Therefore, some patients with transplantation vintage of 10 years or longer benefitted from

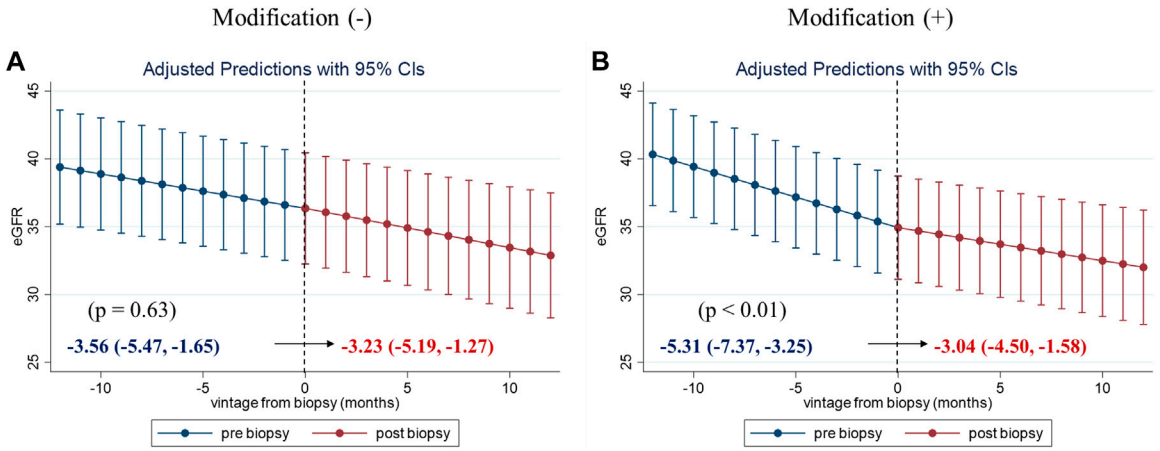


FIGURE 3 | One-year eGFR trajectory, pre- and post-biopsy, with slopes stratified by the treatment modification. One-year eGFR slopes for patients without treatment modification **(A)** and for those with treatment modification **(B)**. The magnitude of eGFR slope improvement was significantly pronounced in patients with treatment modification [2.27 (95% CI: 0.66, 3.89)]. Abbreviations: eGFR, estimated glomerular filtration ratio; CI, confidence interval.

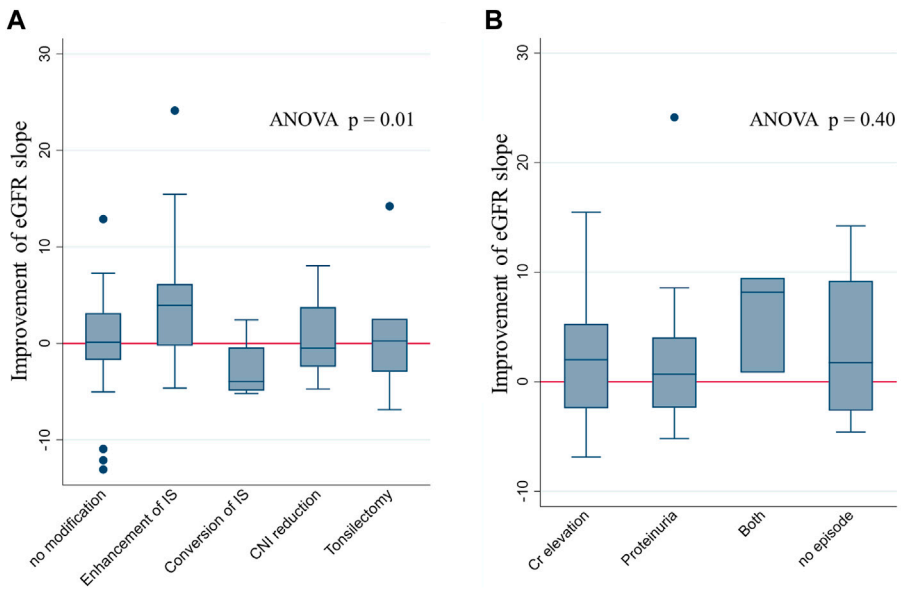


FIGURE 4 | eGFR slope improvement after the index biopsy stratified by types of modification and indications of biopsy. eGFR slope improvement after biopsy, stratified by the type of modification **(A)** and the indications for biopsy **(B)**. Improvement in eGFR was most prominent in patients with IS enhancement. No significant difference in the eGFR slope change was found among the groups stratified according to biopsy indications. Abbreviations: eGFR, estimated glomerular filtration rate; ANOVA, analysis of variance; IS, immunosuppression; CNI, calcineurin inhibitor.

renal biopsy through treatment modifications, resulting in graft survival prolonged by 4 years.

Patients with IS enhancement showed significantly higher g, ptc, and cg scores than those without. The scores are reported to be prognostic factors [18–21] and can be used to diagnose antibody-related rejection (ABMR) [22]. The Banff classification defines g and cg scores as active and chronic scores, respectively. Furthermore, active glomerulitis is described to progress to transplant glomerulopathy (TG) [23, 24]. Among the scores, only the g score was significantly associated with improved eGFR slope after biopsy. Moreover, among those with positive g scores, the ones with mild chronicity (cg ≤ 1) experienced greater improvement in eGFR than those with severe chronicity (cg ≥ 2). The findings overall suggested that active glomerulitis without chronic TG can be treated with IS, even long after transplantation. This was in line with the fact that once TG is established, the lesion resists various treatments. In randomized placebo-controlled clinical trials, intravenous immunoglobulin plus rituximab [25] and bortezomib [26]

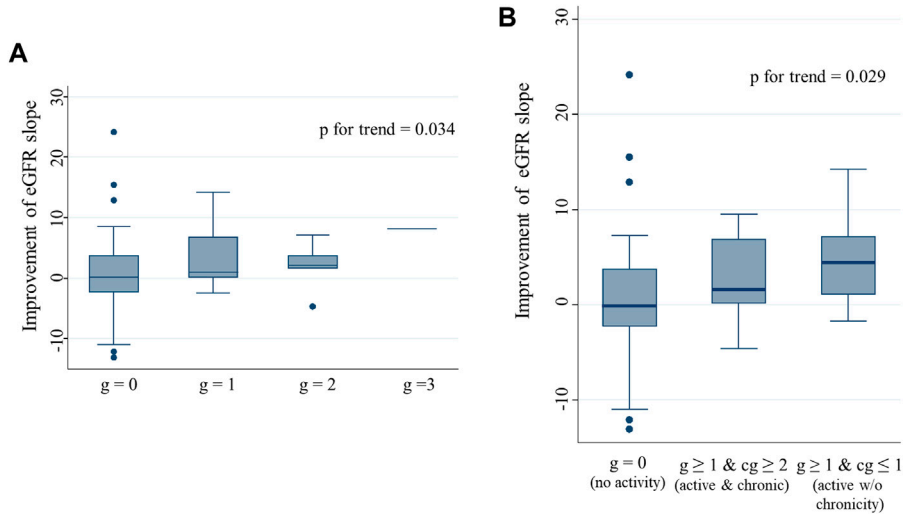


FIGURE 5 | eGFR slope improvement after biopsy stratified by g and cg scores Improvement in eGFR slope after biopsy in patients, stratified by g score **(A)**, and by combination of g and cg scores **(B)**. A significant positive trend was observed between the g scores and eGFR slope improvement after biopsy. Patients with active glomerulitis showed greater improvement in the eGFR slope after biopsy than those without. Among patients with active glomerulitis, a more prominent improvement in the eGFR slope was found in patients without chronicity than in those with. Abbreviations: eGFR, estimated glomerular filtration rate.

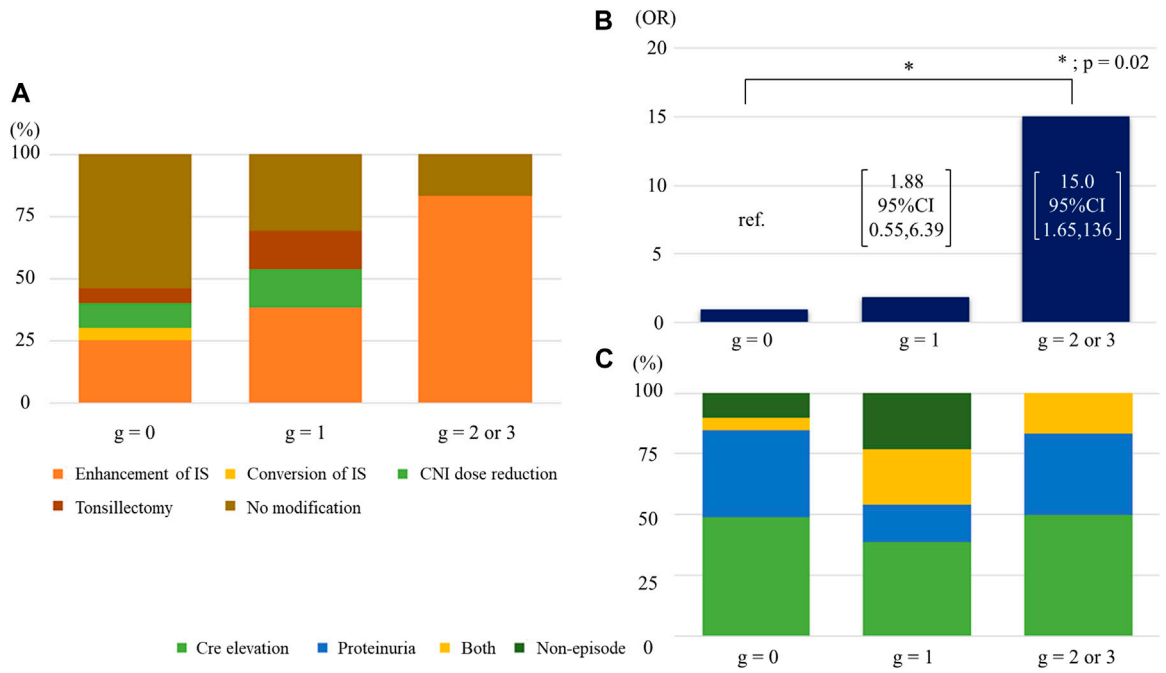


FIGURE 6 | Association of g score with IS enhancement **(A)** Breakdown of treatment modifications by g scores. **(B)** The odds ratios of receiving increased immunosuppressants across g scores. **(C)** Breakdown of indications of biopsy by g scores. Abbreviations: CNI, calcineurin inhibitor; IS, immunosuppression; OR, odds ratio; Cre, creatinine.

failed to improve eGFR or ABMR features in patients with TG or late ABMR, respectively. In contrast, cg1a lesions are potentially treatable. This lesion is defined as an early glomerular membrane duplication, detected only by electron microscopy, in the Banff classification [12] and is known as the very early stage of TG. Indeed, patients with mvi and cg1a lesions developed TG within 18 months without treatment, whereas those treated with IVIg and rituximab, with or

without plasmapheresis, did not develop overt TG for up to 4 years [27].

Stringer D et al. reported that interventions to improve adherence and optimize immunosuppression did not delay renal transplant failure after the development of DSA in a prospective, randomized, multicenter study [28]. In our study, interventions were based on kidney biopsy results, whereas their study relied on HLA antibody testing for interventions. Therefore, the findings of Stringer et al. do not negate our conclusion on the clinical significance of biopsies. Although our study was observational, it suggests that biopsies may provide better assessments of underlying pathology in transplant patients than clinical information alone.

The lack of DSA testing and C4d staining did not allow us to diagnose ABMR accurately in patients with microvascular inflammation. Among patients with intensified immunosuppression, 48.3% had mvi score of 2 or higher. Since not all of them had ABMR, borderline changes for TCMR or recurrent glomerulitis (immunoglobulin A nephropathy; IgAN) might explain the observed therapeutic effect of immunosuppressant enhancement.

It was reported that the recurrence of IgAN after renal transplantation is an important cause of graft failure [29]. No established therapy is currently available for recurrent IgAN. However, some Japanese researchers reported the efficacy of tonsillectomy with or without methylprednisolone pulse therapy for recurrent IgAN [30–33]. These studies show that tonsillectomy has improved hematuria, proteinuria, and pathological manifestations. In our study, seven patients underwent tonsillectomy after the indication biopsy. The improvement in eGFR slope in the entire group of seven individuals was not evident; however, there was a single notable case demonstrating significant improvement.

In this study, a higher percentage of patients received CsA than tacrolimus (TAC), since many patients underwent transplantation before 2000. Previous randomized controlled trials had shown that TAC is associated with less rejection [34–36], better graft function, and better graft prognosis than CsA [34]. Since most of the patients in our study had received CsA with underuse of MMF (approximately 50%), the eGFR slope improvement with the addition of immunosuppressive agents may be attributed to inadequate IS before biopsy.

CNI toxicity is a common cause of allograft injuries. In the current study, more than 80% of patients had positive aah scores, a characteristic of CNI toxicity. Moreover, no improvement in the eGFR slope was observed in patients for whom the CNI doses were reduced. This may be due to the irreversible nature of CNI toxicity. In line with our study, the prevalence of arterial hyalinosis in a 10-year graft biopsy was as high as 80%–90% in patients receiving CsA [37, 38]. Arterial hyalinosis due to CNI toxicity has several effects on allografts. Blood flow in arteries with severe hyalinosis due to CNI toxicity has been reported to be approximately 20% of that in normal arteries [38], leading to interstitial fibrosis. Indeed, associations between the duration of CsA administration and graft loss/poor graft function had been reported previously [31]. The prevalent interstitial fibrosis observed in our study (~70% with positive ci scores), probably due to CNI, can explain the steepness of eGFR slopes (~−3 mL/min/1.73 m^2/year) after biopsy even in patients with treatment modification.

The current study has several limitations. First, DSA measurement and C4d staining were not performed in most cases. This was because most biopsies are performed before insurance coverage of DSA measurements in Japan. Second, the histological improvement after treatment modification was not re-evaluated. Therefore, we could not discuss the relationship between various treatment modifications and histological alterations. Third, the usefulness of the Japanese standard eGFR formula used in this study has not been evaluated yet in relation to renal prognosis in Japanese kidney transplant recipients. Given that the estimation of glomerular filtration rate is affected by differences in creatinine generation among ethnicities, it would not be appropriate to apply the CKD-EPI equation to our study population, which consists predominantly of Japanese patients.

In conclusion, our study demonstrated the clinical significance of renal biopsy performed long after transplantation. Even in the chronic phase, biopsy results changed the treatment strategy in nearly half of the patients. IS enhancement led to an improved eGFR slope, indicating that a substantial proportion of patients experience latent rejection. This disagrees with the perspective of the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, which suggest a gradual reduction of IS owing to the adaptive responses of the immune system in KTRs towards foreign antigens within the graft [39].

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by The Ethics Committee of Osaka University Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because of the retrospective study design.

AUTHOR CONTRIBUTIONS

TN-H and TH conceptualized and designed the study. TN-H and TH analyzed the data and drafted the manuscript. YD, AH, HY, SS, AT, MM, SN, KY, YK, RI, NN, and YI reviewed and edited the manuscript. TN-H was the principal investigator of the study. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13022/full#supplementary-material

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ORIGINAL RESEARCH
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Characteristics of Early Antibody Mediated Rejection in Antibody Incompatible Living Donor Kidney Transplantation

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Antibody incompatible transplantation (AIT) may be an only option for highly sensitized patients. Severe form of early antibody mediated rejection (AMR) adversely affects graft survival after AIT. The aim of this study was to identify individuals at risk of AMR. We analyzed 213 living donor AITs performed at our center. Among 120 ABOi, 58 HLAi and 35 DSA + FCXM-negative cases, the rates of early AMR were 6%, 31%, and 9%, respectively ($p < 0.001$). On multivariate analysis for graft loss, early AMR had a HR of 3.28 ($p < 0.001$). The HLAi group had worse death-censored graft survival ($p = 0.003$). In the HLAi group, Patients with aggressive variant AMR (AAMR) had greater percentage of C3d complement fixing DSA, higher baseline class I and total DSA MFI levels and B-cell FCXM RMF. C1q and C3d complement fixing DSA and strong positivity of baseline B- or T-cell FXCM as predictors of AAMR had 100% sensitivity. Early AMR is of significant clinical concern in AIT as it results in poor graft survival and is not well described in literature. An aggressive variant is characterized by massive rise in DSA levels at rejection. Baseline DSA, C1q, and C3d and baseline FCXM values can be used to risk-stratify candidates for AIT.

Keywords: ABOi, HLAi, kidney transplantation, AMR, memory cells

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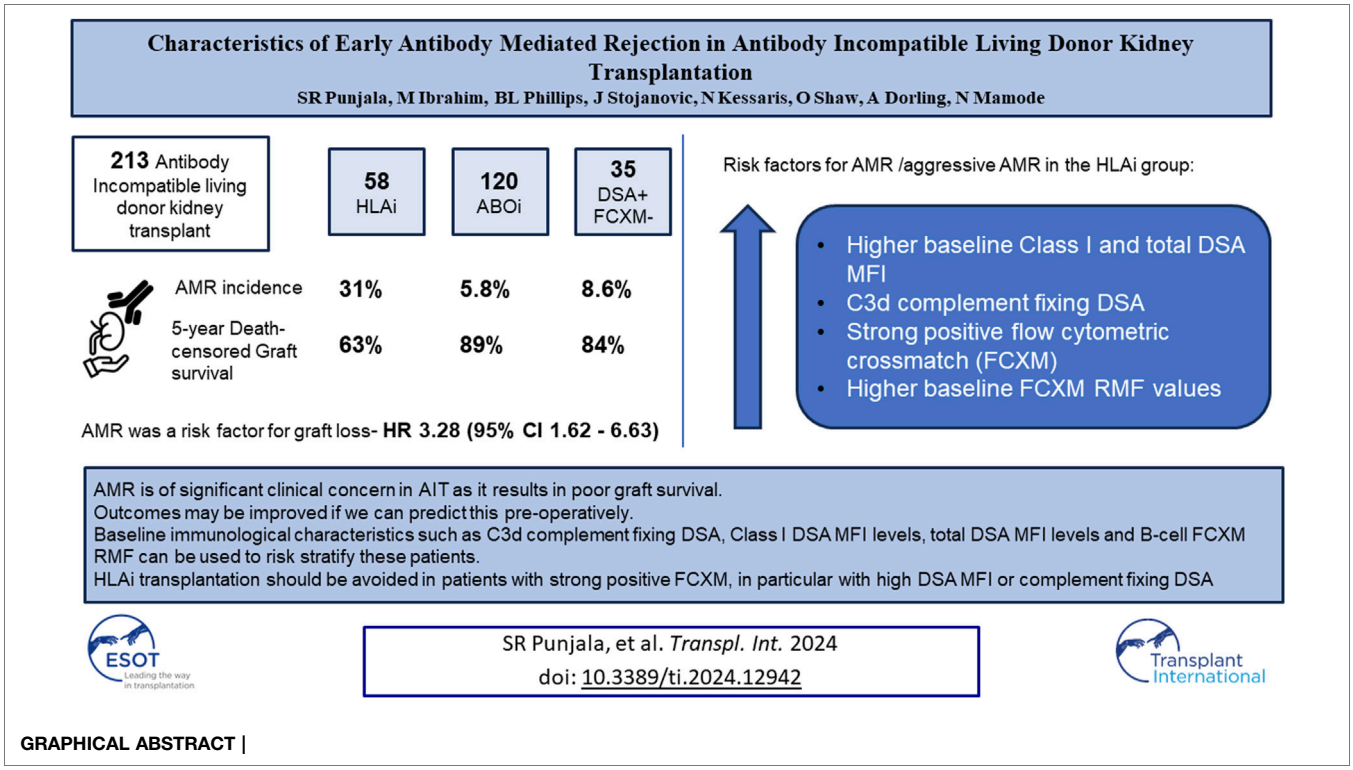
Abbreviations: ABOi, ABO blood group incompatibility; aHUS, atypical hemolytic uremic syndrome; AIT, Antibody incompatible transplantation; AM, Acceptable mismatch program; AMR, Antibody mediated rejection; CDC, Complement-dependent cytotoxicity crossmatch; CPRA, Calculated panel reactive antibody; cRF, calculated reaction frequency; DCGS, Death-censored graft survival; DFPP, Double-filtration plasmapheresis; DSA, Donor specific antibody; EAAMR, Early Aggressive Antibody Mediated Rejection; FCXM, Flow cytometry crossmatch; HLAi, Human leukocyte antigen incompatibility; HSP, Highly sensitized patients; IA, Immunoadsorption; IVIg, Intravenous immunoglobulins; MFI, Mean fluorescence index; NAMR, Non-aggressive AMR; PEX, Plasma exchange; PNF, Primary non function; RMF, Relative mean fluorescence ratio; TMA, Thrombotic microangiopathy.



GRAPHICAL ABSTRACT |

INTRODUCTION

ABO-blood group incompatibility (ABOi) and Human Leukocyte antigen (HLA) sensitization have been barriers to direct kidney transplantation. The degree of sensitization is measured as calculated reaction frequency (cRF) in the UK [1], and as calculated panel reactive antibody (CPRA) in the US [2]. Kidney sharing schemes (KSS), prioritization of highly sensitized patients (HSP, cRF> 85% or CPRA> 80%) in national kidney allocation schemes, acceptable mismatch (AM) programs [3], and antibody incompatible kidney transplantation (AIT) have been successful in overcoming these barriers.

In the recent times, the number of annual kidney AITs has been in decline [4]. This can be attributed to the success of KSS which enable direct compatible transplantation. Although KSS have enabled a steady rise in the number of transplants performed every year, the number of transplants performed in individuals with a cRF 100% or CPRA 98%–100% have been very low [5, 6]. In the U.K, among the patients who wait for >7 years on the kidney only transplant waiting list, 98% are HSP [7]. Therefore, kidney allocation schemes have made provisions to prioritize HSP on the deceased donor waiting list to improve their transplant rates. Despite these provisions in the US, transplant rates remained extremely low in individuals with CPRA ≥99.9%. Any further modifications to the allocation policy may not improve the transplant rates [8]. Furthermore, allocation of organs with a poor HLA mismatch would increase the degree of sensitization in these recipients, rendering them more difficult to match for a future transplant.

Antibody mediated rejection (AMR) is now the most common cause of graft loss in kidney transplant recipients [9]. As anti-donor antibodies are responsible for AMR [10], the rates of AMR are higher in antibody incompatible kidney transplants compared to antibody compatible kidney transplantation [11]. AMR has been broadly classified based on the timing of rejection after transplantation, as early (<30 days) and late (>30 days). Early rejection usually occurs in individuals who undergo transplantation with preformed antibodies to donor antigens or in individuals who have an immunological anamnestic response from previous allo-sensitization [12].

Reports suggest that some patients suffering AMR within the first 2 weeks after transplantation are at particularly high risk of early graft loss [13, 14]. We hypothesize that this phenomenon, poorly described in literature, most likely occurs in patients with strong reactivation of their memory T and B cell responses, leading to a rapid increase in alloantibody production. The aim of this study was to identify, the incidence of AMR within the first 2 weeks after transplantation, and those at risk of early graft failure. Further, to better define the donor, recipient and baseline immunological characteristics associated with early AMR and its outcomes in antibody incompatible living donor kidney transplantation. This may help to risk stratify patients prior to transplantation.

MATERIALS AND METHODS

This study was a retrospective analysis of all antibody incompatible living donor kidney transplants performed at a

UK Transplant center between 2005 and 2019. All blood group incompatible transplants with or without baseline DSA were grouped into ABOi transplants. All Flow Cytometry crossmatch (FCXM) positive transplants (relative mean fluorescence ratio, RMF >2.3) with or without blood group incompatibility were grouped into HLAi transplants, and all DSA positive but FCXM negative transplants (RMF <2.3) were grouped into "high-risk" transplants.

Our desensitization protocols for ABOi [15] and HLAi [16] kidney transplants have been published in the past. To summarize, we undertook antibody removal in ABOi patients with baseline titers of >64 with Glycosorb immunoadsorption (IA) columns, and in individuals with baseline titers between 16 and 64 we used double-filtration plasmapheresis (DFPP). No pre-operative antibody removal was performed in individuals with titers ≤8. In the HLAi group, antibody removal was carried out until a negative FCXM (RMF <2.3) was achieved. If multiple sessions of antibody removal were necessary to achieve a negative FCXM, immunoadsorption using Therasorb columns was preferred due to its minimal effect on coagulation. In all other cases, plasma exchange (PEX) or DFPP was used.

Our immunosuppression protocols have undergone modifications over the course of this study. In the ABOi transplants with no DSA, rituximab was given 4 weeks before a transplant at a dose of 375 mg/m^2. In the initial period of this study, rituximab was given to all patients irrespective of their baseline ABO titers. This was later modified, and the new threshold for rituximab induction was set at ABO titers ≥8. All ABOi transplants received basiliximab induction on the day of transplant. Alemtuzumab induction was used in place of rituximab and basiliximab in ABOi transplants who have DSA. In the HLAi patients, basiliximab was used in the initial period of the study. Alemtuzumab induction has been used since July 2010. All HLAi transplants received low dose intravenous immunoglobulin (IVIg), at 0.5 gm/kg, 1 day before transplant following the last session of antibody removal, unless contraindicated. Patients in the "high-risk" group received alemtuzumab at induction from February 2011; prior to this, basiliximab induction was used. All patients in this study received standard triple maintenance immunosuppression (tacrolimus, mycophenolate mofetil and prednisolone). Participants of a randomized controlled trial looking into safety and efficacy of eculizumab in the prevention of AMR in antibody incompatible living donor kidney transplantation (NCT01399593) were included in this study. The impact of eculizumab on graft survival in multivariate analysis was not studied as some of the patients in the study received prophylactic eculizumab in the treatment arm.

Ethnic groups other than white were grouped together as ethnic minorities. Estimated glomerular filtration rate (eGFR) was calculated by using the Modification of Diet in Renal Disease equation [17]. MDRD eGFR was not collected in pediatric recipients as it is not an accurate marker of graft function in this population. RMF >2.3 but <2.8 were considered weak positive FCXM, and RMF >2.8 was considered as strong positive FCXM.

All FCXM negative, blood group incompatible transplants with or without DSA have been grouped into the ABOi group. We have defined HLAi as DSA positive and FCXM positive cases; and labelled all DSA positive but FCXM negative cases as "high-risk," using this as a control group for comparison of outcomes. Moreover, all blood group incompatible cases who also had a positive crossmatch, are grouped into the HLAi group as these cases behave more like HLAi rather than an ABOi transplant.

The primary outcome of interest was AMR within the first 2 weeks after transplantation. Cases were identified based on for-cause biopsies or on clinical diagnosis. The secondary outcome of interest was to look at the impact of early AMR on patient and graft outcomes; and to predict recipient, donor and immunological factors associated with early AMR.

Statistical analyses were performed using IBM SPSS Statistics for Mac, Version 26.0. Armonk, NY: IBM Corp. Normality of the data was determined using Shapiro-Wilk test. Comparisons for continuous variables were performed with parametric (Student's t-test, ANOVA) and non-parametric tests (Mann-Whitney, Kruskal-Wallis rank sums test), depending on distribution. Categorical variables were compared with Fisher's exact test or χ^2 test. We used Kaplan-Meier, and the log-rank test to compare death-censored graft and patient survival between groups. Risk associations were estimated with the use of multivariable Cox proportional-hazards models. Clinically important factors were tested and fit into a cox model. This study was conducted as an audit under the auspices of hospital audit committee and was exempt from institutional review board approval as it was a retrospective observational study. This study was conducted in accordance with the standards laid down by Declaration of Helsinki.

RESULTS

During this study period, a total of 213 antibody incompatible living donor kidney transplants were performed at our center. Of these, 120 were ABOi (111- DSA negative, 9- DSA positive), 58 were HLAi (50- HLAi, 8- combined HLAi and ABOi) and 35 were high-risk kidney transplants. Demographic data are shown in **Table 1**.

A total of 29 patients were treated for AMR within the first 2 weeks after transplantation. After examining individual cases, one case was excluded from our analysis as review of allograft biopsy suggested recurrence of primary disease (Henoch-Schölen purpura) and no evidence of AMR (**Supplementary Table S1**), giving an overall early AMR rate of 13%.

Among the ABOi, HLAi and high-risk groups, the rates of AMR within 2 weeks were 5.8%, 31% and 8.6%, respectively ($p < 0.001$); the median days to AMR after transplantation were 6 (IQR, 6-7), 6.5 (IQR, 5–8.5) and 8 (IQR, 7–11), respectively ($p = 0.447$). The rates of graft survival at 1, 3 and 5 years were worse in the HLAi group, but there was no difference in 1, 3, and 5-year patient survival between the ABOi, HLAi and high-risk groups (**Table 2**).

Early mortality (patient death <90 days from transplantation) was observed in 7 cases, and sepsis was the most common cause ($n = 4/7$, 57%). Incidentally, we noticed sudden unexpected death in 3 cases, all of whom received eculizumab. On univariate analysis, 30-day death-censored graft survival (DCGS) was worse in patients with AMR (97% vs. 75%, log rank

TABLE 1 | Baseline demographics according to ABOi, HLAi and High-risk groups.

		Total (N = 213)	ABOi (N = 120)	HLAi (N = 58)	High-risk (N = 35)	*p*-value
Recipient age, years		45 (33–55)	47 (28–56)	43 (35–54)	50 (43–56)	0.60
Recipient gender, n (%)	Male	106 (50)	71 (59)	22 (38)	13 (37)	0.008
	Female	107 (50)	49 (41)	36 (62)	22 (63)	
Recipient ethnicity, n (%)	White	180 (84.5)	101 (84)	49 (84.5)	30 (86)	0.98
	Ethnic minorities	33 (15.5)	19 (16)	9 (15.5)	5 (14)	
Donor age, years		44 (36–53)	46 (38–53)	40 (30–46)	49 (37–57)	0.003*
Donor gender, n (%)	Male	100 (48)	54 (45)	32 (57)	14 (44)	0.28
	Female	108 (52)	66 (55)	24 (43)	18 (56)	
Donor ethnicity, n (%)	White	176 (86)	105 (87.5)	47 (85.5)	24 (80)	0.57
	Ethnic minorities	29 (14)	15 (12.5)	8 (14.5)	6 (20)	
Dialysis status pre-transplant, n (%)	Pre-emptive	53 (25)	41 (34)	3 (5)	9 (26)	<0.001
	HD	126 (59)	57 (48)	46 (79)	23 (66)	
	PD	34 (16)	22 (18)	9 (16)	3 (8)	
Duration on dialysis, months		19 (0–51)	10 (0–31)	60 (29–129)	17 (2–43)	<0.001
Previous transplant, yes, n (%)		72 (34)	27 (37.5)	31 (43)	14 (19)	<0.001
Median peak cRF, %		28.5 (0–95)	0 (0–17)	98 (91–100)	87 (55–99)	<0.001[§]
Baseline, total DSA MFI			3,718 (2,210–4,710)	15,530 (9,630–25,849)	5,091 (3,300–8,777)	<0.001[Φ]
Blood group incompatibilities, n (%)	A into O		62 (51.6)	4 (50)		
	B into O		19 (15.8)	2 (25)		
	AB into O		1 (0.8)			
	B into A		13 (10.8)	1 (12.5)		
	AB into A		8 (6.6)			
	A into B		14 (11.6)	1 (12.5)		
	AB into B		3 (2.5)			

*Values expressed as Median (IQR), unless otherwise stated. Abbreviations: ABOi, ABO, blood group incompatible kidney transplantation; HLAi, Human Leukocyte antigen incompatible kidney transplantation; High-risk, DSA, positive and crossmatch negative transplant; IQR, inter quartile range; HD, hemodialysis; PD, peritoneal dialysis; cRF, calculated reaction frequency; DSA MFI, donor specific antibody median fluorescence index. *p = 0.007 and p = 0.016 when HLAi, group is compared to ABOi, and high-risk groups, respectively. [§]p < 0.001 when ABOi group is compared to HLAi and high-risk groups; p = 0.309 when HLAi, and high-risk groups were compared. [Φ]DSA, in ABOi, group included only nine patients who were DSA, positive.*

TABLE 2 | Rejection rates, graft and patient survival rates.

	ABOi (N = 120)	HLAi (N = 58)	High-risk (N = 35)	*p*-value
AMR, n (%)	7 (5.8)	18 (31)	3 (8.6)	<0.001
Median days to AMR, (IQR)	6 (6–7)	6.5 (5–8.5)	8 (7–11)	0.45
1-year DCGS	94% (88–97) n/N = 113/120	82% (69–90) n/N = 48/58	91% (75–97) n/N = 32/35	0.05
3-year DCGS	92% (85–96) n/N = 111/120	66% (51–77) n/N = 40/58	84% (66–93) n/N = 30/35	<0.001
5-year DCGS	89% (81–94) n/N = 108/120	63% (49–75) n/N = 39/58	84% (66–93) n/N = 30/35	<0.001
1-year patient survival	93% (87–97) n/N = 112/120	91% (80–96) n/N = 53/58	97% (81–100) n/N = 34/35	0.56
3-year patient survival	90% (83–94) n/N = 108/120	89% (77–95) n/N = 52/58	97% (81–100) n/N = 34/35	0.42
5-year patient survival	88% (80–93) n/N = 106/120	81% (67–89) n/N = 48/58	93% (73–98) n/N = 33/35	0.29

Graft survival and patient survival rates are expressed as mean survival rates (95% confidence interval). Abbreviations: ABOi, ABO, blood group incompatible kidney transplantation; HLAi, Human Leukocyte antigen incompatible kidney transplantation; high-risk, DSA, positive and crossmatch negative transplant; IQR, inter quartile range; DCGS, death-censored graft survival.

p = <0.001). 10-year DCGS was inferior in the HLAi group compared to ABOi and high-risk groups (p = 0.003) (**Figure 1A**). There was no difference in the 10-year patient survival between the groups (p = 0.148) (**Figure 1B**). On the multivariable Cox regression modelling, AMR (hazard ratio (HR) 3.28; 95% confidence interval (CI) 1.62 to 6.63, p = <0.001) and second or more kidney transplant status (HR, 2.49; 95% CI 1.40 to 4.44; p = 0.002) were associated with overall DCGS. The following independent predictors of patient survival were identified: older recipient age (HR, 1.05; 95% CI 1.02 to 1.08; p < 0.001), longer duration spent on dialysis prior to transplant (HR, 1.009; 95% CI 1.002 to 1.01; p = 0.01) and eculizumab use for AMR treatment (HR, 2.77; 95% CI 1.01to 7.54; p = 0.04) (**Table 3**).

Twenty-eight cases of early AMR were compared to 185 cases without AMR to characterize which patients were at an increased risk of early AMR (**Table 4**). Individuals in the early AMR group, were more likely to have received their kidney from a male donor (67% vs. 45%, p = 0.04), been on dialysis prior to their transplant (93% vs. 72%, p = 0.03), were on dialysis for a longer duration of time (44 vs. 17 months, p = 0.002), had higher baseline class I DSA MFI levels (18,700 vs. 9,127, p = 0.005), had a high-risk relation with the donor (husband to wife or child to mother) (39% vs. 20%, p = 0.03) and a greater percentage had DSA to repeat mismatches (62% vs. 20%, p = <0.001).

Since the HLAi group had greater percentage of cases with AMR (n/N = 18/28, 64%) and had worse overall graft survival,

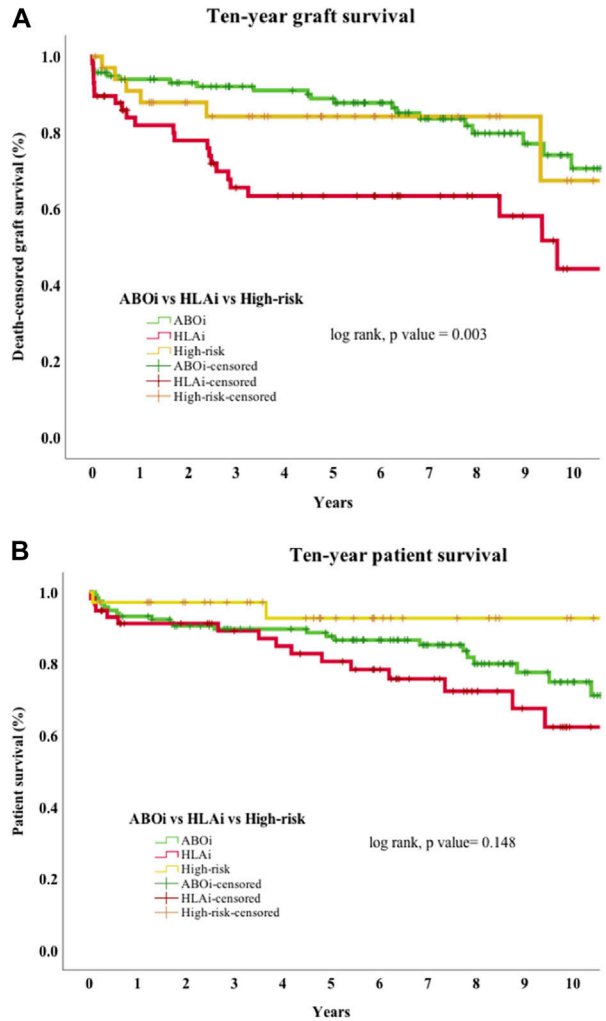


FIGURE 1 | (A) Death-censored graft survival. Ten-year death-censored graft survival of HLAi group was worse compared to the ABOi and high-risk kidney transplant groups. Abbreviations: ABOi, ABO blood group incompatible kidney transplantation; HLAi, Human Leukocyte antigen incompatible kidney transplantation; high-risk, DSA positive and crossmatch negative transplant. **(B)** Patient survival. Ten-year patient survival of ABOi, HLAi and high-risk kidney transplants shows no difference in survival.

subgroup analysis of the HLAi group was performed to characterize which patients were at an increased risk of early AMR. Donor and recipient characteristics were not statistically different in cases with AMR when compared to cases without AMR. However, we observed that cases with AMR had significantly higher baseline class I DSA MFI levels (15,272 vs. 9,422, $p = 0.03$), baseline total DSA MFI levels (24,448 vs. 13,814; $p = 0.01$), pre-transplant class I DSA MFI levels (10,286 vs. 3,459, $p = 0.03$), greater percentage of cases with pre-transplant strongly positive FCXM (47% vs. 14%, $p = 0.01$), higher baseline T-cell FCXM RMF (RMF 3.22 vs. 2.41, $p = 0.047$) and baseline B-cell FCXM RMF (RMF 5.69 vs. 3.70, $p = 0.03$) and strong positivity of baseline B or T cell FXCM as predictors of early AMR had sensitivity of 100% (**Table 5**). A cut-off value of baseline total DSA MFI of 24,000 as predictor of AMR in the HLAi group has a sensitivity and specificity of 50% and 85%, respectively (ROC AUC = 0.70).

There was no significant difference in the MDRD eGFR between ABOi, HLAi and high-risk groups at 1, 3, and 5-year post-transplant. There was also no observed difference in MDRD eGFR at 1, 3, and 5-year, among individuals with and without AMR.

In an *ad hoc* analysis, we attempted to differentiate cases of AMR based on outcomes. Cases of AMR leading to graft loss, or not responding to initial therapy and subsequently needing eculizumab, or with thrombotic microangiopathy (TMA) on biopsy were grouped together as Aggressive AMR (AAMR). The rest of the cases were grouped together as non-aggressive AMR (NAMR) (**Supplementary Table S1**). Subgroup analysis was performed to characterize AAMR in HLAi group. In the AAMR group (n = 11), a massive increase in DSA MFI levels were observed at rejection when compared to baseline levels (DSA MFI 65797 vs. 32,519, $p = 0.01$). In the NAMR group (n = 7), no significant increase in DSA MFI levels were observed at rejection when compared to baseline levels (DSA MFI 36293 vs. 14,805, $p = 0.06$) (**Figure 2**). We observed that cases with AAMR had significantly higher baseline class I DSA MFI levels (17,872 vs. 9,422, $p = 0.01$), baseline total DSA MFI levels (32,519 vs. 14,583, $p = 0.001$), higher baseline B-cell FCXM RMF (RMF 6.44 vs. 3.86, $p = 0.02$) and pre-transplant B-cell FCXM RMF (RMF 3.93 vs. 2.86, $p = 0.03$) and a greater percentage had C3d complement fixing DSA (100% vs. 38%, $p = 0.03$). Strongly positive B/T cell FCXM, C1q and C3d complement fixing DSA as predictors of AAMR have a 100% sensitivity (**Table 6**). A cut-off value of baseline total DSA MFI of 23,000 as predictor of AAMR in the HLAi group had a sensitivity and specificity of 82% and 83%, respectively (ROC AUC = 0.81).

DISCUSSION

This study identifies that early AMR occurs more commonly in HLAi transplants as compared to ABOi and DSA positive FCXM negative transplants, at around 1-week post-transplant. An aggressive form of AMR (AAMR) (AMR leading to graft loss, or TMA on biopsy, or resistant to standard treatment) presents with massive antibody rise at rejection, far beyond baseline levels. It is likely a memory response, with B and T cell activation leading to increased antibody production. Patients with early AMR had higher baseline class I and total DSA, higher pre-transplant class I DSA, greater percentage of strongly positive pre-transplant FCXM, higher baseline T and B cell FCXM RMF, and strong positivity of baseline B or T cell FXCM as predictors of early AMR had sensitivity of 100%. Patients with aggressive AMR had a greater percentage of C3d complement fixing DSA, higher baseline Class I, total DSA MFI levels and B-cell FCXM RMF. Strongly positive B/T cell FCXM, C1q and C3d complement fixing DSA have 100% specificity as predictors of AAMR.

The main limitations of this study are the single center and retrospective observational nature of this study. Early AMR is relatively uncommon; given the low number of AMR events in

TABLE 3 | Multivariable analysis on Cox regression model.

Cox regression model	Variable	HR	95% CI	*p*-value
Death-censored graft survival	No rejection	reference		
	AMR	3.278	1.62 to 6.63	<0.001
	Recipient previously on dialysis, yes	1.33	0.55 to 3.22	0.52
	Duration on dialysis	1	0.99 to 1	0.28
	Previous transplant (yes)	2.49	1.40 to 4.44	0.002
	Donor age	1.00	0.98 to 1.02	0.66
	Recipient age	1.00	0.98 to 1.02	0.66
Patient survival	Recipient age	1.05	1.02 to 1.08	<0.001
	Recipient previously on dialysis, yes	0.88	0.37 to 2.08	0.77
	Previous transplant (yes)	0.84	0.35 to 1.99	0.69
	Recipient gender	1.07	0.55 to 2.09	0.83
	Duration on dialysis	1.009	1.002 to 1.01	0.01
	Donor age	0.99	0.96 to 1.01	0.57
	AMR	1.26	0.48 to 3.30	0.62
	No eculizumab use	reference		
	Eculizumab use	2.77	1.01 to 7.54	0.04

This analysis includes all patients in the study, from all three groups. Choice of risk factors studied in this model was limited by the small sample size and limited number of significant events; therefore, only clinically significant risk factors, which are independent to classification of three subgroups, were included in this analysis. Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval; AMR, antibody mediated rejection; HLA, human leukocyte antigen.

TABLE 4 | Comparison between cases with and without AMR in the whole cohort of patients.

		No AMR (n = 185)	AMR (n = 28)	*p*-value
Donor age, years		45 (36–55)	42 (30–46)	0.07
Donor gender, n (%) (missing cases = 5)	Male	82 (45)	18 (67)	0.04
	Female	99 (55)	9 (33)	
Donor ethnicity, n (%) (missing cases = 8)	White	156 (88)	20 (74)	0.07
	Ethnic minorities	22 (12)	7 (26)	
Recipient age, years		45 (35–55)	43 (31–48)	0.18
Recipient gender, n (%)	Male	97 (52)	9 (32)	0.06
	Female	88 (48)	19 (68)	
Recipient ethnicity, n (%)	White	160 (87)	20 (71)	0.05
	Ethnic minorities	25 (13)	8 (29)	
Recipient dialysis status, n (%)	Pre-emptive	51 (28)	2 (7)	0.03
	HD or PD	134 (72)	26 (93)	
Duration on dialysis, months		17 (0–47)	44 (12–104)	0.002
Peak cRF		97 (81–99)	98 (95–100)	0.15
Total DSA MFI, baseline		9,125 (5,355–15530)	18,700 (7,017–40007)	0.005
Number of previous transplants, n (%)	First transplant	123 (66)	18 (64)	0.83
	Second or more	62 (34)	10 (36)	
High risk relation between recipient and donor, n (%)	No	147 (80)	17 (61)	0.03
	Yes	38 (20)	11 (39)	
HLA mismatch level, n (%)	Level 0, 1 and 2	37 (20)	7 (25)	0.61
	Level 3 and 4	148 (80)	21 (75)	
DSA to repeat mismatch, n (%) (missing cases = 6)	No	145 (80)	10 (38)	<0.001
	Yes	36 (20)	16 (62)	

*Values expressed as Median (IQR), unless otherwise stated. Abbreviations: HD, hemodialysis; PD, peritoneal dialysis; cRF, calculated reaction frequency; HLA, human leukocyte antigen; DSA MFI, Donor specific antibody-mean fluorescence index. *DSA MFI, and cRF, values are presented only for HLAi, and high-risk transplant.*

the study population, interpretation of factors that may have influenced outcomes such as allograft loss, long-term allograft and patient survival may be difficult, as they can be influenced by various external factors. For the same reasons, direct comparisons between NAMR and AAMR were not made in the HLAi group. A low number of AMR events precluded us from performing a subgroup analysis in the ABOi and DSA positive FCXM negative groups. Analyses stratifying risk factors for AMR/AAMR in the HLAi subgroup are hampered by very low sample size, missing data and low number of significant events. This data should be interpreted with caution due to a high risk of both type I and type II errors. However, we do feel it is important to have these data in the manuscript, as the clinical implications of AMR/AMMR are quite devastating. A prospective national registry analysis may overcome some of these limitations. Further

TABLE 5 | Subgroup analysis- Comparison between cases with and without AMR in the HLAi group.

		No AMR (n = 40)	AMR (n = 18)	*p*-value
Donor age, years		40 (27–47)	43 (30–46)	0.92
Donor gender, n (%) (missing cases n = 2)	Female	18 (46)	6 (35)	0.56
	Male	21 (54)	11 (65)	
Donor ethnicity, n (%) (missing cases n = 3)	White	34 (90)	13 (77)	0.23
	Ethnic minorities	4 (10)	4 (23)	
Recipient age, years		42 (35–54)	45 (39–54)	0.45
Recipient gender, n (%)	Female	22 (55)	14 (78)	0.14
	Male	18 (45)	4 (22)	
Recipient ethnicity, n (%)	White	36 (90)	13 (72)	0.12
	Ethnic minorities	4 (10)	5 (28)	
Recipient dialysis status, n (%)	Pre-emptive	3 (7)	0	0.54
	HD or PD	37 (93)	18 (100)	
Duration on dialysis, months		57 (26–131)	75 (41–128)	0.45
Previous transplant, n (%)	No	17 (42)	10 (56)	0.40
	Yes	23 (58)	8 (44)	
High risk relation*, n (%)		9 (23)	8 (44)	0.12
Peak cRF		97 (87–99)	98.5 (95–100)	0.12
HLA mismatch level, n (%)	Level 0, 1 and 2	6 (15)	1 (6)	0.41
	Level 3 and 4	34 (85)	17 (94)	
DSA to repeat mismatches, n (%) (missing cases n = 4)	No	17 (45)	4 (25)	0.23
	Yes	21 (55)	12 (75)	
C1q complement fixing DSA, n (%) (missing cases n = 20)	No	16 (55)	2 (22)	0.18
	Yes	13 (45)	7 (78)	
C3d complement fixing DSA, n (%) (missing cases n = 20)	No	19 (65)	2 (22)	0.05
	Yes	10 (35)	7 (78)	
Class I DSA MFI, baseline		9,422 (5,516–12831)	15,272 (9,976–24867)	0.03
Class II DSA MFI, baseline		3,138 (0–9,565)	10,301 (0–26489)	0.15
Total DSA MFI, baseline		13,814 (9,069–22320)	24,448 (14,735–42491)	0.01
Class I DSA MFI, pre-transplant (missing cases n = 13)		3,459 (1,426–7,478)	10,286 (2,620–13953)	0.03
Class II DSA MFI, pre-transplant (missing cases n = 13)		1,412 (0–5,159)	2,958 (0–8,516)	0.48
Total DSA MFI, pre-transplant (missing cases n = 5)		6,946 (3,538–10728)	11,686 (5,432–20283)	0.08
FCXM, B or T cell, baseline, n (%) (missing cases n = 1)	Negative[μ]	2 (5)	0	0.08
	Weak positive	7 (18)	0	
	Strong positive	30 (77)	18 (100)	
FCXM, B or T cell, pre-transplant, n (%) (missing cases n = 5)	Negative	24 (67)	9 (53)	0.01
	Weak positive	7 (19)	0	
	Strong positive	5 (14)	8 (47)	
Baseline T-cell FCXM, RMF		2.41 (1.52–3.36)	3.22 (2.12–6.23)	0.047
Baseline B-cell FCXM, RMF		3.70 (2.81–6.18)	5.69 (4.35–9.75)	0.03
Pre-transplant T-cell FCXM, RMF (only 1 case in each group)		N/A	N/A	
Pre-transplant B-cell FCXM, RMF (among positive cases)		2.7 (2.47–3.27)	3.85 (3.03–3.95)	0.04

Values expressed as Median (IQR), unless otherwise stated.

*[μ]These two cases were grouped in HLAi, as their pre-transplant FCXM, was weakly positive, *High risk relation between donor and recipient includes child to mother, or husband to wife relationship. Abbreviations: HLAi, Human Leukocyte antigen incompatible kidney transplantation; AAMR, aggressive antibody mediated rejection; cRF, calculated reaction frequency; DSA, donor specific antibody; MFI, median fluorescence index; FCXM, flow cytometry crossmatch; RMF, relative mean fluorescence ratio.*

limitations of this study are the heterogeneity of the immunosuppression protocols and the treatment regimens used over time.

It is clear that ABOi and HLAi transplants are different entities, and rejection episodes in these groups should be usually discussed separately; ABOi transplants have a better graft survival and lower rejection rates. Risk aversion to unfavorable patient and graft outcomes have led to a decline in the number of AITs across different centers [18]. However, it is observed that the graft outcomes of HLA incompatible kidney transplants are comparable to compatible deceased donor kidney transplants [19]. Also, patient survival in individuals who undergo HLA incompatible living donor kidney transplantation is better than [20] or comparable to [21] individuals who wait on dialysis for a compatible transplant. In our study, the 5-year patient survival was 81%, which is much higher than the 5-year patient survival on dialysis. In individuals who are very highly sensitized (cPRA ≥98%) or unsuccessful in the kidney sharing schemes, antibody incompatible living donor kidney transplantation is sometimes the only option [22]. However, the long-term graft outcomes of antibody incompatible living donor kidney transplants are worse when compared to antibody compatible living donor kidney transplants [5]. This difference in long-term graft outcomes

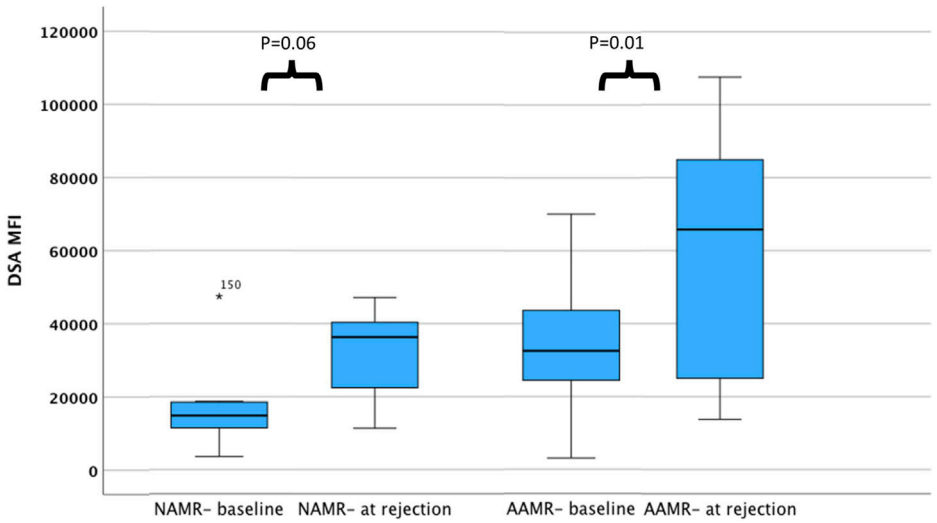


FIGURE 2 | DSA levels among cases with AMR at baseline and at rejection. The bar chart illustrates DSA MFI levels among cases with AAMR and NAMR, at baseline and at rejection. Compared to baseline, DSA MFI levels are higher at rejection for cases with aggressive AMR. Abbreviations: DSA MFI: DSA, donor specific antibody median fluorescence index; AAMR, aggressive AMR; NAMR, non-aggressive AMR.

between ABOi and ABO-compatible living donor kidney transplants has been attributed to increased risk of graft loss within the first 14 days due to antibody mediated rejection (AMR) in the ABOi transplants [23]. In HLA incompatible (HLAi) living donor kidney transplants [24], this has been attributed to AMR occurring at different time periods [25] i.e., initial graft loss from early acute AMR (<30 days post-transplant) [14], and long-term graft loss from late acute AMR (>30 days post-transplant) [26, 27] or chronic AMR (CAMR) [28, 29].

Previous reports suggest accelerated acute rejection occurs around a week after transplant and represents an anamnestic response [13, 14]. Locke et al suggest early severe AMR after crossmatch positive live donor kidney transplant results in sudden onset oliguria/anuria with a rise in DSA; and may lead to graft loss if treated only with plasmapheresis and IVIg. In this series of five cases, splenectomy in addition to standard rescue therapy was able to salvage all kidney allografts [13]. Orandi et al report early severe AMR in 24 (9%) of their patients at a median of 6 days after HLA-incompatible living donor kidney transplantation. Sudden onset oliguria, rapid rise in serum creatinine and marked rebound of DSA were observed in these patients. This study reports 100% 1-year graft survival in patients treated with combined splenectomy and eculizumab, compared to 78% and 30% when treated with splenectomy alone and eculizumab alone, respectively. They suggest that while splenectomy debulks the active plasmablasts, high levels of antibodies still persist. Eculizumab, a monoclonal antibody that cleaves C5 complement, renders these antibodies ineffective, which may otherwise take days to be cleared by plasmapheresis [14].

We used eculizumab only in severe forms of rejection (AAMR) which were refractory to treatment with plasma exchange and IVIg. We feel that pre-emptive PEX in this group of patients is unlikely to make a difference, as the data that appears in this entity suggests that the antibody titers rise very quickly and manyfold. In our study, we observed that patients with AAMR had greater than two-fold increase in DSA MFI levels at rejection. Eculizumab is known to reduce the rates of early humoral rejection in sensitized individuals [30, 31], and has been recommended as adjunctive treatment therapy for early acute humoral rejection according to expert consensus from the transplantation society working group [12]. Of note, the use of eculizumab was associated with worse overall patient survival (**Table 4**). An important finding that needs to be further explored is the occurrence of sudden-onset early death (<90 days) due to a suspected cardiac cause in three individuals (11, 18, and 44 years old) who have been treated with eculizumab. These individuals have had no other identifiable cause of death. Eculizumab treatment should be initiated immediately after rejection, as its protective effect may not be durable if strong DSA is allowed to persist for long periods of time [32]; however, its risks and benefits should be assessed.

The long-term graft survival outcomes in our study were comparable to other large studies which looked at long term graft outcomes in ABOi [33] and HLAi kidney transplants [19]. Data suggests that C1q and C3d complement fixing DSA negatively affects long term graft survival [34, 35]. We looked at pre-transplant complement fixing DSA in only 38 (65%) patients in the HLAi group and found that patients with pre-transplant C3d complement fixing DSA were at an increased risk of AAMR. Also, patients with C1q and C3d complement fixing DSA had a sensitivity of 100% as predictors of AAMR. Massive increase of DSA MFI was observed in AAMR (N = 11) at rejection as compared to baseline (65,797 vs. 32,519,

TABLE 6 | Subgroup analysis- Comparison between cases with and without AAMR in the HLAi group.

		No AAMR (n = 47)	AAMR (n = 11)	*p*-value
Donor age, years		40 (31–46)	43 (27–51)	0.87
Donor gender, n (%) (missing cases n = 2)	Female	18 (42)	5 (45)	1.0
	Male	26 (58)	6 (55)	
Donor ethnicity, n (%) (missing cases n = 3)	White	37 (84)	10 (90)	1.0
	Ethnic minorities	7 (16)	1 (10)	
Recipient age, years		43 (35–54)	42 (35–56)	0.70
Recipient gender, n (%)	Female	28 (60)	8 (73)	0.50
	Male	19 (40)	3 (27)	
Recipient ethnicity, n (%)	White	39 (83)	10 (91)	1.0
	Ethnic minorities	8 (17)	1 (11)	
Recipient dialysis status, n (%)	Pre-emptive	3 (6)	0	1.0
	HD or PD	44 (94)	11 (100)	
Duration on dialysis, months		60 (31–133)	66 (16–96)	0.71
Previous transplant, n (%)	No	21 (45)	6 (55)	0.74
	Yes	26 (55)	5 (45)	
High risk relation*, yes, n (%)		12 (25)	5 (45)	0.27
Peak cRF		97 (89–99)	99 (95–100)	0.11
HLA mismatch level, n (%)	Level 0, 1 and 2	6 (13)	1 (9)	1.0
	Level 3 and 4	41 (87)	10 (91)	
DSA to repeat mismatches, n (%) (missing cases n = 4)	No	19 (43)	2 (20)	0.23
	Yes	25 (57)	8 (80)	
C1q complement fixing DSA, n (%) (missing cases n = 20)	No	18 (53)	0	0.11
	Yes	16 (47)	4 (100)	
C3d complement fixing DSA, n (%) (missing cases n = 20)	No	21 (62)	0	0.03
	Yes	13 (38)	4 (100)	
Class I DSA MFI, baseline		9,422 (5,516–13814)	17,872 (14,514–26958)	0.01
Class II DSA MFI, baseline		3,138 (0–9,565)	11,760 (773–26489)	0.12
Total DSA MFI, baseline		14,583 (9,037–21691)	32,519 (23,371–45928)	0.001
Class I DSA MFI, pre-transplant (missing cases n = 13)		3,663 (1,498–7,749)	11,686 (2,334–17249)	0.056
Class II DSA MFI, pre-transplant (missing cases n = 13)		1,299 (0–5,153)	5,770 (0–8,768)	0.33
Total DSA MFI, pre-transplant (missing cases n = 5)		7,080 (3,752–11359)	12,883 (4,034–23991)	0.10
FCXM, B or T cell, baseline, n (%) (missing cases n = 1)	Negative[µ]	2 (4)	0	0.43
	Weak positive	7 (15)	0	
	Strong positive	37 (81)	11 (100)	
FCXM, B or T cell, pre-transplant, n (%) (missing cases n = 5)	Negative	27 (64)	6 (54)	0.14
	Weak positive	7 (17)	0	
	Strong positive	8 (19)	5 (46)	
Baseline T-cell FCXM, RMF		2.5 (1.73–3.42)	3.42 (2.2–9.64)	0.07
Baseline B-cell FCXM, RMF		3.86 (2.82–6.3)	6.44 (5.34–10.10)	0.02
Pre-transplant T-cell FCXM, RMF (only 1 case in each group)		N/A	N/A	
Pre-transplant B-cell FCXM, RMF (among positive cases)		2.86 (2.49–3.3)	3.93 (3.65–4.08)	0.02

Values expressed as Median (IQR), unless otherwise stated. [µ]*These two cases were grouped in HLAi, as their pre-transplant FCXM, was weakly positive,* *High risk relation between donor and recipient includes child to mother, or husband to wife relationship. Abbreviations: HLAi, Human Leukocyte antigen incompatible kidney transplantation; AAMR, aggressive antibody mediated rejection; cRF, calculated reaction frequency; DSA, donor specific antibody; MFI, median fluorescence index; FCXM, flow cytometry crossmatch; RMF, relative mean fluorescence ratio.*

p = 0.01), whereas the non-aggressive AMR (N = 7) do not have a significant increase in MFI (36,293 vs. 14,805, p = 0.06). This data should be interpreted with caution. The DSA MFI approximately doubles in the two subcategories. What differentiates the two types of AMR is the higher baseline value in the aggressive AMRs. The borderline statistical significance is the result of the very low sample size of this subgroup analysis. In practice, we may avoid considering individuals with very high baseline total DSA MFI levels for an AIT as they may be at an increased risk of AAMR. We also advocate assessing complement data in more detail and avoid an AIT if the recipient has complement fixing DSA, pre-transplant.

We believe AAMR may be an anamnestic response from memory B and T-cells. One of the mechanisms of DSA formation is from interactions between $CD4^+$ T-cells and donor HLA, via indirect pathway. Signals from these activated T-cells differentiates naïve B cells into antibody producing B-cells and antigen-specific memory B-cells. Memory B-cell survival is not dependent on continued exposure of antigen, and their threshold for activation is low. They rapidly expand and differentiate into short-lived

antibody producing plasma cells on exposure to antigens [36]. Methods to identify HLA-specific memory B cells pre-operatively may risk-stratify candidates for AIT and prevent an anamnestic response [37]. Quantification of memory B-cells can be achieved using HLA specific B cell ELISpot assay. Interestingly, these donor specific memory B-cells may be present pre-transplant, despite the absence of circulating DSA [38]. Inflimidase is a protease that cleaves IgG antibodies. Our study identifies that high baseline DSA MFI levels, complement fixing DSA, and DSA against repeat mismatches with a previous failed transplant are risk factors for AAMR. Also, AAMR presents with significant rise in DSA MFI levels at rejection. The role of Inflimidase in desensitization protocols and treatment of AAMR needs to be further explored. Encouraging short-term graft and patient survivals were observed in HSP kidney transplant recipients with Inflimidase desensitization [39]. By routinely assessing memory B cells and Inflimidase use, we may be able to perform AIT with low short-term risk.

In conclusion, AAMR is of significant clinical concern in AIT as it results in poor graft survival and is not well described in literature. Outcomes may be improved if we can predict this pre-operatively. Baseline immunological characteristics such as C3d complement fixing DSA, Class I DSA MFI levels, total DSA MFI levels and B-cell FCXM RMF can be used to risk stratify these patients. HLAi transplantation should be avoided in patients with strong positive flow crossmatch, in particular with high DSA MFI or complement fixing DSA or DSA against repeat mismatches with a previous failed transplant. Complement inhibition can be successful if initiated early after rejection, but its use should be considered on an individual basis. AAMR may be due to T or B cell memory response, and methods to identify this pre-operatively would be an important area of future research.

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 was conducted as an audit under the auspices of hospital audit committee and was exempt from institutional review board approval as it was a retrospective observational study. This study was conducted in accordance with the standards laid down by Declaration of Helsinki. Written informed consent was not required for this study because it was a retrospective observational study.

AUTHOR CONTRIBUTIONS

SP, Acquisition, analysis and interpretation of data; Drafting the manuscript. MI, Analysis of data and drafting of manuscript. BLP, Analysis of data and critical revision of manuscript. JS, Acquisition of data and critical revision of manuscript. NK, Interpretation of data and critical revision of manuscript. OS, Acquisition of data and critical revision of manuscript. AD, Interpretation of data and critical revision of manuscript. NM, Conception of study, interpretation of data and critical revision of manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

Author AD is a consultant for Hansa Biopharma, and is on the scientific advisory board for Verici Dx Ltd. Author NM receives honoraria from Hansa Biopharma, Takeda and Novartis. The rest of the author(s) of this study have no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated. Author OS was employed by Viapath.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12942/full#supplementary-material

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Glycolysis Changes in Alloreactive Memory B Cells in Highly Sensitized Kidney Transplant Recipients Undergonig Desensitization Therapy

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Despite the growing use of desensitization strategies, hyperimmune patients remain at high risk of antibody-mediated rejection suggesting that, even when donor-specific antibodies (DSA) are effectively depleted, anti-donor specific B cells persist. We included 10 highly sensitized recipients that underwent desensitization with plasmapheresis and B cell depletion prior to kidney transplantation. We quantified changes in DSA (luminex), total B-cell subsets (flow cytometry), anti-donor HLA B cells (fluorospot), and single-cell metabolism in serially collected samples before desensitization, at the time of transplant, and at 6 and 12 months thereafter. Desensitization was associated with a decrease in DSA and total memory B cell and naive B cell percentage, while plasma cells and memory anti-donor HLA circulating B cells persisted up to 12 months after transplant. At 12-month post-transplantation, memory B cells increased their glycolytic capacity, while proliferative KI67+ plasma cells modified their metabolism by increasing fatty acid and amino acid oxidation capacity and decreasing their glucose dependence. Despite effective DSA depletion, anti-donor B cells persist in kidney transplant recipients. Due to the reliance of these cells on glycolysis, glycolysis-targeting therapies might represent a valuable treatment strategy.

Keywords: donor-specific antibody, desensitization, kidney transplantation, metabolism, memory B cells, glycolysis

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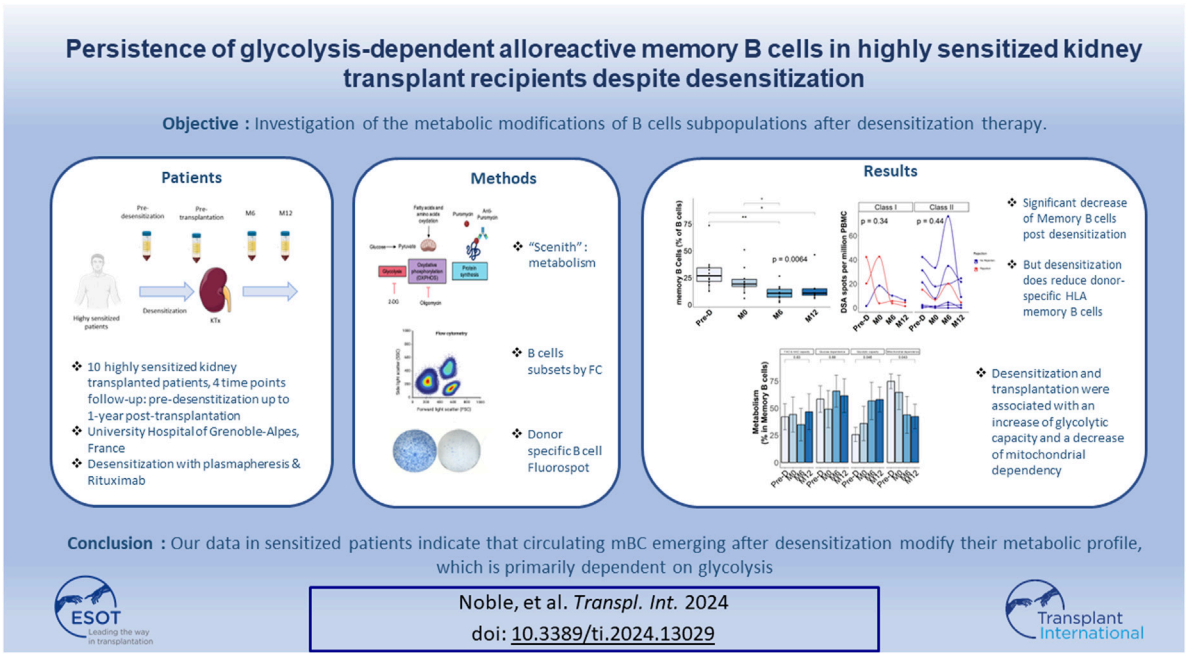
Abbreviations: FAO/AAO, fatty acid and amino acid oxidation; HLA, Human Leukocyte Antigen; IL-2, interleukin 2; mBC, memory B cells; Oligo, Oligomycin; OXPHOS, Oxidative phosphorylation; PBMC, Peripheral Blood Mononuclear Cells; SCENITH, Single-Cell ENergetIc metabolism by profiling Translation inhibition; 2-DG, 2-deoxyglucose.



GRAPHICAL ABSTRACT

INTRODUCTION

One of the main obstacles to access kidney transplantation (KT) in a context of graft shortage is the presence of anti-human leukocyte antigens (anti-HLA) antibodies. Highly-sensitized patients waiting for a KT represent approximately 10% of the waitlisted individuals and their number is increasing every year [1]. Highly sensitized patient remains on the waiting list longer than non-sensitized patients, despite different national prioritization programs for these patients [2]. This prolonged time on the waiting list and in dialysis is responsible for an increase in patient morbidity and mortality as well as a significant cost to society [3].

To increase the access to transplantation for highly sensitized patients, anti-HLA desensitization may be proposed. Anti-HLA desensitization involves the use of treatments that remove anti-HLA antibodies from plasma and prevent the formation of new anti-HLA antibodies. The most commonly used strategy relies on plasmapheresis and B cell depletion with anti-CD20 monoclonal antibodies (Rituximab) [4–7].

However, despite these techniques that allows KT without hyper-acute rejection, the risk of antibody-mediated rejection (AMR) remains high varying between 15% and 43% depending on the populations, the desensitization techniques, systematic biopsies, and the duration of follow-up [6, 8, 9].

The dynamic changes of B cell compartments, including memory B cells (mBC) after desensitization and post KT are still poorly defined.

Immune cell survival and function are dependent on the adaptability of their metabolism which may be modified by many external factors, including, among many others, inflammation or immunosuppression [10]. Therefore, B cell metabolisms may serve as a surrogate marker for rejection risk. Moreover, metabolic changes of B cells may provide new therapeutic targets to prevent antibody-mediated rejection (AMR) in high-risk patients [11]. This formed the background for the present cohort study aimed at deciphering changes in B cell subsets, including donor-reactive mBCs, and their metabolic profile in highly sensitized kidney transplant recipients undergoing desensitization.

MATERIALS AND METHODS

Patients and Study Design

In this monocentric study, we included highly sensitized (historical PRA ≥85%) adult patients that received a KT at Grenoble University Hospital, Grenoble, France, between January 2015 and November 2022 post-desensitization therapy. Patients had to be on the KT waiting list for at least 3 years before inclusion, to have no history or ongoing severe infectious or neoplastic disease and to have a satisfying cardiac check-up within the previous 3 months. Peripheral Blood Mononuclear Cells (PBMC) were collected at 4 timepoints: before desensitization, the day of transplantation, at 6-month (M6) post-KT and at 12-month (M12) post-KT (**Supplementary Figure S1**). Donor-specific antibodies (luminex) were quantified at the same timepoints.

The protocol was approved by investigational review board at Grenoble University Hospital (AC-2019-3627) and by French National committee for data protection (CNIL; approval number 1987785v0). All patients signed written informed consent.

Desensitization Procedure and Post-Transplant Immunosuppression

Desensitization protocol consisted of *i*) two Rituximab i.v., (375 mg/m^2), 2 weeks apart; *ii*) serial apheresis sessions were performed by immunoadsorption or double-filtration plasmapheresis and a plasma-exchange before the transplantation; *iii*) oral immunosuppression started the first day of first apheresis. The immunosuppression regimen included prednisone (0.5 mg/kg), mycophenolate mofetil (500 mg x2/day), and tacrolimus (0.1 mg/kg/day, then adapted to an 8–10 ng/mL target trough concentration). Post-transplantation, all recipients received anti-thymocyte globulin induction therapy (1 mg/kg/day for 5 days). Tacrolimus and mycophenolate mofetil were continued post-transplantation and steroids were tapered at 3 months post-transplantation (10 mg/day).

Phenotypic Analysis of B Cell Subpopulations

PBMC were isolated by density gradient centrifugation (Ficoll®) separation and stored at −120°C before use. After thawing, PBMC were incubated at 37°C between 2 and 4 h to allow them recovering from cryopreservation. Dead cells were stained with LIVE/DEAD™- Yellow LDFixable-575 (BD Biosciences) at room temperature during 15 min. Then, PBMC were stained with the mix of fluorochrome-labelled anti-human antibodies during 20 min at 4°C. The panel included following markers: CD20-BV421, CD38-BV510, CD3-BV650, CD14-BV650, CD24-BV711, CD27-BV786, IgM-BB515 and CD19-APC-R718 (BD Biosciences) antibodies.

After washing, cells were fixed and permeabilized using fixation and permeabilization buffer following manufacturer instructions. Intracellular staining was performed by incubating cells during 30 min at 4°C with intracellular antibodies: Puromycin-AF647 (Merck), Ki67-PE and IgD-BB700 (BD Biociences). Stained cells were then directly analysed using the 4-laser BD FACSymphony A3 flow cytometer, data were analysed by BD FACSDiva™ software (BD Biosciences), FCS Express-7 software and Cytobank. A minimum of one million PBMC of cells were used in each experiment. The gating strategy of B cell subpopulations was shown in **Supplementary Figure S2**.

Analysis of Cell Metabolism

We used the SCENITH (Single-Cell ENergetIc metabolism by profiling Translation inhibition) technology to assess the metabolic features of B cell subsets at a single cell level [12]. Before antibody staining, PBMC were resuspended in RPMI 10% FCS and were treated during 30 min at 37°C with DMSO (Control), 2DG (100 mM, Sigma), Oligo (1mM, Ozyme), or both (DG + Oligo). Puromycin (Puro, 10 mg/mL, Cayla) was then added during 20 min and cells were washed in cold PBS. The metabolic profiles allowed to distinguish: "glucose dependency" and "mitochondrial dependency" (i.e., the proportion of protein synthesis dependant on glucose and oxidative phosphorylation [OXPHOS], respectively), "glycolytic capacity" showing the maximal capacity of glycolysis to compensate OXPHOS inhibition and "fatty acid and amino acid oxidation (FAO/AAO) capacity" showing FAO/AAO maximal capacity to compensate both glycolysis and OXPHOS inhibition.

HLA Donor Specific B cells

PBMCs were stimulated at a concentration of 1.5×10^6 PBMC/mL [13, 14]. The non-specific stimulation cocktail consisted in recombinant interleukin 2 (IL-2) at 10 ng/mL and R848 (Resquimod) at 1 µg/mL from Mabtech®. After 6 days, cells were added in ELISpot wells at $4.5.10^6$/mL (100 µL) and incubated at 37°C (5% CO_2) for 24 h in plate coated with anti-human IgG. Anti-HLA specific IgG-antibody secreting cells were detected using fluorescent dye labeled class I and II HLA dextramers and tetramers to cover at least 1 donor specific HLA corresponding to immunodominant DSA in recipients (PE-tagged B51 dextramers, FITC-labelled dextramer A24, PE-labelled dextramer A2) (Immudex®, Cy5-labelled DR9 tetramers, Cy3-labelled DQ2 tetramers). FluoroSpot enhancer was added during 15 min before reading. Spot quantification was made on IRIS™ reader (Mabtech®). We presented the findings as the proportion of HLA-specific memory B cells (mBC) relative to polyclonal IgG (used as positive controls) for each HLA antigen [15].

IgG Total Secretion by Antibody Secreting Cells Assessed by B Cell ELISpot With Metabolism Blockade

To assess the effect of glycolysis and OXPHOS blockade on IgG secretion by activated mBC, we performed modified ELISpot protocol. We stimulated PBMC with the same non-specific stimulation cocktail IL-2 at 10 ng/mL and R848 at 1 µg/mL (Mabtech®) in addition to 2- DG (100 mM, Sigma), Oligo (1 mM, Ozyme) or both (DG + Oligo). Positive control consisted in Cycloheximide (100 mg/mL, Sigma) and stimulated PBMC cultured without metabolism blockers were used as negative. PBMC were stimulated for 4 days (comparable results *versus* 6 days, data not shown) and added in the anti-IgG coated wells at $4.5.10^4$/mL (100 µL) and incubated at 37°C (5% CO_2) for 24 h. IgG secretion by polyclonal antibody secreting cells was then revealed colorimetrically after addition of Streptavidin-ALP complex and BCIP/NTB-plus substrate.

Statistical Analysis

All numerical data were presented as mean ± standard deviation or median [Q1Q3] according to distribution. All categorical variables were presented as number (percentage).

TABLE 1 | Baseline patients' characteristics.

	Total n = 10
Donor	
Age (year); median (Q1; Q3)	59.3 ± 11
Gender, male; N (%)	4 (40.0%)
Type, deceased; N (%)	2 (20%)
Recipient	
Age (year); median (Q1; Q3)	48 ± 14
Gender, male; N (%)	4 (40)
Delay between desensitization and transplantation (day); median (IQR)	28.5 (23; 31)
Time on the transplant waiting list (year); median (Q1; Q3)	3.8 (3.0; 5.5)
Initial nephropathy	
- Hypertension	4 (40)
- Diabetes	2 (20)
- ADPKD	1 (10)
- Membranous nephropathy	1 (10)
Unknown	2 (20)
Graft number, first; N (%)	6 (60)
Sensitizing events; N (%)	
- Pregnancy	5 (50)
- Blood transfusion	3 (30)
- Transplantation	4 (40)
Panel Reactive Antigen; median (Q1; Q3)	95 (90.5; 97.0)
Mismatch AB; median (Q1; Q3)	3.0 (2.0; 3.0)
- 1; N (%)	2 (20)
- 2; N (%)	2 (20)
- 3; N (%)	5 (50)
- 4; N (%)	1 (10)
Mismatch DQ; median (Q1; Q3)	1.5 (0.0; 2.0)
- 0; N (%)	4 (40)
- 1; N (%)	1 (10)
- 2; N (%)	5 (50)
Mismatch DR; median (Q1; Q3)	0.5 (0.0; 1.5)
- 0; N (%)	5 (50)
- 1; N (%)	3 (30)
- 2; N (%)	2 (20)
Crossmatch positivity on historical sera; N (%)	2 (20)
- LCT	3 (30)
- FACS	5 (50)
Desensitization protocol; N (%)	
- RTX + maintenance therapy + plasmapheresis	9 (90)
- RTX + maintenance therapy	1 (10)
Plasmapheresis type; N (%)	
- PE	3 (30)
- DFPP	6 (60)
- IA	4 (40)
Number of plasmapheresis session; median (Q1; Q3)	8 (7; 12)
Induction therapy, ATG; N (%)	10 (100)
Maintenance therapy, Tac MMF Cs; N (%)	10 (100)

ADPKD, Autosomal dominant polycystic kidney disease LCT, lymphocytotoxicity cross-math; FACS, Flow-cytometry cross-match; RTX, rituximab; PE, plasma exchange; DFPP, double-filtration plasmapheresis; IA, immunoadsorption; ATG, anti-thymoglobulin; Tac, Tacrolimus; MMF, mycophenolate mofetil; CS, corticosteroids.

Wilcoxon test was used to compare continuous variables and Fisher exact test was used to compare categorical variables. A two-sided p-value of <0.05 was considered statistically significant and all p-value <0.1 were shown in the figures. To compare the overall differences of subpopulation over time, we first performed an ANOVA. Then we performed two by two analysis between all-time points using t-test after Bonferroni correction. Statistical analyses and figures were conducted using R statistical software® 0.98.932 (Boston, MA, USA). Flow cytometry FCS files were analyzed using Cytobank software [16].

RESULTS

Patients Baseline Characteristics and Outcomes

We included 10 highly sensitized patients (median PRA: 95% [90.5%–97.0%]) that received a KT post-desensitization at the KT department of Grenoble-Alpes (Grenoble, France). Donor and recipient characteristics are presented in **Table 1**.

Before desensitization, mean number of class I DSA was 2.0 ± 1.0 and mean number of class II DSA was 1.4 ± 0.8. The day of KT, mean number of class I DSA was 0.7 ± 1.2 and mean number of class II DSA was 0.6 ± 0.5 (**Figures 1A, B**). Desensitization therapy was associated with a significant decrease in all DSA MFI and allowed KT with negative crossmatch (**Figure 1C**). Mean immunodominant MFI (Mean Fluorescence Intensity) for class I DSAs before desensitization was 5,564 ± 5,072 and decreased to 1252 ± 1943 the day of transplantation, p = 0.14 (**Figure 1D**). Mean immunodominant class II DSA MFI before desensitization was 4629 ± 526 and decreased to 1694 ± 2774 the day of transplantation, p = 0.024 (**Figure 1D**). The sum of all class I MFI was 8,232 ± 7,903 before desensitization and 6,102 ± 10,570 at 12-month, p = 0.507 (**Figure 1E**) and all class II MFI was 4195 ± 5058 before desensitization and was 586 ± 681 at 12-month, p = 0.170 (**Figure 1F**).

At the end of the follow-up period (61.6 months [49–68]), all patients were alive and none of them had lost their graft. Three patients developed acute rejection: two patients developed AMR and one patient had a cellular borderline rejection. All patients were successfully treated with steroids pulse and plasmapheresis. Although non statistically significant, immunodominant DSA mean MFI was higher in patients with rejection as compared with those without rejection (8,503 ± 5,808 versus 2,866 ± 1,413.8, p = 0.180). Similarly, sum of all DSA MFI was higher in patients with rejection: 12,776 ± 11,173 as compared to those without rejection: 3,619 ± 4,116 (p = 0.086).

Changes in B Cells Subsets

Upon rituximab therapy, $CD19^+$ B cells were effectively depleted and fully recovered at 6 months post-transplant (**Figure 2A**). The percentage of memory B cells (mBC) significantly declined after desensitization and did not fully recover post-transplantation (**Figure 2B**).

The frequency of plasma cells decreased, but not significantly with desensitization therapy. We observed a return to baseline level at 6 months and at 12 months after transplantation (**Figure 2C**).

We observed a marked decrease of the naive B cell ($CD27^-$ IgD^+) after desensitization that did not fully recover post-transplantation (**Figure 2D**). The frequency of transitional B cells did not significantly change after desensitization (**Figure 2E**).

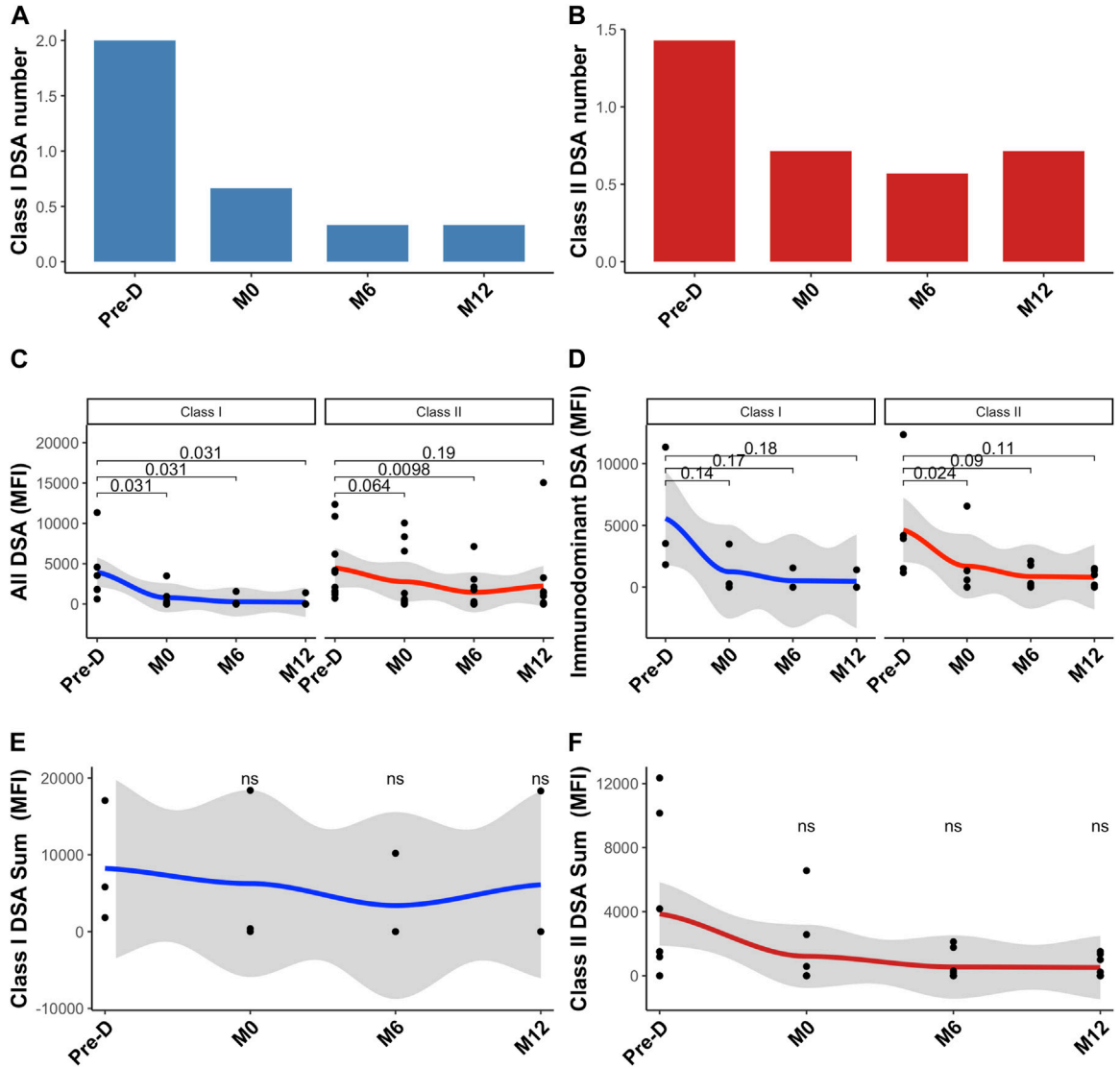


FIGURE 1 | Donor-specific antibodies change post desensitization and transplantation. Trends in the number of class I DSA **(A)**, class II DSA **(B)**, in all class I and II DSA MFI **(C)**, in immunodominant class I and class II DSA **(D)**, in class I sum of MFI **(E)** and in class II sum of MFI **(F)**. Blue is for class I DSAs and Red is for class II DSAs. Pre-D: pre-desensitization.

We found no correlation between DSA and anti-HLA specific B cells.

Changes in Proliferative B Cell Subsets

While the relative percentage of total Ki67$^+$ B cells was not impacted by desensitization and transplantation (**Figure 3A**), desensitization was associated with a decrease of Ki67$^+$ mBC percentage, followed by an increase at M6 and M12 (**Figure 3B**).

The percentage of Ki67$^+$ plasma cells significantly decreased post-desensitization and recovered to pre-desensitization levels at M12 post-transplant (**Figure 3C**).

The percentage of Ki67$^+$ naïve B cells, significantly increased post-desensitization and decreased post-transplantation (**Figure 3D**). The percentage of Ki67$^+$ transitional B cells progressively increase after transplant increased up to M12 (**Figure 3E**).

Proliferative mBC and transitional B cells are the subsets that increased the most after transplant.

Desensitization Does Not Reduce Donor-Specific Anti-HLA B Cells

Next, we focused on the donor-specific anti-HLA B cells by FluoroSpot. The number of total IgG spots secreted by polyclonal antibody secreting cells was positively correlated (p = 0.012) with the percentage of mBC assessed by Flow cytometry (**Supplementary Figure S3**) but not with the other B cells subtypes (data not shown). Median number of donor specific anti-HLA B cells was 17.6 spots [1.7–28.6] before desensitization and

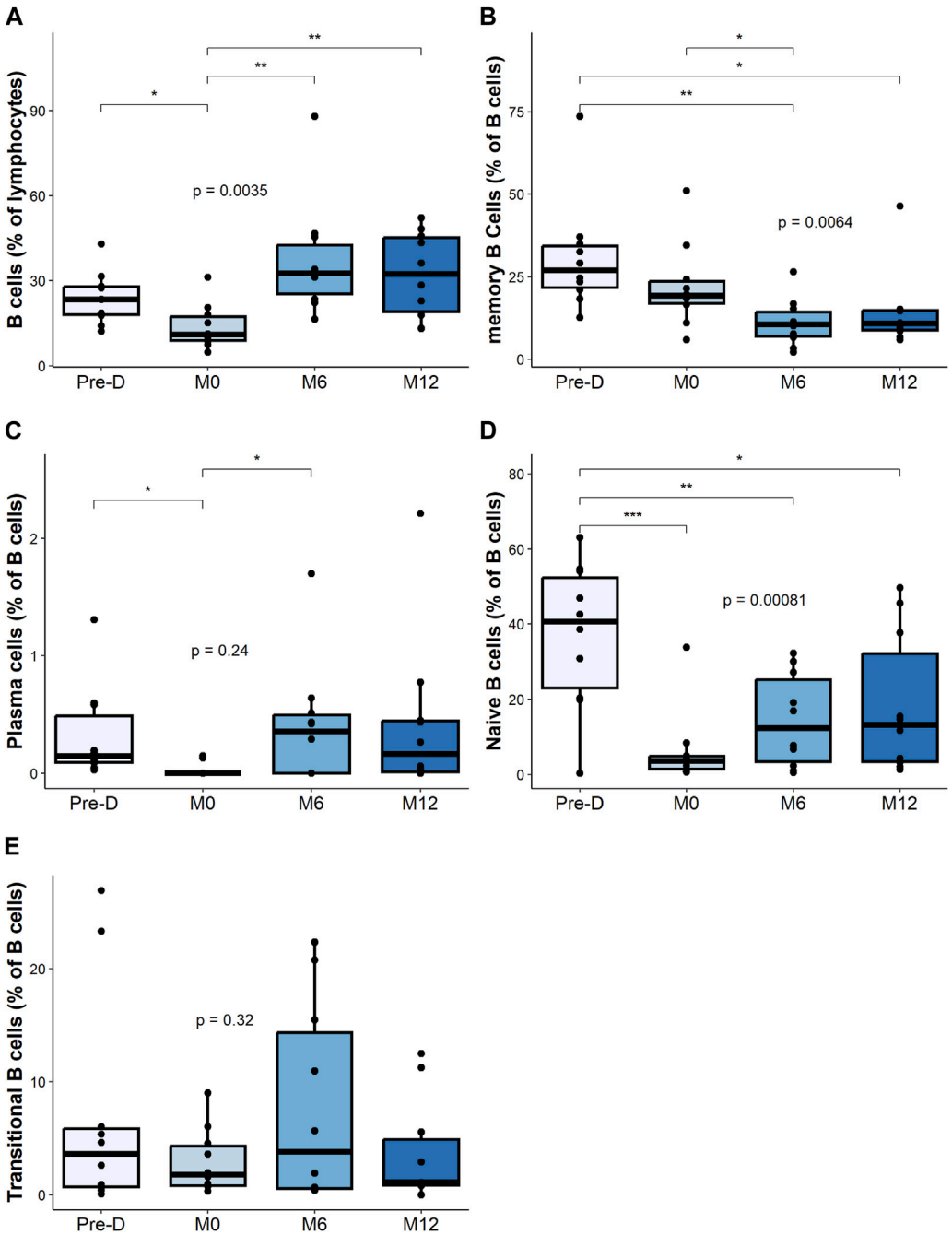


FIGURE 2 | B cell composition and change post desensitization and transplantation. Trends in B cells and plasma cells before and after desensitization and transplantation: total B cells ($CD19^+CD3^-CD14^-$) **(A)**, memory B cells ($CD19^+CD38^-$ $CD24^+$ $CD27^+$) **(B)**, plasma cells ($CD19^+CD27^{hi}$ $CD38^{hi}$) **(C)**, naïve B cells ($CD19^+CD27^-$ IgD^+) **(D)**. Transitional B cells ($CD19^+$ $CD27^-$ $CD24^{hi}$) **(E)**. *$p < .05$ by Anova test. PBMC were also available for all 10 patients at the 4 timepoints.

7.2 spots [1.7–18.2] at pre-transplantation, $p = 0.41$. This secretion remained stable at 6 months: 7.8 spots [3.8–20] and at 12 months: 4.4 spots [1.6–8.2] post-transplantation. **Figure 4** shows the evolution of class I and class II donor-specific anti-HLA B cells. There was no statistical difference between patients with rejection and those without rejection regarding the evolution of donor-specific anti-HLA B cells.

Three patients developed rejection post transplantation. The number of donor-specific anti-HLA IgG was higher in those three patients at pre-desensitization time as compared to the recipients without humoral rejection although this difference did not reach significance: 26 ± 14 spots in patients with rejection, versus 14 ± 17 spots in recipients without rejection, $p = 0.37$ (despite a significantly higher

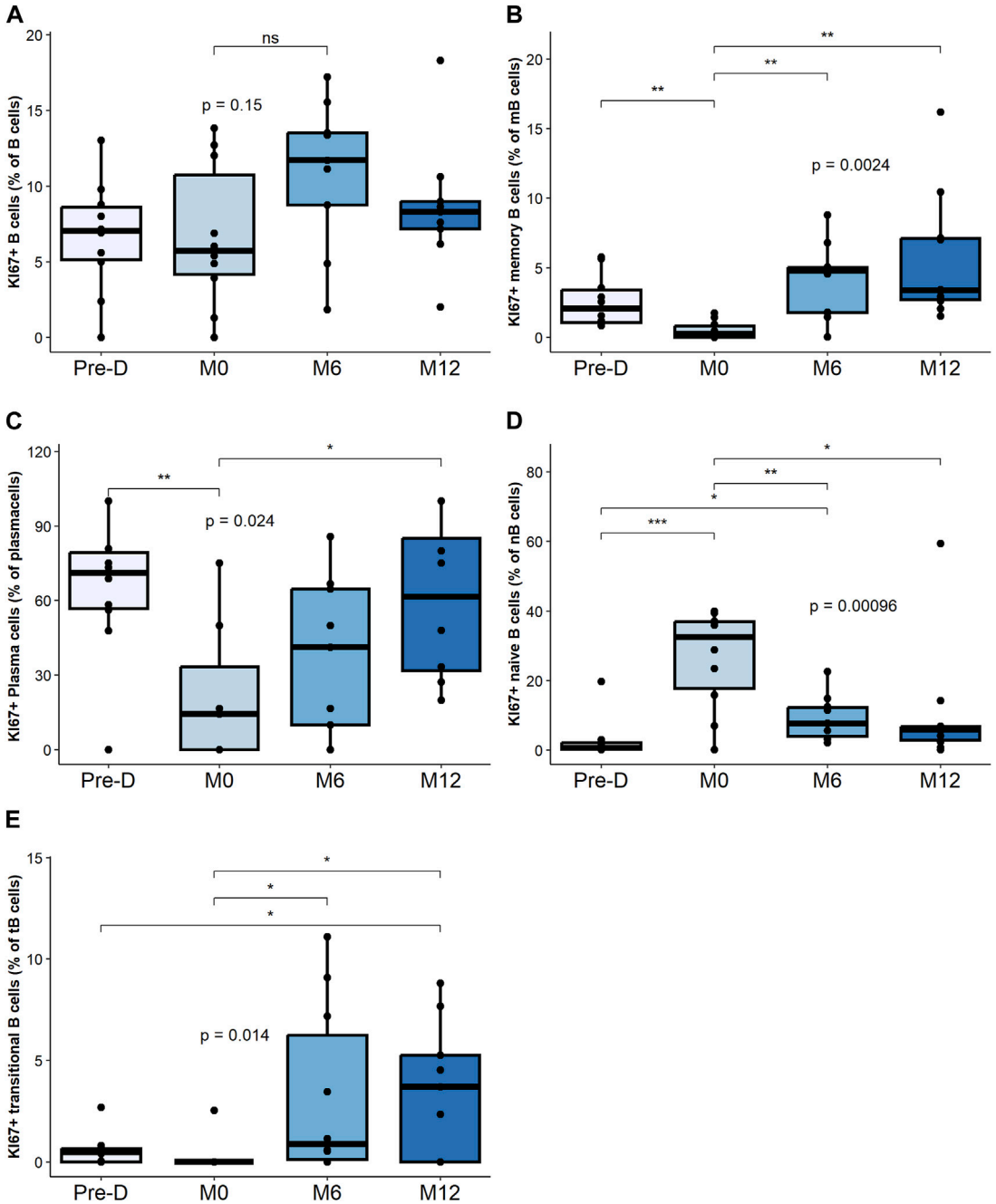


FIGURE 3 | Proliferative B cell composition and change post desensitization and transplantation. Trends in KI67 + B cells and plasma cells before and after desensitization and transplantation: total B cells ($CD19^+CD3^-CD14^-$) **(A)**, memory B cells ($CD19^+CD38^-$ $CD24^+$ $CD27^+$) **(B)**, plasma cells ($CD19^+CD27^{hi}$ $CD38^{hi}$) **(C)**, naïve B cells ($CD19^+CD27^-$ IgD^+) **(D)**. Transitional B cells ($CD19^+$ $CD27^-$ $CD24^{hi}$) **(E)**. *$p < .05$ by Anova test. PBMC were also available for all 10 patients at the 4 timepoints.

total IgG secretion in patients without rejection: 4950 spots per million of PBMC [999–17,238] *versus* 2475 [876–25,825] in patients with AMR, $p < 0.001$). **Supplementary Figure S4** illustrates the stereotypical evolution profile of DSA in relation to similar HLA-specific mBC as measured by FluoroSpot in 4 patients. This data shows that there is no clear correlation between the levels of DSA and the frequency of donor-specific mBC.

Changes in B Cell Metabolic Profile

Before desensitization, the metabolic profile was similar across all B subpopulations (**Supplementary Figure S5A**), were also not

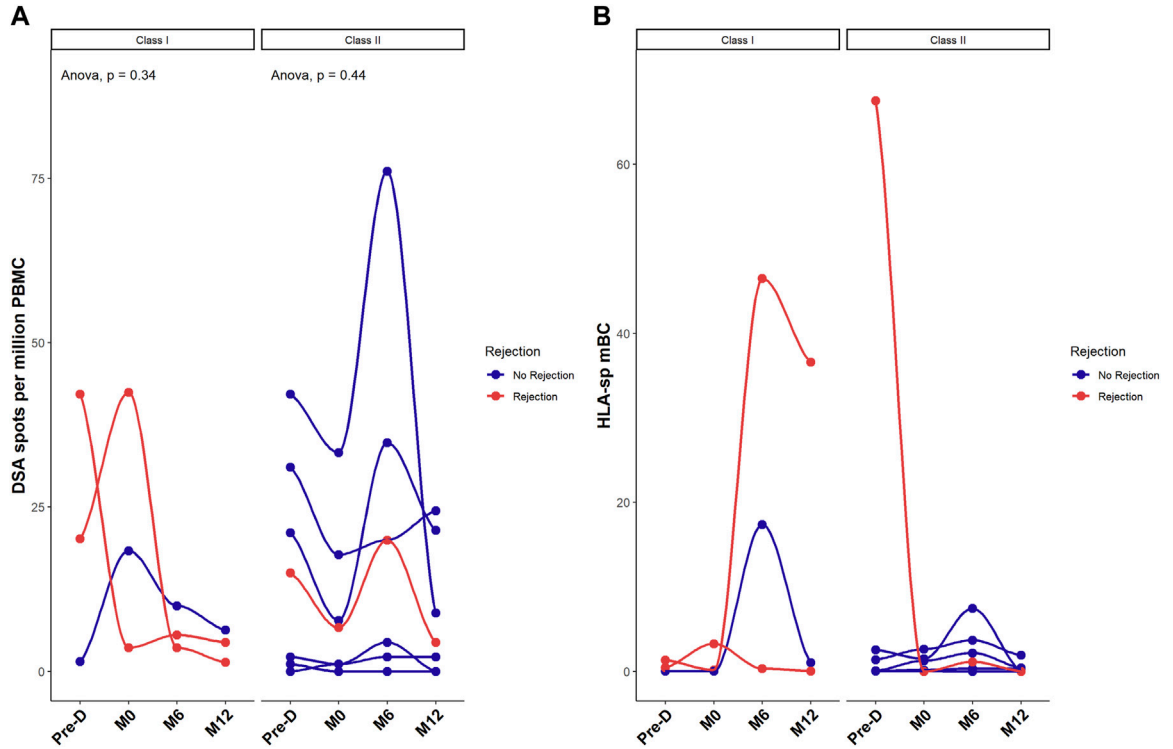


FIGURE 4 | Desensitization does reduce donor-specific HLA memory B cells. Donor-specific memory B cell differentiation into plasma cells assessed by FluoroSpot measuring donor-specific antibodies (spots) and expressed as mean number of spots per million of PBMC **(A)** and ratio between HLA-specific mBCs over the total polyclonal IgG mBCs in each patient **(B)**. Red dots correspond to patients with rejection. *$p < .05$ by Anova test. PBMC were also available for all 10 patients at the 4 timepoints.

statistically different at 12 months post-post-transplantation (M12) (**Supplementary Figure S5B**).

Within the overall B cell population, desensitization and transplantation had no significant impact on cell metabolism (**Figure 5A**). We next zoomed in on B cell subpopulations metabolism.

In mBC, desensitization and transplantation were associated with an increase of glycolytic capacity between pre-desensitization and M12 and a decrease of mitochondrial dependency (**Figure 5B**). FAO/AAO capacity and glucose dependence were not impacted. To further define the dependency of mBC in kidney transplant recipients on glycolysis, we performed ELISpot with different metabolism inhibitors. Selective inhibition of glycolysis resulted in a significant decrease of IgG secretion (spots) by activated memory B cells, while OXPHOS inhibition did not affect it (**Figure 6**).

Metabolic requirements did not significantly change over follow-up period in naïve B cells, transitional B cells and plasma cells (**Figures 5C–E**). In KI67+ plasma cells, percentage of FAO/AAO capacity significantly increased post desensitization and transplantation while glucose dependence percentage significantly decreased (**Supplementary Figure S6**). The metabolic modifications were not statistically significant in KI67+ mBC. The tendency was similar with a median increase of glycolytic capacity from 26% at baseline to 60% at M12, $p = 0.44$ and a median decrease of mitochondrial dependence from 73% at baseline to 31% at M12.

DISCUSSION

Previous studies have shown a clear correlation between circulating HLA-specific mBCs and high risk of antibody-mediated acute and chronic rejection in kidney transplantation [14, 15]. However, no study has formally investigated the changes in circulating HLA-specific mBCs after desensitization. Our data indicate that apheresis and B cell depletion, together with chronic immunosuppression are effective in removing DSA allowing for transplantation procedure, but do not clear HLA-specific mBCs. These cells are mainly located in peripheral lymphoid organs and, upon re-encounter with target antigens, can differentiate into antibody-secreting cells [17]. Therefore, they may account for the high risk of ABMR despite effective DSA removal after desensitization [18].

Long-lived plasma cells are also a major source of alloantibodies. These cells reside primarily in the bone marrow where they continuously secrete antibodies [19]. Our data indicate that desensitization and immunosuppression are not able to reduce circulating plasma cells but reduced the proliferative ratio of plasma cells at the initial phase. A limitation of our study lies in

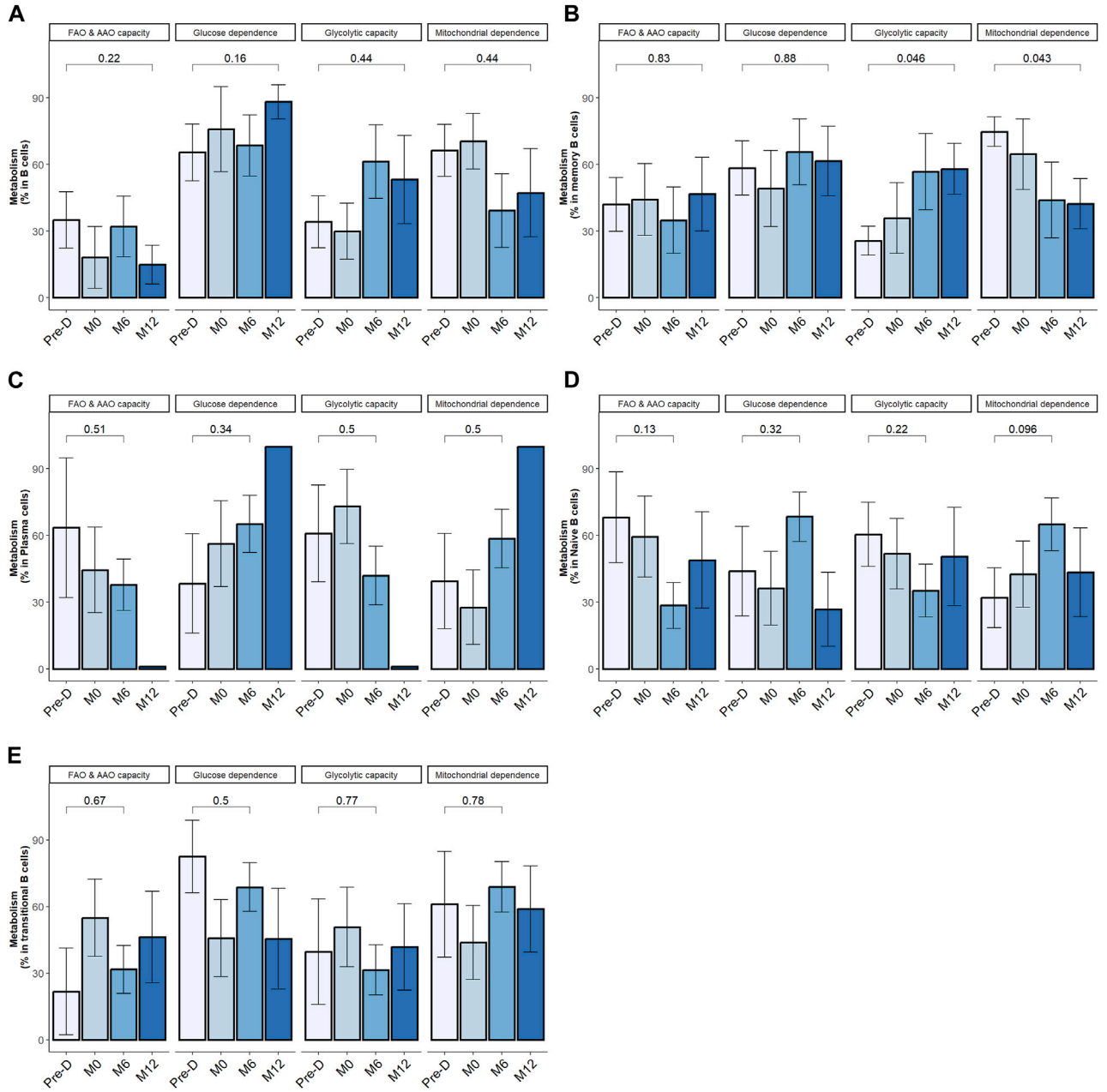


FIGURE 5 | Desensitization and transplantation impact on B cell metabolism. Trends in B cell subsets metabolism profile evolution before desensitization (Pre-D), pre-transplantation (M0), at month-6 (M6) and at month-12 (M12) post-transplantation; total B cells ($CD19^+CD3^-CD14^-$) **(A)**, memory B cells ($CD19^+CD38^-$ $CD24^+$ $CD27^+$) **(B)**, plasma cells ($CD19^+$CD27hi CD38hi) **(C)**, naïve B cells ($CD19^+CD27^-$ IgD^+) **(D)**. Transitional B cells ($CD19^+$ $CD27^-$ CD24hi) **(E)**. The p-value is indicated the evolution of metabolism percentage between Pre-D and M12. *p < .05 by Wilcoxon test. FAO: fatty acids oxidation; AAO: amino acids oxidation. 4 patients had 1 timepoint missing.

the fact that we only analyzed circulating plasma cells and long-lived plasma cells reside in the bone marrow. Some data on long-lived plasma cells suggest that their metabolism requires more glucose and amino acids than short-lived plasma cells (ref lam cell rep). No study has reported on the metabolic changes induced by allostimulation of long-lived plasma cells.

Metabolism has been shown to shape the survival and functionality of innate and adaptive immune cells [20, 21]. However, metabolic profile of B cells has been poorly characterized, especially in the field of solid-organ transplantation. We observed that, in sensitized patients, B cell subsets have a similar baseline metabolism profile characterized by a high glucose and mitochondrial dependency associated with a lower level of FAO, AAO and glycolytic capacities. Interestingly, after desensitization and after transplantation, we observed different metabolic modifications within the B cell subpopulations. After

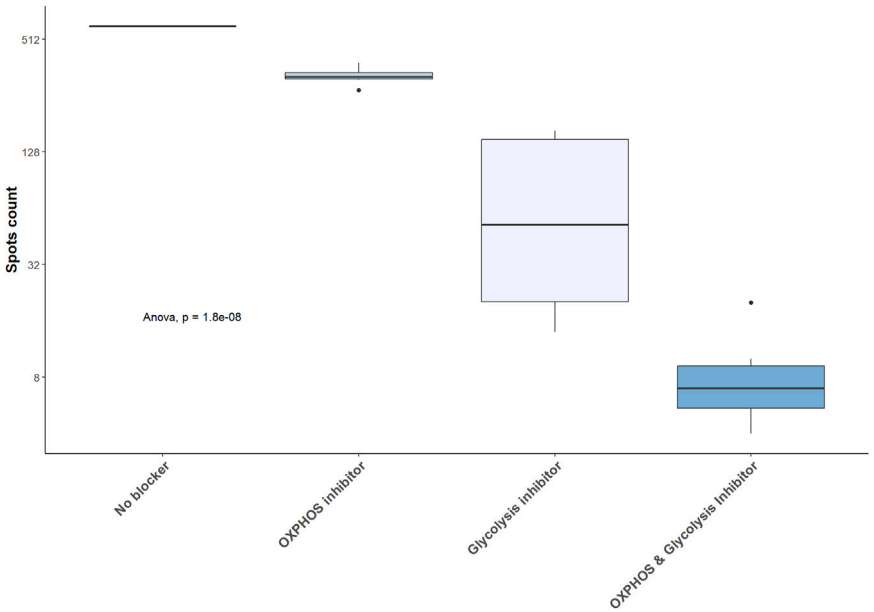


FIGURE 6 | Glycolysis inhibition results in a significant decrease in IgG secretion by activated memory B cell. IgG secretion assessed by ELISpot in 5 kidney transplant recipients and expressed as raw number of spots. OXPHOS: oxidative phosphorylation.

desensitization, total B cells re-emerged to baseline level but with more heterogenicity in their metabolism capacities. mBC percentages did not fully recover after transplant. Of note, those that formed after desensitization had a high glucose dependency, higher glycolytic capacity, and lower OXPHOS metabolism than at baseline. Interestingly, Torigeo *et al.* showed that glucose uptake and glycolysis are important for mBC differentiation into plasma cells [22]. This may at least in part explain the glycolytic capacity increase in mBC post-KT of sensitized kidney recipients and the major impact of antibody secretion by glycolysis inhibitor in the ELISpot results. After desensitization and transplantation, we also observed a re-emergence of proliferative plasma cells with a different metabolic profile, i.e., with higher FAO/AAO capacity and less glucose dependence. This is consistent with Lam *et al.* showing an elevated expression of an amino-acid transported in long-lived plasma cells [23].

Improvement in immunosuppressive strategies have contributed to improve long-term patient and graft survival. Yet, long-term immunosuppression is burdened by increased risk of infections, cancer, and metabolic complications [24–26]. Targeting metabolism, especially by blocking specific pathways may effectively control alloimmune response.

We tried to directly modify the metabolism of antibody-secreting cells using glycolysis and OXPHOS inhibitors. To date, direct modulation of immune cell metabolism has only been assessed in innate immune cells and T lymphocytes [27, 28]. The authors showed that, the adjunction of metabolism inhibitors (glycolysis inhibitor and glutamine inhibitor) on top of immunosuppression, increases skin and heart allograft survival in a mice model [29, 30].

Several limitations exist in our study. Firstly, the sample size of included patients is relatively small. This limitation stems from the infrequent occurrence of desensitization procedures within our patient population. From a technical point of view, cryopreservation may impact the results of metabolism assessment. To minimize this potential confounder, we allowed cells to recover in the incubator for a short period before doing the analyses [31, 32]. However, despite this constraint, our cohort exhibits comprehensive phenotypic characterization. Additionally, we were able to analyze serial samples from all participants, allowing for thorough investigation. Our investigation focused on analyzing B cell metabolism, yet numerous other cell subtypes play crucial roles in the cascade of allograft rejection and merit deeper examination. Recent literature highlights the significance of glycolysis in macrophages that infiltrate the graft (REF transplant). Additionally, metabolic pathways like the polyamine pathway have been implicated in modulating Th17 pathogenicity (ref COMP). There remains ample scope for elucidating the immune-metabolism nuances specific to each cell subtype within the realm of solid-organ transplantation and rejection.

Our data in sensitized patients indicate that circulating mBC emerging after desensitization modify their metabolic profile, which is primarily dependent on glycolysis. Therefore, targeting this pathway specifically in mBC may represent a valuable therapeutic option to deplete mBCs, avoid the antibody rebound and maybe reduce the risk of AMBR.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the protocol was approved by investigational review board at Grenoble University Hospital (AC-2019-3627) and by French National committee for data protection (CNIL; approval number 1987785v0). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JN, LC, and AT performed the experiments. JN, TJ, and ZM-J participated in the study design. JN, LD, and ZM-J participated in the data analysis. CD provided and analyzed the anti-HLA data. JN and PC wrote the manuscript. PM, LR, and PS reviewed and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13029/full#supplementary-material

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ORIGINAL RESEARCH
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Delayed Graft Function After Kidney Transplantation: The Role of Residual Diuresis and Waste Products, as Oxalic Acid and Its Precursors

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Delayed graft function (DGF) after kidney transplantation heralds a worse prognosis. In patients with hyperoxaluria, the incidence of DGF is high. Oxalic acid is a waste product that accumulates when kidney function decreases. We hypothesize that residual diuresis and accumulated waste products influence the DGF incidence. Patients transplanted between 2018–2022 participated in the prospective cohort study. Pre-transplant concentrations of oxalic acid and its precursors were determined. Data on residual diuresis and other recipient, donor or transplant related variables were collected. 496 patients were included, 154 were not on dialysis. Oxalic acid, and glyoxylic acid, were above upper normal concentrations in 98.8%, and 100% of patients. Residual diuresis was ≤150 mL/min in 24% of patients. DGF occurred in 157 patients. Multivariable binary logistic regression analysis demonstrated a significant influence of dialysis type, recipient BMI, donor type, age, and serum creatinine on the DGF risk. Residual diuresis and glycolic acid concentration were inversely proportionally related to this risk, glyoxylic acid directly proportionally. Results in the dialysis population showed the same results, but glyoxylic acid lacked significance. In conclusion, low residual diuresis is associated with increased DGF incidence. Possibly accumulated waste products also play a role. Pre-emptive transplantation may decrease the incidence of DGF.

Keywords: delayed graft function, kidney transplantation, residual diuresis, accumulated waste products, oxalic acid, glyoxylic acid, deceased donor, living donor

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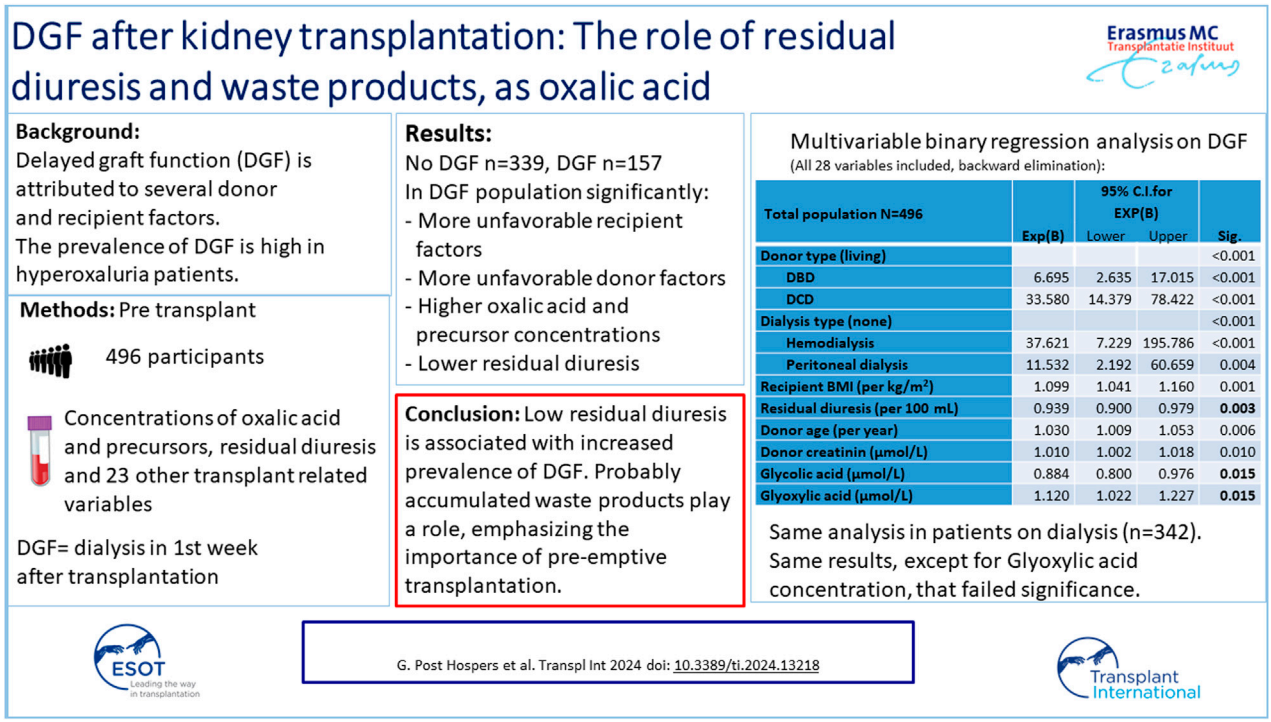
Abbreviations: AGTX, Alanine glyoxylate aminotransferase; CAPD, Continuous ambulant peritoneal dialysis; DGF, Delayed graft function; DBD, Donation after brain death; DCD, Donation after cardiac death; eGFR, estimated Glomerular filtration rate; GO, Glycolate oxidase; HD, Hemodialysis; HLA, Human leucocyte antibody; IQR, interquartile range; LDH, Lactic dehydrogenase; vPRA, virtual Panel reactive antibodies.



GRAPHICAL ABSTRACT |

INTRODUCTION

Delayed graft function (DGF) after kidney transplantation has been associated with donor, recipient and transplant related factors [1–3]. However, DGF most probably is multifactorially determined while the contribution of those factors varies in different studies. DGF occurs in about 30% of recipients of a deceased donor organ [2, 3] and 3.6% of recipients of a living donor kidney [1]. DGF is associated with decreased short and long term graft survival, partly due to an increased rejection risk [4, 5]. This means that DGF should be prevented when possible.

Acute tubular necrosis (ATN) in DGF differs from native kidney ATN in several ways, but the most striking is the prevalence of polarizable crystals consistent with calcium oxalate in DGF [6]. Calcium oxalate deposition in the transplanted kidney heralds a bad prognosis [7–9]. Experience with transplantation in patients with primary or secondary hyperoxaluria demonstrated that this population had higher rates of DGF, partially accompanied by biopsy-proven calcium oxalate deposition, compared to the non-hyperoxaluria population [10].

Primary hyperoxaluria is a group of autosomal recessive genetic disorders of the glyoxylate metabolism (**Figure 1**) [14, 15]. Oxalic acid is the end product of many metabolic processes and cannot be metabolized in the human body. Apart from oxalic acid, the nephrotoxic glyoxylic acid concentration is high in all types of primary hyperoxaluria [15]. In type 1 primary hyperoxaluria, glycolic acid is also high, in type 2 glyceric acid concentration is high.

High plasma oxalic acid concentrations may also be caused by several disorders associated with fat malabsorption (non-inherited, secondary or enteric hyperoxaluria) and that may lead to kidney injury and insufficiency as well [10, 16, 17].

Both primary and enteric hyperoxaluria may be associated with kidney stone formation and with oxalate crystal deposition, CKD and kidney failure [16, 18]. There is a high oxalate nephropathy (recurrence) rate after kidney transplantation in patients with primary and enteric hyperoxaluria [10, 15, 16, 19, 20]. Apart from crystal deposition, oxalic acid and its precursor glyoxylic acid have been shown to cause inflammation and tubulotoxicity [12, 13]. This means that kidney damage may be caused without or before tubular crystal depositions occur.

Finally, high plasma oxalic acid concentrations may be caused by kidney insufficiency and failure *per se* since the main excretion pathway is glomerular filtration and tubular secretion [21,22]. As urinary oxalic acid concentrations are unreliable in CKD stage 4 and 5, in analogy to primary hyperoxaluria, plasma oxalic acid concentrations are used instead [23]. Oxalic acid is easily removed by hemodialysis, but rebounds to pre dialysis concentrations within 48 h [24]. Clearance of oxalic acid is highest in the first hours of dialysis. Residual diuresis is superior to dialysis in removing various (non-urea) solutes and even clinically negligible residual kidney function has been shown to provide non-urea solute clearance [25–29]. With decreasing residual diuresis, accumulation of waste products increases even further. This means that almost all pre-transplant patients have high plasma oxalic acid concentrations.

We hypothesize that DGF is associated with high pre-transplantation concentrations of waste products, such as oxalic acid and its precursors. Under unfavorable conditions, these may lead to inflammation, tubular toxicity and in worse cases even depositions in the transplanted kidney.

Residual diuresis was included in our study in order to exclude the possibility that the effect of oxalic acid and precursors may represent the effect of a whole collection of waste products that have accumulated as a result of reduced residual diuresis. In that case residual diuresis may be a better representative for the whole collection of waste products.

PATIENTS AND METHODS

All patients referred for kidney transplant work-up between September 2018 and January 2022 were asked to participate in this study. Follow-up was until January 1st, 2023. The study conforms with the principles outlined in the Declaration of Helsinki. It was approved by the medical ethics committee of Erasmus University Medical Center Rotterdam, and all patients gave their written informed consent before inclusion (MEC 2018-044). Participation comprised a 10 mL blood sample drawn on the operation ward immediately before transplantation. Oxalic acid and substrates in the metabolic pathway of oxalic acid (precursors, see **Figure 1**); glyoxylic acid, glycolic acid and glyceric acid concentrations were determined. Residual diuresis (remaining urine volume) was based on the patient's last reported 24 h urine volume submitted. Besides, a questionnaire on dietary habits was filled in. Results of this food frequency questionnaire will be described separately.

Kidney function related variables were collected. Recipient variables studied were: age and gender, body mass index (BMI), pre-transplant CRP, pre-transplant vPRA (% panel reactive antibody), use of diuretics (yes versus no), pre-transplant oxalic acid, glyoxylic acid, glycolic acid, glyceric acid concentrations, cardiac disease, diabetes mellitus, vascular disease, cerebrovascular event, previous kidney transplantations (yes versus no), kidney function replacement therapy (none/hemodialysis/peritoneal dialysis), and time between start dialysis and current transplantation (months). Donor and transplantation related variables were: donor type (living versus donation after brain death (DBD) and donation after cardiac death (DCD)), donor age, gender, serum creatinine, BMI, hypertension, diabetes mellitus, HLA mismatches (A, B, DR; 1–6), and cold ischemia period. Delayed graft function (DGF) was defined as dialysis treatment in the first week after transplantation.

To quantify the plasma organic acids (oxalic acid, glyoxylic acid, glycolic acid and glyceric acid), blood was drawn and placed on ice followed by centrifugation at 4°C without delay. Heparinized plasma samples were de-proteinized by addition of 75 µL 37% hydrochloric acid to 0.5 mL plasma followed by centrifugation. The supernatant was stored at −70°C until analysis. For quantification, a gas chromatography mass spectrometry (GC-MS) method was used.

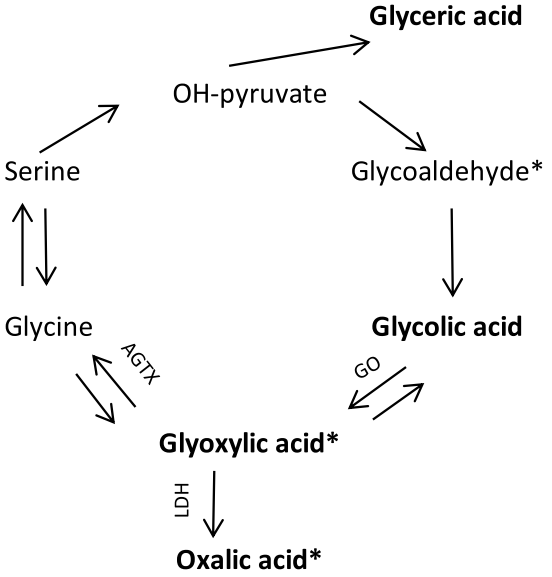


FIGURE 1 | Endogenous oxalic acid synthesis pathway [11]. In bold are the substances whose concentrations were determined in this study. * known to be nephrotoxic, tubulotoxic [12, 13]. GO, glycolate oxidase; AGTX, Alanine glyoxylate aminotransferase; LDH, Lactic dehydrogenase.

Data Analysis

Statistical analysis was performed using IBM SPSS Statistics 24. Baseline characteristics and outcomes were described as counts and percentages for categorical variables. For continuous variables, medians and interquartile ranges (IQR) were given for skewed continuous variables. Differences between continuous variables were studied using Mann-Whitney-U test. Differences between categorical variables were studied using Chi-square test.

Multivariable binary logistic regression analysis with backward elimination was used to study the influence of variables on the incidence of DGF, both in the total population (N = 496) and in the selection of patients on dialysis (n = 342). Kaplan Meier survival curves of the DGF and non-DGF populations were performed.

Spearman correlation analyses were performed to obtain correlation and 95%-confidence intervals between residual diuresis and plasma oxalic acid concentrations and residual diuresis and oxalic acid precursors. Correlation analyses were also performed for oxalic acid and its precursors and between precursors. Correlation values lower than 0.5 were considered weak. A p-value <0.05 was considered statistically significant.

RESULTS

512 patients consented and underwent a kidney transplantation. In 16 patients concentrations of oxalic acid and/or its precursors were missing. Results of 496 patients were available for analysis. **Table 1** shows patient characteristics, there were no missing

TABLE 1 | Patient characteristics.

	Total population N = 496	no DGF n = 339	DGF n = 157	*p*-value no DGF versus DGF
Recipient characteristics				
Gender male n (%)	300 (60.5)	197 (58.1)	103 (65.6)	0.068
Age (years) median (IQR)	62 (51; 69)	60 (49; 68)	64 (55; 70)	0.008
BMI median (IQR)	27 (24; 31)	26 (23; 30)	30 (25; 34)	<0.001
CRP mg/L median (IQR)	3 (1; 8)	3 (1; 6)	5 (2; 12)	<0.001
Medical history				
Cardiac event n (%)	89 (17.9)	51 (15.0)	38 (24.2)	0.010
Cerebrovascular accident n (%)	60 (12.1)	38 (11.2)	22 (14.0)	0.227
Vascular event n (%)	43 (8.7)	22 (6.5)	21 (13.4)	0.011
Diabetes mellitus n (%)	167 (33.7)	88 (26.0)	79 (50.3)	<0.001
Residual diuresis in mL/day median (IQR)	1000 (200; 2000)	1500 (500; 2000)	250 (0; 875)	<0.001
Use of diuretics, yes n (%)	184 (37.1)	128 (37.8)	56 (35.7)	0.365
Dialysis n (%)				<0.001
No	154 (31.0)	152 (44.8)	2 (1.3)	
PD	105 (21.2)	78 (23.0)	27 (17.2)	
HD	237 (47.8)	109 (32.2)	128 (81.5)	
Time between last dialysis and transplantation (days)				
PD only median (IQR)	0.39 (0.21; 0.71)	0.39 (0.23; 0.90)	0.35 (0.20; 0.59)	0.217
HD only median (IQR)	1.34 (0.80; 1.95)	1.33 (0.98; 2.09)	1.32 (0.64; 1.77)	0.035
Time on dialysis in months median (IQR)	15(0; 30)	6.8 (0; 21.8)	28 (16; 44)	<0.001
Hyperoxaluria, non-renal cause n (%)	20 (4.0)	7 (2.1)	13 (8.3)	0.002
Oxalic acid in µmol/L median (IQR)	33 (18; 57)	25 (14; 48)	46 (32; 64)	<0.001
Glycolic acid in µmol/L median (IQR)	5.7 (5.0; 6.7)	5.5 (4.8; 6.4)	6.0 (5.3; 7.0)	<0.001
Glyoxylic acid in µmol/L median (IQR)	2.0 (1.4; 2.8)	1.8 (1.2; 2.5)	2.3 (1.7; 3.3)	<0.001
Glyceric acid in µmol/L median (IQR)	2.6 (2.2; 3.1)	2.4 (2.1; 2.8)	2.9 (2.5; 3.4)	<0.001
vPRA median (IQR)	4 (0; 5)	4 (0; 5)	4 (0; 22)	0.055
vPRA n (%)				0.163
<4	229 (46.2)	166 (49.0)	63 (40.1)	
4–84	230 (46.4)	150 (44.2)	80 (51.0)	
≥85	37 (7.5)	23 (6.8)	14 (8.9)	
First kidney transplantation n (%)	421 (84.9)	297 (87.6)	124 (79.0)	0.010

IQR, interquartile range; HD, hemodialysis; CAPD, continuous ambulatory peritoneal dialysis; vPRA, virtual panel reactive antibodies.

TABLE 2 | Donor and transplantation characteristics.

	Total population N = 496	no DGF n = 339	DGF n = 157	*p*-value no DGF versus DGF
Donor characteristics				
Donortype				<0.001
Living donor n (%)	230 (46.4)	220 (64.9)	10 (6.4)	
DBD n (%)	88 (17.7)	57 (16.8)	31 (19.7)	
DCD n (%)	178 (35.9)	62 (18.3)	116 (73.9)	
Age donor years median (IQR)	58 (48; 67)	56 (48; 65)	62 (51; 69)	0.003
Donor gender male n (%)	253 (51.0)	159 (46.9)	94 (59.9)	0.005
Donor BMI median (IQR)	26 (23; 29)	26 (23; 29)	26 (24; 29)	0.445
Donor comorbidity				
hypertension n (%)	155 (31.3)	99 (29.2)	56 (35.7)	0.091
Diabetes mellitus n (%)	23 (4.6)	12 (3.5)	11 (7.0)	0.073
Donor creatinine (µmol/L) median (IQR)	72 (59; 83)	73 (63; 83)	66 (53; 84)	0.006
Transplantation characteristics				
HLA A, B, DR mismatches				0.362
0–3	296 (59)	200 (59)	96 (61.1)	
4–6	6200 (40.3)	139 (41.0)	61 (38.9)	
HLA mismatches median (IQR)	3 (3; 6)	3 (2; 5)	3 (2; 4)	0.660
Cold ischemia time (min)	389 (120; 707)	136 (111; 493)	693(534; 797)	<0.001

IQR, interquartile range; DBD, donation after brain death; DCD, donation after cardiac death; HLA, Human leukocyte antigen.

values in these 496 patients. Residual diuresis was 150 mL/day or less in 121 patients (24%). **Table 2** shows donor and transplantation characteristics. There were 230 (46%) living donor transplantations, 88 (18%) donation after brain death transplantations and 178 (36%) donatio after cardiac death transplantations.

TABLE 3 | Median values of oxalic acid, glycolic acid, glyoxylic acid and glyceric acid in the total study population and subgroup population on dialysis and not on dialysis.

	Normal values	Total study population (N = 496)	Subgroup population not on dialysis (n = 154)	Subgroup population on dialysis (n = 342)	*p*-Value
Oxalic acid in µmol/L *median (IQR)*	2.5–7.0	33.1 (18.0; 56.6)	14.5 (11.3; 21.0)	45.8 (30.1; 65.0)	<0.001
Glycolic acid in µmol/L *median (IQR)*	3.6–7.6	5.7 (5.0; 6.7)	5.0 (4.5; 5.6)	6.0 (5.3; 7.0)	<0.001
Glyoxylic acid in µmol/L *median (IQR)*	0.2–0.4	2.0 (1.4; 2.8)	1.3 (1.0; 1.8)	2.3 (1.7; 3.3)	<0.001
Glyceric acid in µmol/L *median (IQR)*	1.3–2.1	2.6 (2.2; 3.1)	2.2 (2.0; 2.5)	2.8 (2.4; 3.3)	<0.001

IQR: interquartile range.

p *values measured with Mann-Whitney U test for the difference between the subgroups of patients on dialysis and patients not on dialysis.*

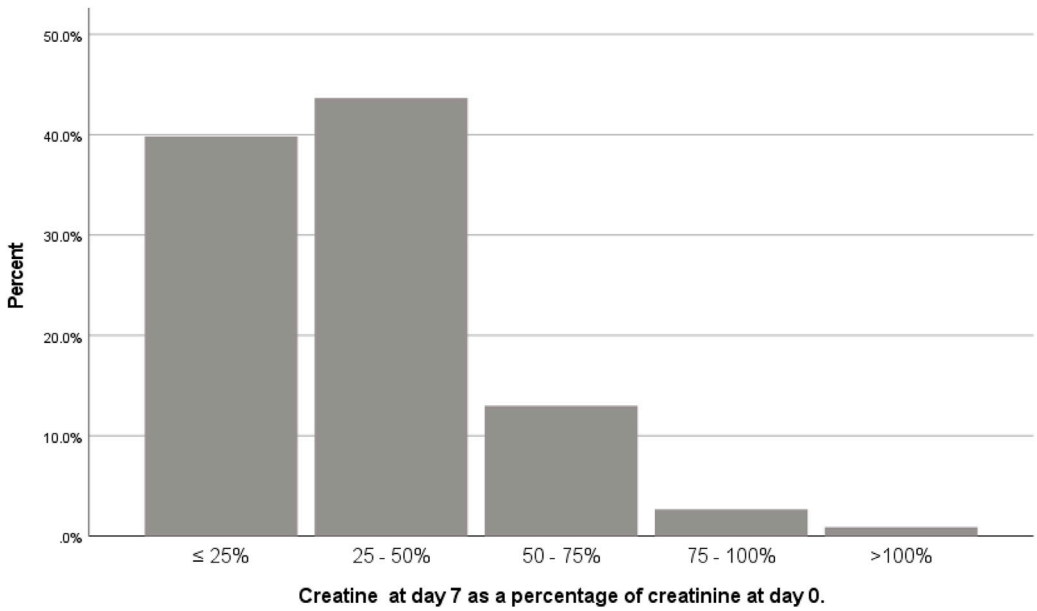


FIGURE 2 | Ratio of serum creatinine on day 7 after transplantation and serum creatinine on day of transplantation in patients without delayed graft function (n = 339).

Table 3 shows the concentrations of oxalic acid, glycolic acid, glyoxylic acid, and glyceric acid in the total patient population, the pre-dialysis population and in the population on dialysis. Only 1.2% of the patients had pre-transplant oxalic acid concentrations within the normal range. All glyoxylic acid concentrations were above the upper limit of normal. Glycolic acid and glyceric acid concentrations were within the reference range in 87% and 22% of cases respectively. Patients on dialysis had significantly higher oxalic acid, glycolic acid, glyoxylic acid and glyceric acid concentrations compared to pre-dialysis patients.

Delayed graft function occurred in one-third (n = 157; 32%) of the population. There were 339 patients without DGF. In 84% of patients without DGF, serum creatinine at day 7 was at least halved compared to pre-transplant serum creatinine (**Figure 2**). Only 3 patients had an increase of serum creatinine on day 7 compared to day 0. One of them had a surgical complication with temporary increase in serum creatinine on day 7. Consequently, two patients without the diagnosis DGF that had an increase in serum creatinine on day 7, but adequate residual diuresis, ruling out the necessity for dialysis. **Tables 1**, **2** show that there are large differences between the populations with versus without DGF. The influence on the DGF risk of all variables shown in **Tables 1**, **2** was tested in binary logistic regression analysis. In univariable analysis, recipient variables with significant effect on DGF risk were: age, BMI, CRP, cardiac event, vascular disease, diabetes mellitus, residual diuresis, dialysis type, dialysis vintage, oxalic acid, glyoxylic acid and glyceric acid concentration, and number of previous kidney transplants. Donor variables with significant influence in univariable analysis were: donor type, age, gender, and cold ischemia time. In multivariable analysis, after backward

TABLE 4 | Multivariable binary logistic regression analysis on delayed graft function, using backward elimination. Total population N = 496, events = 157.

	Exp(B)	95% C.I.for EXP(B)		Sig
		Lower	Upper	
Donor type (living)				<0.001
DBD	6.695	2.635	17.015	<0.001
DCD	33.580	14.379	78.422	<0.001
Dialysis type (none)				<0.001
Hemodialysis	37.621	7.229	195.786	<0.001
Peritoneal dialysis	11.532	2.192	60.659	0.004
Recipient BMI (kg/m^2)	1.099	1.041	1.160	0.001
Residual diuresis (per 100 mL)	0.939	0.900	0.979	0.003
Donor age (years)	1.030	1.009	1.053	0.006
Donor creatinin (μmol/L)	1.010	1.002	1.018	0.010
Glycolic acid (μmol/L)	0.884	0.800	0.976	0.015
Glyoxylic acid (μmol/L)	1.120	1.022	1.227	0.015
HLA mismatches	0.819	0.664	1.009	0.061
Donor gender (male)	0.583	0.321	1.056	0.075
Constant	0.000			0.000

TABLE 5 | Multivariable binary logistic regression analysis on delayed graft function, using backward elimination. Dialysis population n = 342, events = 155.

	Exp(B)	95% C.I.for EXP(B)		Sig
		Lower	Upper	
Donor type (living)				<0.001
DBD	6.031	2.385	15.248	<0.001
DCD	36.257	15.215	86.398	<0.001
Dialysis type (HD)	0.336	0.172	0.657	0.001
Recipient BMI (kg/m^2)	1.119	1.057	1.184	<0.001
Residual diuresis (per 100 mL)	0.993	0.989	0.997	0.001
Donor creatinin (μmol/L)	1.012	1.004	1.020	0.004
Donor age (years)	1.027	1.006	1.049	0.013
Glycolic acid (μmol/L)	0.889	0.804	0.983	0.021
Glyoxylic acid (μmol/L)	1.087	0.993	1.191	0.071
Constant	0.001			0.000

DBD, donation after brain death; DCD, donation after cardiac death; HD, hemodialysis.

elimination, categorical variables that remained in the model with a significant influence on the DGF risk were: donor type, and dialysis type. Besides, recipient BMI, donor age, donor serum creatinine, and glyoxylic acid concentration were significantly and directly proportionally related to the DGF risk, while residual diuresis and glycolic acid concentration were inversely proportionally related the DGF risk (**Table 4**). There was no interaction between any combination of residual diuresis, glyoxylic acid, and glycolic acid. There was no interaction between donor type and glyoxylic acid concentration, glycolic acid concentration, dialysis type, residual diuresis, and recipient BMI.

The same analysis was performed in the population restricted to patients on dialysis (n = 342). Glyoxylic acid concentration failed significance, but all other variables with a significant influence in the total population also had a significant influence in the population on dialysis (**Table 5**).

In a follow up period of almost 5 years, the survival curve shows significantly worse results in patients with DGF compared to those without DGF ($p < 0.001$; **Figure 3**).

The relationship between residual diuresis and oxalic acid and its precursors was studied using Spearman's correlation. It showed a significant, moderate correlation between oxalic acid and residual diuresis (N = 496; r = −0.529; $p < 0.001$). Correlation of residual diuresis with glycolic acid (r = −0.287; $p < 0.001$); with glyoxylic acid (r = −0.258; $p < 0.001$); and with glyceric acid (r = −0.260; $p < 0.001$) was weak but significant.

The relationship between oxalic acid and its precursors was studied using Spearman's correlation. Correlation of oxalic acid with glyoxylic acid (r = 0.685; $p < 0.001$); and with glyceric acid (r = 0.570; $p < 0.001$) was significant. Correlation of oxalic acid with glycolic acid (r = 0.472; $p < 0.001$) was weak, but statistically significant.

Because glyoxylic acid significantly increased the DGF risk and glycolic acid decreased that risk in our multivariable regression analysis, their relationship was studied. Correlation of glyoxylic acid and glycolic acid was weak, but statistically significant (r = 0.370; $p < 0.001$). The scatterplot showed a dichotomy: In the extremes of the graph patients had either high glyoxylic acid or high glycolic acid concentrations, not both (**Figure 4**).

DISCUSSION

Our study shows a significant effect of residual diuresis on the incidence of DGF after kidney transplantation: this holds true for the total population, but also, after exclusion of pre-dialysis patients, for the population on dialysis. Most probably, the association between low residual diuresis and DGF is the result of the accumulation of more or less toxic waste products that were not adequately removed via dialysis when residual diuresis decreased. Our study confirms that DGF is associated with decreased long-term graft survival [1, 5, 30].

There are two studies on the incidence of DGF after kidney transplantation, that also describe a significant influence of residual diuresis [30, 31]. Chaumont et al. studied the incidence of DGF in their center and concluded that perioperative saline loading and higher residual diuresis attributed to a lower risk [30]. Jahn aimed at risk factors for DGF and 1 year graft failure and concluded that residual diuresis influenced the DGF risk [31]. In patients on peritoneal dialysis [27, 32] and hemodialysis [33], patient survival has been shown to be negatively influenced by low residual diuresis. Besides, in dialysis patients, decreasing residual kidney function is associated with serious comorbidities [33–41]. This means that the pre-transplant patients with low or absent residual diuresis, are the less vital patients compared to those with significant residual diuresis volume. The cause of comorbidities is probably associated with accumulation, toxicity and/or deposition of toxic waste products, left behind as a result of failing diuresis [25, 28, 29, 42, 43]. Sudden excretion of these products by the newly transplanted kidney might cause inflammation, toxicity and possibly even depositions, that lead to kidney injury, and to impaired kidney function or even inhibition of the onset of donor kidney function.

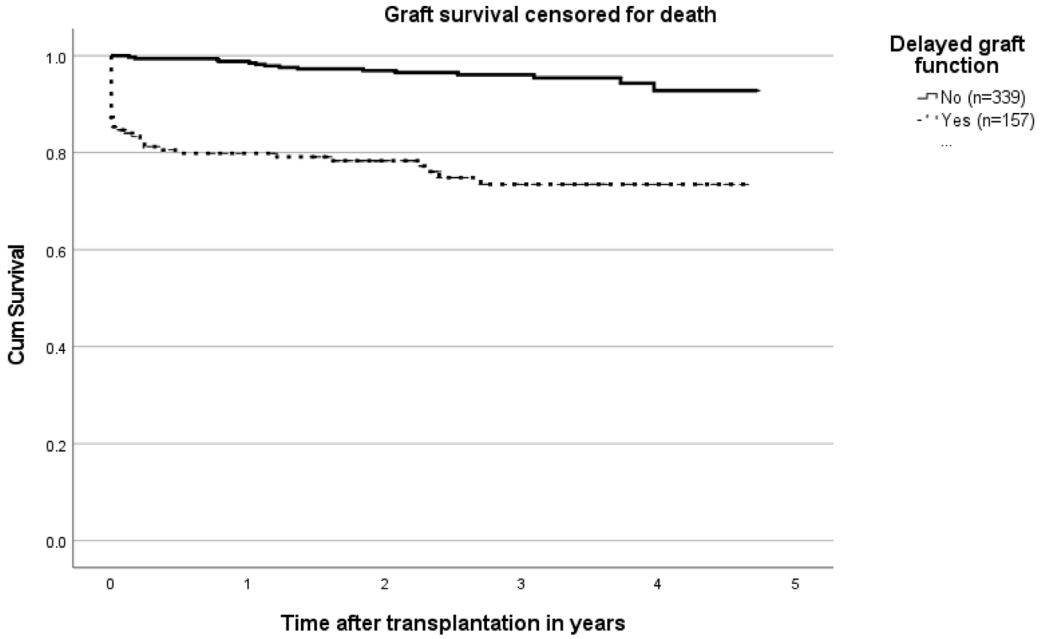


FIGURE 3 | Kaplan Meier curves on graft survival censored for death in patients with and without delayed graft function ($p < 0.001$).

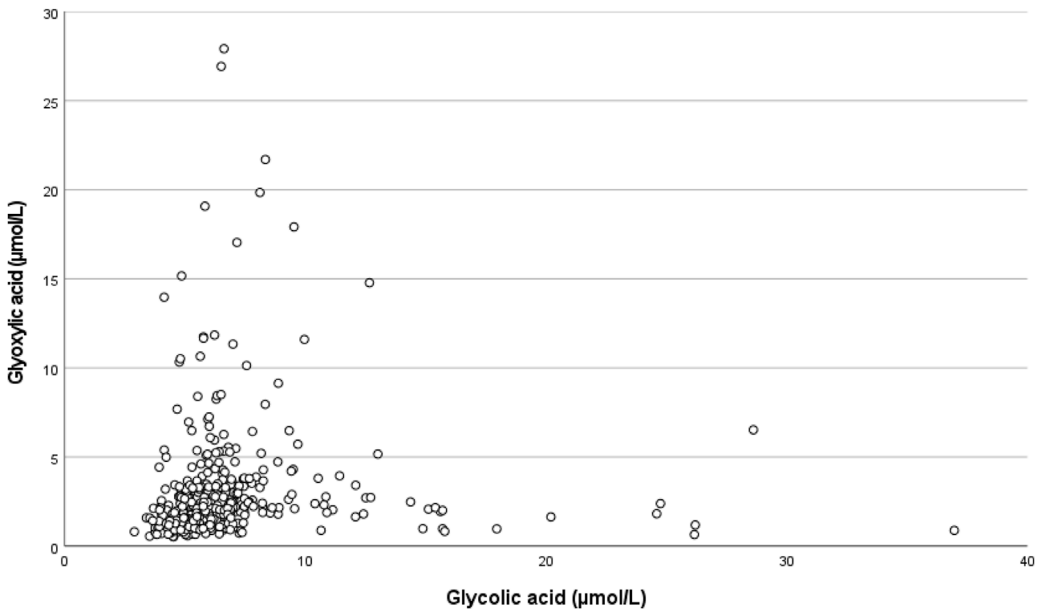


FIGURE 4 | Scatterplot showing the relationship between pre-transplant glyoxylic acid concentration and glycolic acid concentration.

Our study also shows that oxalic acid and its direct precursors glyoxylic acid, glycolic acid and glyceric acid are examples of waste products that accumulate when the kidney fails. In many pre-transplant patients, plasma oxalic acid concentrations are comparable to those of patients with primary hyperoxaluria. The glycolic acid concentrations are above the upper normal value in only 13% of cases (**Table 3**). Recently a small scale study showed normal glycolic acid concentrations in the dialysis population [44].

There was a significant and relevant correlation between oxalic acid concentration and glyoxylic and glyceric acid concentrations implicating that when high oxalic acid concentrations are found in pre-transplant patients, relatively high glyoxylic and glyceric acid concentrations may be expected. Highest concentrations of oxalic acid, glyoxylic acid, glycolic acid and glyceric acid were found in dialysis patients compared to pre-dialysis patients.

Both oxalic acid and its direct precursor glyoxylic acid are known for their tubulotoxicity, [12, 13, 45]. Although concentrations of both oxalic acid and glyoxylic acid significantly influenced DGF risk in univariable binary logistic regression analysis, in multivariable analysis only glyoxylic acid remained in the model and significantly influenced the DGF risk. This effect was independent of the effect of residual diuresis, emphasizing the individual toxic effect of glyoxylic acid. Glycolic acid on the other hand exerted a protective effect. Glycolic acid is not toxic. There is an inverse relationship between glyoxylic acid and glycolic acid as shown in **Figure 4**. The "protective" effect may be the result of shifting to non-toxic glycolic acid instead of toxic glyoxylic acid (**Figure 1**). On the other hand, in the restricted dialysis population glyoxylic acid failed significance, possibly as a result of lower numbers of patients included. When residual diuresis was removed from the model, the influence of glyoxylic acid became significant ($p = 0.017$), indeed suggesting that residual diuresis is a surrogate marker for at least glyoxylic acid, but probably also for many other toxic waste products.

A limitation of our study is that residual kidney function was not available in patients with residual diuresis, thus residual urine volume was used as a representative instead. Residual urine volume was based on the patient's last 24-h urine collection submitted. Last collection may have been a few months before transplantation as 24-h urine collection must be submitted every 3 months for clinical care. Dialysis patients are aware of their 24 h urine production as it determines their fluid restriction.

Another limitation is the definition used for DGF, which is the most commonly used: dialysis in the first week after transplantation. However, fluid overload as the indication for dialysis may not be set in patients with preserved residual diuresis. When assessing transplant function on day 7 post-transplantation, there were only 2 patients without the diagnosis DGF that had an increase in serum creatinine, but adequate residual diuresis, ruling out the necessity for dialysis. All other patients without DGF had a decrease in serum creatinine. Without dialysis, even a small creatinine decrease of 10% is supposed to be the result of function of the transplanted kidney. This means that the definition for DGF turns out to be adequate in our population.

Preservation of residual diuresis, even after start dialysis, is useful and gains attention nowadays. Dietary and pharmacological interventions are defined to ensure optimal native kidney function preserving care [46]. Besides, on top of Kt/V there should be more attention for removal of other waste products, because high concentrations have a negative effect on graft function. This means that more intensive or optimal dialysis treatment, could have a beneficial effect on the prevention of DGF after transplantation. Besides, our study adds an argument to stimulate pre-emptive transplantation in patients who still have adequate diuresis and relatively low concentrations of waste products and thus are in relatively good condition.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the Medical ethics committee of Erasmus University Medical Center Rotterdam (MEC 2018-044). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GP: participated in research design, the performance of the research, data analysis, and the writing of the paper. WV: participated in research design, data collection and the writing of the paper. ML: participated in the performance of the research, data analysis, and the writing of the paper. SB: participated in statistical analysis and the writing of the paper. IM: participated in research design, data collection and the writing of the paper. DH: participated in research design, data collection and the writing of the paper. Participated in requesting patient consent. JV and AM-vE: participated in research design, data collection and the writing of the paper. MB, MvA, DS, JW, RZ, and MK: participated in requesting patient consent and the writing of the paper. MV: participated in data collection and determination of samples and the writing of the paper. IK and MR: participated in the writing of the paper. JR: participated in research design, the performance of the research, data analysis, the writing of the paper and requesting patient consent. All authors contributed to the article and approved the submitted version.

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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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Outcomes of Kidney Transplants From Toxoplasma-Positive Donors: An Organ Procurement and Transplant Network Database Analysis

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There is a need to reconsider the acceptance of organs from donors considered suboptimal, in the absence of data. Toxoplasma antibody-positive donors (TPD) constitute one such group. The objective of our study was to compare graft survival in deceased donor renal transplant (Tx) recipients, stratified by Toxoplasma IgG status, using the Organ Procurement and Transplantation Network (OPTN) database. A log-linear event history regression model for graft failure categorized by Toxoplasma IgG status, adjusting for confounders was applied to first kidney-only Tx recipients from 2018 to 2022. Of the 51,422 Tx, 4,317 (8.4%) were from TPD. Acute rejection and graft failure (5% each) were similar between groups. Crude graft failure was 7.3 failures per 100 person-years for TPD recipients compared to 6.5 failures per 100 person-years for the Toxoplasma-negative group (p 0.008). The crude failure rate ratio was 1.14 with an adjusted hazard rate ratio of 1.04 (95% CI: 0.94, 1.15, p 0.39). In renal Tx recipients, TPD graft recipients have comparable survival to Tx from Toxoplasma-negative recipients. While caution and close monitoring of recipients post-Tx for surveillance of disseminated toxoplasmosis are still warranted, our study suggests that patients can be successfully managed using TPD organs.

Keywords: toxoplasma, kidney, outcomes, survival analysis, infection

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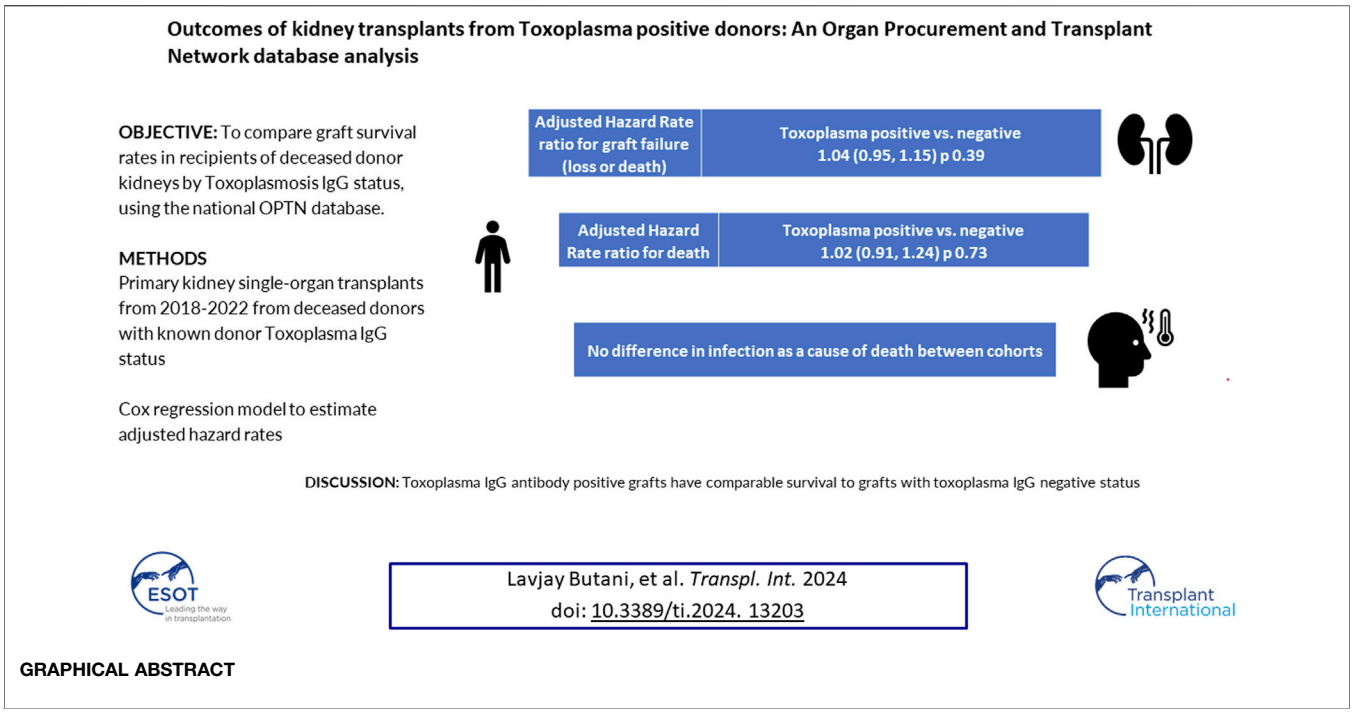
INTRODUCTION

Given the marked shortage of organ donors relative to the number of patients on the waiting list, it behoves the Tx community to systematically review organ acceptance practices which may be based on historical data and anecdotal experience. Moreover, with increasing experience with Tx techniques and management, organ donors previously considered unsuitable for Tx, may no longer be so; examples of such instances include the current practice of utilizing kidneys from donors after circulatory arrest [1], and from donors who have experienced acute kidney injury [2].

Toxoplasma positive donors (TPD) are another such organ donor group, from which Tx has been considered risky and discouraged, based on historical data demonstrating high mortality, especially

Abbreviations: AR, Acute rejection; HLA, Human Leukocyte Antigen; CIT, Cold ischemia time; CKD, Chronic kidney disease; DD, Deceased Donor; DGF, Delayed graft function; OPTN, Organ Procurement and Transplantation Network; TPD, Toxoplasma-positive donor; Tx, Transplantation.



GRAPHICAL ABSTRACT

in heart transplant recipients [3]. Toxoplasma is an intracellular protozoan parasite that is common in humans and animals and that causes mild and self-limited illness in immunocompetent individuals [4]. In its cystic form, it remains latent in various tissues after infection, such as the heart, and can be transmitted through the Tx of such organs. Moreover, Toxoplasma can get reactivated in immunosuppressed states and lead to life-threatening and potentially fatal illnesses [4]. In 2017, based on data from heart Tx recipients, the OPTN issued an advisory and mandated the screening of all deceased organ donors for Toxoplasma, with suggested guidelines for the acceptance of such organs leaving the final decision to individual Tx centers[1].

The goal of our study was to compare outcomes in recipients of TPD renal Tx, to those who received organs from Toxoplasma-negative donors, in a contemporary cohort of patients following the OPTN policy change, in light of advancements in the diagnosis, prevention and treatment of infections in patients receiving Tx. To our knowledge, there are no published data addressing the frequency of TPD organ acceptance in Tx recipients or their outcomes in the current era.

PATIENTS AND METHODS

We conducted a retrospective cohort analysis of the OPTN database, to identify Tx recipients who had received their first deceased donor (DD) kidney-only Tx between 28 February 2018, and 30 June 2022. Donors and recipients who tested positive for HIV were excluded from the analyses because of their higher risk of toxoplasmosis. To be included in the study we also required that the recipient have a graft that had not failed on the day of the surgical procedure in order to be able to analyze our primary outcome measure (time to graft failure or death) using survival analyses. Data on donor and recipient demographics and peri-Tx characteristics were compared among Tx recipients stratified by Toxoplasma IgG antibody status (positive or negative), using Pearson Chi-squared or Fisher exact tests for categorical variables and the Kruskal Wallis test for continuous variables. Kaplan-Meier curves were estimated and categorized for the primary outcome, time to graft failure (graft loss or patient death) by Toxoplasma antibody status. For the primary outcome measure, the following recipient, donor, and Tx-related characteristics were included in the multivariate models-recipient age (pediatric <18 years at the time of Tx) and sex, self-reported race-ethnicity, cause of chronic kidney disease (CKD), donor age and cause of death, OPTN region where the Tx occurred, donor source, need for pre-Tx dialysis, number of HLA mismatches, year of Tx, pre-Tx hypoalbuminemia, and cold ischemia time (CIT). Secondary outcome measures were rates of DGF (defined as the need for dialysis in the first week after Tx), treatment for AR at 1 year, and causes of graft loss/death. We also performed separate univariate and multivariate analyses on outcome measures using a pediatric-only (<18 years) recipient dataset since the pediatric population is at the greatest risk of complications from TPD Tx, due to the lower seroprevalence of toxoplasma in children [5], increasing their risk of developing *de novo* disease.

Datasets were assembled and analyzed using Version 9.4 of SAS (Cary, NC), with multivariate analysis for time to graft failure

[1]https://unos.org/news/guidance-regarding-donor-toxoplasma-screening-and-organ-acceptance/

TABLE 1 | Demographic and Tx characteristics of study subjects (n 51,422).

Demographic and Tx-related characteristics	Frequencies
Recipient sex (female)	20,599 (41%)
Recipient age (pediatric)	2,166 (4.2%)
Recipient race-ethnicity	Black 17,679 (34.4%)
	Non-Hispanic White 17,797 (34.6%)
	Hispanic 10,732 (20.9%)
Recipient cause of CKD	Glomerular 36,204 (70.4%)
	Structural 5808 (11.3%)
Donor Toxoplasma IgG status (positive)	4,317 (8.4%)
Donor cause of death	Anoxia 26,571 (51.7%)
	Head trauma 15,124 (29.4%)
	Stroke/Cerebrovascular 7,764 (15.1%)
HLA mismatch	02,151 (4.2%)
	1,426 (0.8%)
	22,198 (4.3%)
	37,170 (13.9%)
	414,536 (28.3%)
	517,291 (33.6%)
	67,650 (14.9%)
Receipt of pre-Tx dialysis	46,295 (90%)

CKD, chronic kidney disease; HLA, human leukocyte antigen; Tx, Transplant.

modeled using the Cox proportional hazards regression procedure. All available data were used, and the resulting precision of the estimates is reflected by the width of the confidence intervals.

The study was granted exempt status by the University of California Davis Institutional Review Board.

RESULTS

Descriptive Data

During the study period, 51,422 patients received a DD renal Tx; baseline characteristics of the study subjects are displayed in detail in **Table 1**. The majority of the recipients were adults (95.7%), men (59.9%), self-identified as non-Hispanic white (34.6%) and black (34.4%), and had glomerular disease as the cause of their CKD (77.3%), representative of the larger Tx population. The leading cause of death was anoxia (51.7%) followed by head trauma (29.4%). The majority of Tx were poorly matched for HLA antigens (as expected for DD Tx), with 76.8% of Tx having >3 HLA mismatches. The majority of patients had received pre-Tx dialysis (90%). Only a small fraction of the study population received TPD organs (4,317; 8.24%). The rates of DGF, and AR at 1 year, were 28.5% and 4.9% respectively.

Comparing TPD and Toxoplasma-Negative Donor Cohorts

The two cohorts were comparable in most demographic and Tx characteristics with a few exceptions, as outlined in **Table 2**. Causes of donor death differed between the two cohorts ($p < 0.001$), with a disproportionately higher percentage of deaths in the TPD group attributed to stroke/cerebrovascular accident (21.6%) compared to the Toxoplasma-negative group (14.5%), which may be explained by the known association between toxoplasma and stroke [4]. TPD were more likely to be seen in adult Tx recipients (8.5% versus 5.3%, $p < 0.0001$) and in men (8.7% versus 8.0%, p 0.005). Recipient race-ethnicity and causes of CKD were comparable between the two groups. Approximately 9% of each cohort received pre-emptive Tx; HLA mismatches were similar in both groups. Tx from TPD donors occurred in each of the years under study and accounted for approximately 8%–8.5% of all Tx; the largest number of TPD Tx as a fraction of the total Tx, occurred in 2018 (791 of 8,057; 8.9%). Each of the OPTN regions performed TPD Tx with some regional differences ($p < 0.0001$); the majority of TPD Tx occurred in region 3 (n 888) and accounted for 11.3% of all Tx in that region and 20.6% of all TPD Tx nationally. As a fraction of all Tx, region 5 had the lowest number of TPD Tx (5.8%). Delayed graft function was slightly more common in the TPD group (29.9% versus 28.4%, p 0.03) but the rates of AR (5.2% in TPD Tx versus 5.0%, p 0.51) and 1-year graft failure (5.3% in TPD Tx versus 4.9%, p 0.20) were similar between the groups.

Unadjusted graft failure rates per 100 patient-years of follow-up are depicted in **Table 3** and were significantly different in the two cohorts with a higher rate in recipients of TPD organs (7.4/100 patient-years in TPD Tx versus 6.5/100 patient-years, p 0.008), and a failure rate ratio of 1.14 (95th percentile confidence interval of 1.03, 1.25). No differences were noted in the pediatric-only cohort with a failure rate ratio of 1.03 (95th percentile confidence interval of 0.38, 2.82) in the TPD Tx cohort (p 0.95).

Graft Failure

On multivariate regression analyses, several independent predictors of graft failure, previously described, were noted (see **Table 4**). These included recipient sex (higher risk of failure in males), recipient age, with the oldest three recipient age groups in the study having the highest risk of graft loss compared to the youngest group (0–11 years) (adjusted hazard ratio for the 50+ year cohort compared to the 1–11 years group 2.7, 95th percentile confidence interval 1.9, 3.8; $p < 0.001$), recipient ethnicity with white and Hispanic recipients having a lower risk of graft loss compared to Black ethnicity (adjusted hazard ratio for graft loss in the Hispanic cohort 0.80; $p < 0.001$), receipt of pre-Tx dialysis (hazard ratio 1.59, compared to pre-emptive Tx), pre-Tx serum hypoalbuminemia (hazard ratio 0.63 in the cohort with a serum albumin >3.5 g/dL compared to those with serum albumin <2.5 g/dL), increasing donor age (for every 1 year increase in donor age, the hazard ratio for graft loss increased by 1.009; $p < 0.0001$), 5+ HLA mismatches and CIT (for every 1 h increase in CIT, the hazard ratio for graft loss increased by 1.01; $p < 0.001$). Donor Toxoplasma antibody status was not a significant predictor of graft failure (adjusted hazard ratio for TPD Tx 1.04, 95th percentile confidence interval 0.95, 1.15; p 0.39). This was also true in the pediatric-only cohort (adjusted hazard ratio for graft failure for TPD Tx 0.66, 95th percentile confidence interval 0.23, 1.91; p 0.45).

TABLE 2 | Comparing demographic and Tx-related data by Toxoplasma antibody status.

Variables	Toxoplasma IgG status		*p*-value
	Positive (n 4,317)	Negative (n 47,105)	
Recipient age (n, %)			
Pediatric (<18 years)	116 (5.3%)	2050 (94.7%)	<0.0001
Adult (18–50 years)	4,201 (8.5%)	45,055 (91.5%)	
Recipient sex (%)			
Female	8.0%	92%	0.005
Male	8.7%	91.3%	
Recipient ethnicity (%)			
Non-Hispanic White	8.1%	91.9%	0.29
Hispanic	8.1%	91.9%	
Black	8.8%	91.2%	
Causes of CKD (%)			
Glomerular	78.0%	77.2%	0.27
Structural	11.2%	11.3%	
Donor cause of death (%)			
Anoxia	43.3%	52.4%	<0.001
Head trauma	31.4%	29.3%	
Cerebrovascular/stroke	21.6%	14.5%	
Pre-emptive Tx (%)	9.9%	9.8%	0.89
HLA mismatch (%)			
0	4.7%	4.1%	0.31
3	13.3%	14%	
6	15.1%	14.9%	
DGF (%)	29.9%	28.4%	0.03
Treatment for rejection at 1 year (%)	5.2%	5.0%	0.51
1 year graft failure rate	5.3%	4.9%	0.20

CKD, chronic kidney disease; DGF, delayed graft function; Tx, Transplant; HLA, human leukocyte antigen.

TABLE 3 | Unadjusted Graft failure Rates by donor Toxoplasma status.

	Failure rate per 100 patient-years	95th% confidence intervals	*p*-value
Toxoplasma positive	7.4	6.7, 8.1	0.008
Toxoplasma negative	6.5	6.3, 6.7	
Rate ratio	1.14	1.03, 1.25	

Causes of Death/Graft Failure

For patients receiving organs from Toxoplasma-negative donors, we observed 3,435 deaths in 69,739 years of follow-up, for a crude rate of 4.93 deaths per 100 years of follow-up (95% CI: 4.76, 5.09). For recipients of TPD organs, we observed 357 deaths in 6,355 years of follow-up, a crude rate of 5.64 deaths per 100 years of follow-up (95% CI: 5.06, 6.23). The unadjusted rate ratio was 1.14 (1.02, 1.27); $p = 0.02$. However, when we adjusted the estimate, using the same covariates as were used to model graft loss, the adjusted rate ratio was 1.02 (0.91, 1.24); $p = 0.73$. Secondary outcomes were infection as the cause of death, infection as the cause of graft failure and a composite of infection as the cause of either death or graft failure, compared to all other known causes of death. As shown in **Table 5**, there were no statistically significant differences in any of these secondary outcome measures between the Toxoplasma-negative and TPD cohorts.

DISCUSSION

Based on our study, the largest to date exploring the outcomes of renal Tx recipients categorized by donor Toxoplasma status, we can reasonably recommend that TPD Tx is safe to perform with close monitoring, and that such organs should not be reflexively discarded, with the caveats discussed below. We would like to note here that we are not aware of any published data on whether Tx centers routinely use or discard TPD kidneys and how, if at all, they decide to triage such organs. This is a gap in our current understanding but based on the experience at our own transplant center and those in our immediate region, we know that there is significant center variation among centers and that some centers have varying degrees of concern about accepting such organs. Our analyses demonstrate that when adjusted for other covariates known to be associated with graft survival, TPD Tx had comparable survival to those from Toxoplasma-negative

TABLE 4 | Multivariate analyses on key predictors of graft loss.

Predictor variable	Adjusted hazard ratio for graft loss	95th% confidence intervals	*p*-value
Recipient sex (male)	1.07	1.01, 1.14	0.02
Recipient age (compared to 1–11 years)			0.16
12–18 years	1.47	0.96, 2.25	0.08
19–32 years	2.38	1.55, 3.64	<0.001
25–50 years	1.52	1.06, 2.19	0.02
50+ years	2.27	1.86, 3.83	<0.001
Recipient race-ethnicity (Black)			
White	0.89	0.83, 0.96	0.001
Hispanic	0.81	0.74, 0.88	<0.0001
Donor age	1.009	1.007, 1.012	<0.0001
Receipt of pre-Tx dialysis	1.59	1.41, 1.79	<0.0001
Serum albumin at Tx (compared to <2.5 g/dL)			
2.5–3.4 g/dL	0.64	0.50, 0.83	0.0007
3.5 + g/dL	0.63	0.49, 0.81	0.0003
HLA mismatches (Compared to zero)			
5	1.25	1.06, 1.46	0.006
6	1.24	1.05, 1.47	0.01
Donor Toxoplasma status (positive versus negative)	1.04	0.95, 1.15	0.39
CIT	1.01	1.01, 1.01	<0.0001

CIT, cold ischemia time; HLA, human leukocyte antigen; Tx, Transplant.

TABLE 5 | Infection as a cause of death or graft loss.

	Infection as a cause of death # (% of all deaths in a row)	Infection as a cause of graft loss # (% of all graft losses in a row)	Infection as a cause of graft loss or death (n, % of all losses + deaths in row)
Toxoplasma positive	153 (58.2%)	15 (8.3%)	167 (40.1%)
Toxoplasma negative	1,420 (55.2%)	124 (7.5%)	1,506 (37.7%)
Toxoplasma positive versus negative row percentage difference (95% Miettinen-Nurminen Confidence Interval)	2.9 (–3.3, 9.1)	–0.8 (–2.7, 5.9)	2.5 (–3.6, 7.4)
p-value	0.36	0.70	0.33

donors. While this was not statistically significant, the confidence intervals suggest that graft failure in recipients of TPD Tx could be as much as 5% lower to as much as 15% higher, compared to Toxoplasma-negative donor Tx. Since the majority of cases of donor-derived Toxoplasmosis would be expected to occur shortly after Tx [6], it is unlikely that longer follow-up would yield different results. However, whether a larger sample size would change the results is worth exploring through ongoing studies, especially prospective studies of TPD Tx recipients.

From our study it was encouraging to note that Tx from TPD occurred in all OPTN regions and in each year under study with some geographic and temporal variations that are of unclear significance but may represent geographic differences in Toxoplasma seropositivity in the United States (US) based on sociodemographic factors [7] and unique practices and preferences of centers in accepting such donors. However, the number of such Tx is quite small, both in absolute numbers and as a fraction of all Tx, accounting for only 8.4% of all the Tx during our study period. This compares to a prevalence of Toxoplasma of approximately 11% in the general US population [8] and a prevalence of 17.2% in renal Tx donors based on single-center studies [9].

Patients who are seronegative for Toxoplasma and received TPD organs have been noted to have higher seroconversion rates [6] and, while infrequent, also develop life-threatening and fatal infectious complications [6, 10, 11], especially in heart Tx recipients [11]. In the current era, based on the routine use of trimethoprim-sulfamethoxazole (TMP-SMX) prophylaxis in the post-Tx period in all patients to prevent Pneumocystis, a drug that is also effective against Toxoplasma [12], some have suggested not even checking Toxoplasma antibody status in non-heart Tx recipients and in geographic areas with a low Toxoplasma seroprevalence [9] while others have recommended close monitoring and follow-up [6]. Even in the setting of post-Tx Toxoplasmosis infections, outcomes have been favorable with early detection and treatment, even in the highest risk groups [9, 11, 13], further justifying the use of TPD for Tx. Further supporting our recommendations was our observation that infections as a cause of death or graft loss (or a composite of both) were not significantly higher in the TPD cohort, as might be expected if the Toxoplasma positivity were expected to have a detrimental effect on survival.

The limitations of our study pertain to the limited data available in the OPTN database. These include restriction of

analyses to recipients of DD renal Tx only (since testing for Toxoplasma is neither required by nor reported to the OPTN), lack of availability of recipient Toxoplasma status to assess donor-recipient mismatch (although based on the aforementioned literature, this may not be as relevant), and the use of antimicrobial prophylaxis in the post-Tx period. We acknowledge that there may be selection bias introduced, since Tx centers may selectively opt for Tx TPD organs in seropositive recipients as they are at lower risk of post-Tx toxoplasmosis. To account for this to the best of our ability, we analyzed outcomes in a pediatric-only subset of Tx recipients. Children are more likely to be Toxoplasma naïve and therefore at the greatest risk of developing post-Tx complications from Toxoplasmosis. We did not find any differences in outcomes in this population, which is reassuring.

In spite of these limitations, and in support of the smaller studies discussed above, our data confirm that Tx from TPD occur in all geographic regions of the US and are associated with comparable graft failure rates. We do strongly advocate for ongoing donor testing for Toxoplasma, testing of Tx recipients for Toxoplasma, universal TMP-SMX prophylaxis if either the donor or recipient is positive, and close monitoring of patients, especially after discontinuation of prophylaxis, as late-onset Toxoplasmosis may occur [10, 11]. Based on our data, we suggest that Tx centers re-evaluate their current policy on the acceptance of TPD organs in light of recent data, and not discard such organs without considering the pros and cons of doing so, for each individual potential Tx recipient. Even if all of our recipients were Toxoplasma seropositive (which is unlikely), we believe that this study adds to the literature and would be of practical value and benefit in that at least in the recipient cohort that is seropositive for Toxoplasma, the use of TPD organs should not be a cause for concern.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Datasets are only available to member organizations. Requests to access these datasets should be directed to https://optn.transplant.hrsa.gov/data/about-data/optn-database/.

ETHICS STATEMENT

The study was granted an exempt status from the University of California Davis Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

LB was responsible for conceptualizing the study, guiding analyses and preparing the initial draft of the manuscript. DT conducted all the statistical analyses. Both authors reviewed and edited the final version of the submitted manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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The Utility of the Vasoactive-Inotropic Score and Its Nomogram in Guiding Postoperative Management in Heart Transplant Recipients

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Background: In the early postoperative stage after heart transplantation, there is a lack of predictive tools to guide postoperative management. Whether the vasoactive-inotropic score (VIS) can aid this prediction is not well illustrated.

Methods: In total, 325 adult patients who underwent heart transplantation at our center between January 2015 and December 2018 were included. The maximum VIS (VIS_{max}) within 24 h postoperatively was calculated. The Kaplan-Meier method was used for survival analysis. A logistic regression model was established to determine independent risk factors and to develop a nomogram for a composite severe adverse outcome combining early mortality and morbidity.

Results: VIS_{max} was significantly associated with extensive early outcomes such as early death, renal injury, cardiac reoperation and mechanical circulatory support in a grade-dependent manner, and also predicted 90-day and 1-year survival ($p < 0.05$). A VIS-based nomogram for the severe adverse outcome was developed that included VIS_{max}, preoperative advanced heart failure treatment, hemoglobin and serum creatinine. The nomogram was well calibrated (Hosmer-Lemeshow $p = 0.424$) with moderate to strong discrimination (C-index = 0.745) and good clinical utility.

Conclusion: VIS_{max} is a valuable prognostic index in heart transplantation. In the early post-transplant stage, this VIS-based nomogram can easily aid intensive care clinicians in inferring recipient status and guiding postoperative management.

Keywords: heart transplantation, survival, nomogram, vasoactive-inotropic score, early outcome

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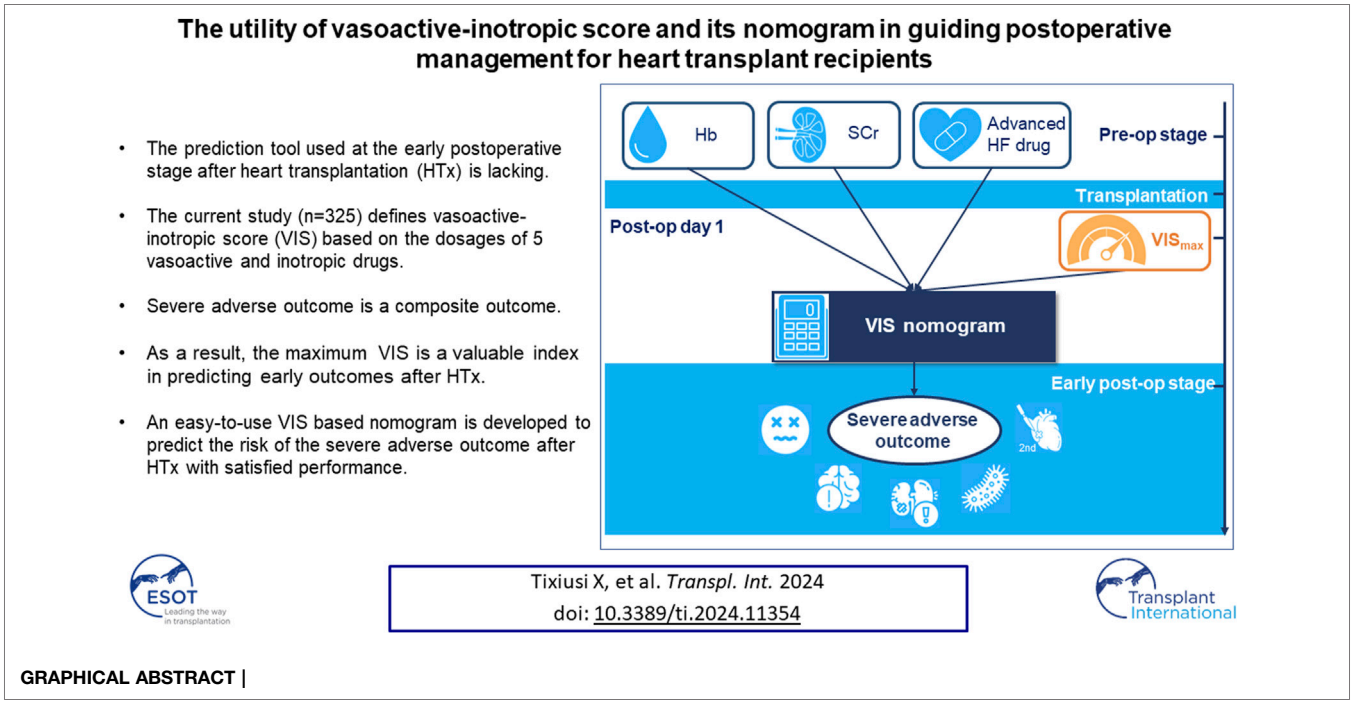
Abbreviations: AUC, Area under the curve; CI, Confidence interval; CRRT, Continuous renal replacement therapy; ECMO, Extracorporeal membrane oxygenation; IABP, Intra-aortic balloon pump; ICU, Intensive care unit; IDI, Integrated discrimination improvement; IQR, Interquartile range; NRI, Net reclassification index; OR, Odds ratio; PGD, Primary graft dysfunction; ROC, Recipient operating characteristics; VIS, Vasoactive-inotropic score.



GRAPHICAL ABSTRACT |

INTRODUCTION

Heart transplantation is currently the final treatment for end-stage heart failure [1]. Developments in surgical technique and perioperative management have led to a significant decrease in post-transplant mortality [2]. However, 30-day mortality has remained unchanged at approximately 7% over the past decade [3]. Post-transplant morbidities are common and consistently worsen early recovery and long-term survival [2, 4, 5]. Thus, it is important to predict early mortality and morbidity in heart transplant recipients. While many models have been developed to predict the outcome of heart transplantation [6–8], only a few of them have been established for early outcomes in the hospital or within 90 days after transplant [9–11].

Compared with the preoperative prediction, which the majority of models perform to aid clinicians in making transplant decisions for a specific patient, the prediction in the early postoperative stage is also important. First, there is more information related to the transplant procedure and early postoperative recovery that can be used to improve outcome prediction in the early post-transplant stage than in the preoperative stage [6]. Second, a prediction model in the early postoperative stage can be utilized by intensive care unit (ICU) clinicians to infer the early recovery status of the recipient and guide subsequent management [12]. Nevertheless, relevant studies in heart transplantation are limited and a prediction tool early after transplantation is warranted.

The vasoactive-inotropic score (VIS) is a weighted sum of the doses of common vasoconstrictors and inotropes and is calculated during the first postoperative day or two [13]. It is considered a prognostic index of short-term outcomes in cardiac surgery patients [12, 14]. A VIS greater than 10 within the first 24 h post-transplant has been proposed as a criterion for primary graft dysfunction (PGD) by the consensus of the International Society of Heart and Lung Transplantation (ISHLT) [15]. Since PGD remains the leading cause of early mortality [16], the VIS index is thus expected to be useful in the outcome prediction of heart transplantation. However, the independent role of the VIS index in predicting outcomes after adult heart transplantation has not been adequately studied. The VIS index has been previously reported to be associated with early morbidities in adult and pediatric heart transplantation cohorts of small sample size [17, 18], but its relationship with mortality in different time scales was ambiguous [18]. Based on the above facts, we hypothesize that the VIS index can be used to develop an effective prediction model in the early postoperative stage for subsequent early outcomes after heart transplantation. Thus, we aim to explore the clinical value of VIS in predicting post-transplant outcomes and to construct an easy-to-use VIS-based nomogram for an early composite outcome in our heart transplant cohort that can be used by ICU clinicians to guide postoperative management of recipients.

METHODS

Study Population

We included all adult patients who underwent orthotopic heart transplantation at our center between 1 January 2015 and 31 December 2018. Patients were excluded for: (1) Re-transplantation or multi-organ transplantation; (2) Immediate death within the first postoperative day; (3) Extreme body weight

(<40 kg or >130 kg); (4) Lack of sufficient data on vasoactive-inotropic agents. After exclusion, 325 patients qualified for further analyses (**Supplementary Figure S1**). The donor hearts were all procured from voluntary donations after brain death and allocated using the China Organ Transplant Response System. The organs of executed prisoners were not used. Our research work conformed to the Declarations of Helsinki and Istanbul, and was approved by the Institutional Review Boards of Tongji Medical College. The requirement for patient consent was waived because the study's nature was retrospective.

Data Collection

We acquired patient data from the electronic medical record system. Among them, advanced heart failure treatment was defined as the preoperative administration of levosimendan or a recombinant human brain natriuretic peptide. The VIS was calculated using the formula modified from the inotrope score formula in the PGD consensus definition [16]: VIS = dopamine + dobutamine + 15 × milrinone + 100 × epinephrine + 100 × norepinephrine. Each item denotes the quotient of the drug dose (μg/min) divided by body weight (kg). Within the first 24 postoperative hours, the VIS at each hour was calculated and the maximum VIS (VIS_{max}) [12] was obtained. Survival information was obtained through follow-up with the recipients and consultation with the related responsible doctors.

Outcome Definitions

The primary outcome was the severe adverse outcome, a composite of early outcomes including early death, neurological complications, renal injury, septic shock and cardiac reoperation, which are commonly studied in cardiac patients [5, 12, 13]. The development of at least one of the above early outcomes was defined as the severe adverse outcome. Secondary outcomes were 90-day, 1-year and 6-year survival.

Early death was defined as in-hospital death or out-of-hospital death within 30 days of discharge [13]. Other complications all occurred in the hospital. Neurological complications were defined as the combination of stroke, as demonstrated by new cerebral deficits on radiological imaging, and seizure episodes requiring intervention. Renal injury was defined as newly initiated continuous renal replacement therapy (CRRT). Septic shock was defined as hypotension or hypoperfusion status with an infectious etiology. Cardiac reoperation was defined as a second thoracotomy after the initial transplantation.

Statistical Analysis

Descriptive data were presented as "median (interquartile range)" or "mean (standard deviation)" for continuous variables, and as "number (percentage)" for categorical variables. Comparisons were performed by t-test or Mann-Whitney U-test for continuous data, and by Pearson χ^2 test, continuity-adjusted χ^2 test or Fisher's exact probability test for categorical data. Survival curves were generated by the Kaplan-Meier method and their differences were examined using the Log rank test. Landmark analysis was undertaken for crossed survival curves. A logistic regression model was used to determine the independent risk factors for the severe adverse outcome. Clinical variables were selected according to clinical importance and the significance level in the univariate analysis of $p < 0.1$. All predictors were preoperative or intraoperative except VIS_{max}. A correlation matrix was generated to assess all the continuous variables for collinearity. A forward stepwise method was used to screen variables for the multivariate model. The missing values for each variable were imputed using the multiple imputation method. A nomogram was constructed based on the multivariate logistic model. The regression coefficients in the model were used to derive linear predictors and allocate points in the nomogram.

The model's performance was evaluated by calibration, discrimination and clinical utility. The calibration was assessed using a calibration plot and the Hosmer-Lemeshow test. The discrimination was assessed using the C-index or area under the curve (AUC) in the receiver operating characteristics (ROC) plot. The difference between the two AUCs was examined using DeLong's method. The net reclassification index (NRI) and the integrated discrimination index (IDI) were calculated to determine whether the addition of a new index to the original model would improve the prediction. A decision curve analysis was performed to evaluate the clinical utility of the nomogram. Statistical analyses were conducted using SPSS v22.0 (SPSS, Chicago, IL, United States) and R v4.2.1 (The R Foundation for Statistical Computing, Vienna, Austria[1]). Figure plotting was completed using the same R software and GraphPad Prism v8.3.0 (GraphPad Software, San Diego, CA, United States). A p-value <0.05 was required for statistical significance.

RESULTS

Demographic and Clinical Characteristics

The median age of our cohort was 50 years (IQR, 39.5–57 years), and the proportion of male patients was 78.46% (255/325). The median BMI was 22.81 kg/m^2 (IQR, 19.86–25.35 kg/m^2). After transplantation, the median VIS_{max} was 17.50 (12.92–24.90), and the rates for postoperative IABP and ECMO use were 37.23% (121/325) and 4.94% (16/325) respectively. Other demographic and clinical characteristics of the total cohort are summarized in **Table 1**. To explore the clinical value of VIS_{max}, the cohort was divided into two groups according to its median. The high VIS_{max} group (VIS_{max} >17.5) had baseline variables that were overall comparable with the low VIS_{max} group (VIS_{max} ≤17.5) except for the ratios of lung disease history and preoperative dopamine usage (**Table 1**).

VIS_{max} and Post-Transplant Survival of 90-Day to 6-Year

The survival curves of the two VIS_{max} groups intersected at approximately day 20 within a 90-day and 1-year follow-up (**Figures 1A, B**). In the landmark analysis, no significant survival difference was observed before the intersection, while

[1]http://www.r-project.org

TABLE 1 | Clinical characteristics and outcomes in different VIS_{max} groups.

Characteristics	Total cohort (n = 325)	VIS_{max}		*p*-value
		Low (n = 163)	High (n = 162)	
Baseline				
Age (year)	50.00 (39.50–57.00)	51.00 (39.00–59.00)	49.00 (40.00–56.00)	0.315
Male patients	255 (78.46)	121 (74.23)	134 (82.72)	0.079
BMI (kg/m^2)	22.81 (19.86–25.35)	22.83 (19.71–25.23)	22.77 (20.07–25.35)	0.947
Primary diagnosis				0.366
Non-ischemic cardiomyopathy	201 (61.85)	97 (59.51)	104 (64.20)	
Ischemic cardiomyopathy	67 (20.62)	33 (20.25)	34 (20.99)	
Valvular heart disease	40 (12.31)	21 (12.88)	19 (11.73)	
Others	17 (5.23)	12 (7.36)	5 (3.09)	
Diabetes mellitus	47 (14.46)	20 (12.27)	27 (16.67)	0.274
Lung disease	9 (2.77)	1 (0.61)	8 (4.94)	0.042
Kidney disease	20 (6.15)	8 (4.91)	12 (7.41)	0.367
Dopamine	184 (56.62)	82 (50.31)	102 (62.96)	0.025
Epinephrine	23 (7.08)	9 (5.52)	14 (8.64)	0.289
Advanced heart failure treatment	69 (21.23)	29 (17.79)	40 (24.69)	0.128
Hemoglobin (g/L)	136.00 (120.00–147.00)	136.00 (121.00–149.00)	137.00 (119.00–147.00)	0.885
Albumin (g/L)	39.45 (4.83)	39.60 (37.15–42.40)	39.00 (36.70–42.50)	0.406
Serum creatinine (μmol/L)	88.60 (71.30–105.30)	89.45 (72.48–107.63)	88.00 (71.05–105.15)	0.744
Total bilirubin (μmol/L)	21.20 (13.10–36.43)	19.90 (12.85–33.10)	23.40 (13.70–38.05)	0.123
Left ventricular ejection fraction (%)	26.00 (20.00–31.00)	26.00 (20.55–33.00)	25.55 (20.00–30.00)	0.306
Donor age (year)	35.00 (23.50–44.00)	35.00 (23.00–44.00)	35.50 (24.00–44.25)	0.507
Male donors	289 (89.20)	146 (90.12)	143 (88.27)	0.721
Donor BMI (kg/m^2)	22.04 (20.76–23.88)	21.97 (20.76–24.22)	22.04 (20.76–23.63)	0.868
Cold ischemia time (min)	360.00 (300.00–404.00)	359.00 (289.25–411.00)	360.00 (300.00–400.00)	0.758
Postoperative				
VIS_{max}	17.50 (12.92–24.90)	12.96 (10.26–15.38)	24.90 (20.51–31.63)	<0.001
IABP	121 (37.23)	29 (17.79)	92 (56.79)	<0.001
ECMO	16 (4.94)	2 (1.23)	14 (8.70)	0.002
Cardiac reoperation	14 (4.31)	3 (1.84)	11 (6.79)	0.031
CRRT	36 (11.08)	7 (4.29)	29 (17.90)	<0.001
Mechanical ventilation duration (h)	38.00 (24.00–59.48)	27.58 (21.40–41.50)	45.80 (33.83–89.91)	<0.001
ICU stay (h)	218.50 (168.00–281.00)	204.50 (158.75–253.50)	236.50 (180.50–321.75)	0.001
Respiratory complication	179 (55.08)	76 (46.63)	103 (63.58)	0.003
Neurological complication	16 (4.92)	6 (3.68)	10 (6.17)	0.319
Septic shock	9 (2.77)	2 (1.23)	7 (4.32)	0.173
Postoperative hospital stay (d)	31.00 (24.00–42.00)	29.00 (24.00–37.00)	34.00 (24.00–48.00)	0.005
Early death	32 (9.85)	10 (6.13)	22 (13.58)	0.026
Severe adverse outcome	63 (19.4)	21 (12.9)	42 (25.9)	0.003

Note: BMI, body mass index; VIS_{max}, maximal vasoactive-inotropic score; IABP, Intra-aortic balloon pump; ECMO, extracorporeal membrane oxygenation; CRRT, continuous renal replacement therapy; ICU, intensive care unit.

TABLE 2 | Multivariate logistic model predicting severe adverse outcomes after heart transplantation.

Variables	β	Odds ratio (95% CI)	*p*-value
VIS_{max}	0.054	1.055 (1.027–1.084)	<0.001
Hemoglobin (g/L)	–0.019	0.981 (0.967–0.996)	0.013
Serum creatinine (μmol/L)	0.012	1.012 (1.005–1.019)	0.001
Advanced heart failure treatment	0.916	2.499 (1.265–4.939)	0.008

Note: VIS_{max}, maximum vasoactive-inotropic score.

the survival of the low VIS_{max} group was evidently higher than that of the high VIS_{max} group after the intersection within a 90-day ($p = 0.005$) and 1-year follow-up ($p = 0.039$) (**Figures 1D, E**). Subsequently within a 6-year follow-up, the intersection became negligible and the survival difference between groups became not significant (**Figure 1C**). These results show that VIS_{max} is useful in predicting post-transplant survival in the short term rather than the long term.

VIS_{max} Predicts Early Post-Transplant Mortality and Morbidity

High VIS_{max} was significantly associated with various early post-transplant outcomes such as intra-aortic balloon pump (IABP), extracorporeal membrane oxygenation (ECMO), cardiac reoperation, secondary intubation, CRRT, respiratory system syndrome, early death, prolonged duration of mechanical ventilation, ICU stay and hospital stay (**Table 1**). We further divided our cohort into 5 groups with different VIS_{max} grades. Grades 1 to 5 corresponded to a VIS_{max} of: <=10, 10–15, 15–20, 20–25 and >25 respectively [13]. Significant increasing trends along with VIS_{max} grade existed in the rates of CRRT, mechanical circulatory support

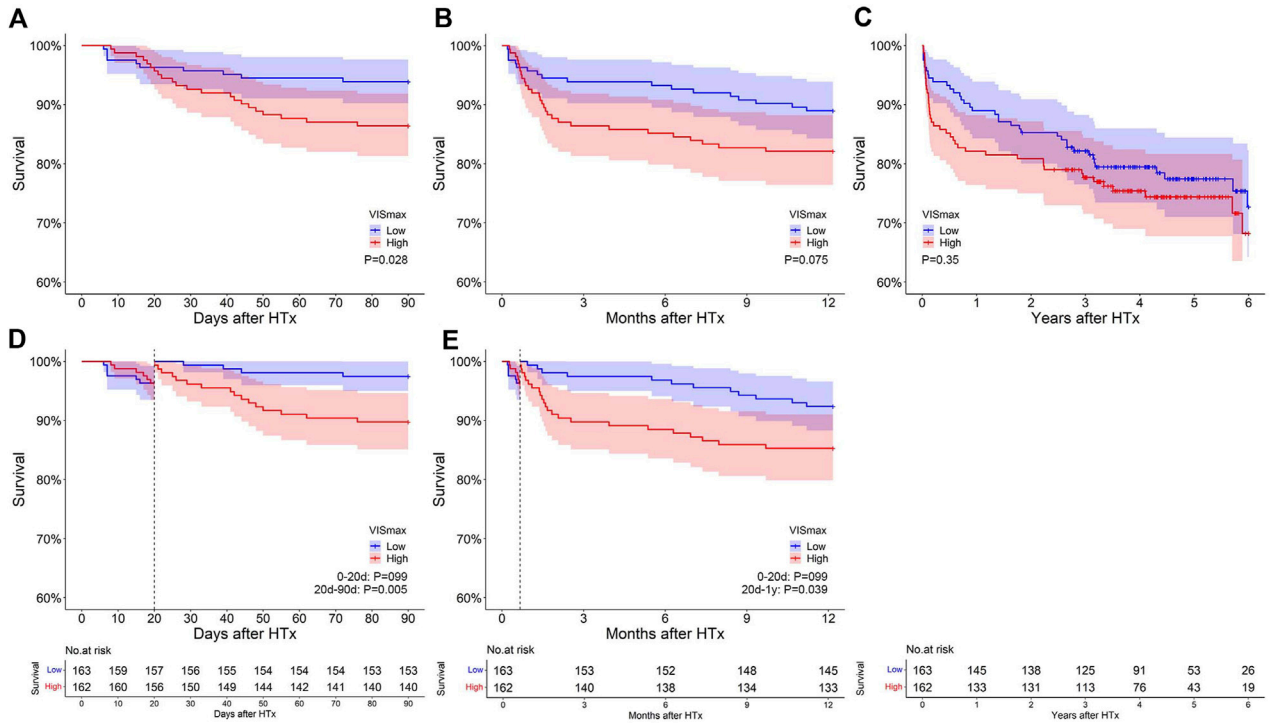


FIGURE 1 | The impact of VIS_{max} on survival after heart transplantation at different follow-up periods. **(A–C)**: Original survival curves within 90 days, 1 year and 6 years after transplantation. **(D–E)**: Landmark analysis within 90 days and 1 year after transplantation.

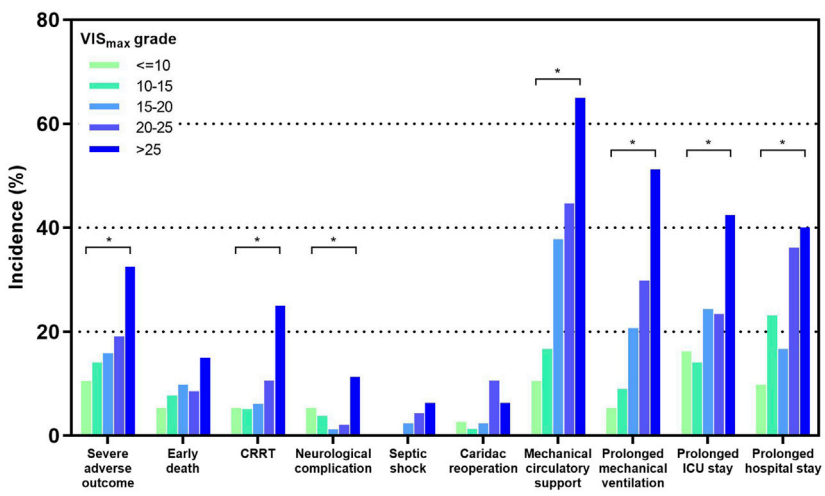


FIGURE 2 | The incidences of early outcomes after heart transplantation in different VIS_{max} grade groups. *: $p < 0.05$ for multiple categorical comparisons. More details are presented in the **Supplementary Material**.

(IABP or ECMO), prolonged mechanical ventilation, ICU stay and hospital stay ($p < 0.05$), while a tendency for this trend existed for other outcomes such as early death, septic shock and cardiac reoperation ($p > 0.05$) (**Figure 2**). The above results show that VIS_{max} is associated with extensive early outcomes in a grade-dependent manner, indicating its predictive ability for an early composite outcome. The severe adverse outcome occurred in 19.4% of our patients and was also significantly associated with VIS_{max} in a grade-dependent manner ($p < 0.05$) (**Figure 2**).

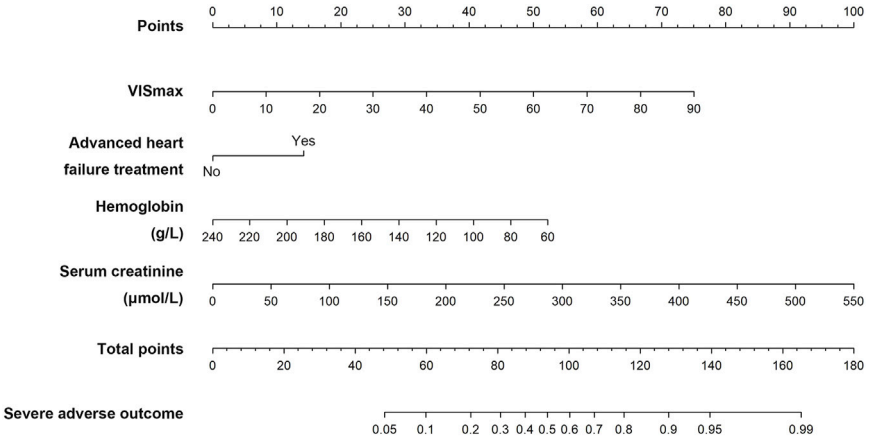


FIGURE 3 | The VIS-based nomogram predicting the severe adverse outcome after heart transplantation.

Establishment of a VIS-Based Predictive Model

For model establishment, a set of candidate variables included common preoperative variables such as recipient age, sex, BMI, diagnosis, and donor age, sex, BMI and cold ischemia time; intraoperative variables such as CPB duration and operation length; and VIS_{max} (Details are in **Supplementary Table S1**). The univariate logistic regression analyses were conducted to determine whether each candidate variable had a potential association with the severe adverse outcome (**Supplementary Table S1**). Forward stepwise selection in multivariate logistic modeling identified the following 4 variables independently related to the severe adverse outcome: VIS_{max} (OR: 1.055; 95% CI: 1.027–1.084; $p < 0.001$), hemoglobin (OR: 0.981; 95%CI: 0.967–0.996; $p = 0.013$), serum creatinine (OR: 1.012; 95%CI: 1.005–1.019; $p = 0.001$) and advanced heart failure treatment (OR: 2.499; 95%CI: 1.265–4.939; $p = 0.008$) (**Table 2**). This model established from the complete variable set was called the "complete model". Next, by excluding VIS_{max} from the variable set of the complete model, a simplified set was generated and used to construct a control model. Similarly, in multivariate modeling, we identified 3 independent variables for the same outcome: hemoglobin (OR: 0.982; 95%CI: 0.968–0.997; $p = 0.015$), serum creatinine (OR: 1.012; 95%CI: 1.005–1.019; $p = 0.001$), advanced heart failure treatment (OR: 2.318; 95%CI: 1.208–4.448; $p = 0.011$) (**Supplementary Table S2**).

VIS-Based Nomogram and Its Performance

The VIS-based nomogram for the severe adverse outcome in heart transplant recipients is shown in **Figure 3**. The points for each variable were summed up to generate a total score. A higher total score was related to a higher risk of the severe adverse outcome after heart transplantation. For example, a patient with a VIS_{max} of 7.62, hemoglobin of 96 g/L, serum creatinine of 70.7 μmol/L and no advanced heart failure treatment, would have 61.5 points (6.5 points for VIS_{max}, 42 points for hemoglobin, 13 points for serum creatinine and 0 points for advanced heart failure treatment), for a predicted risk of the severe adverse outcome of 10.7%.

The calibration curve of the VIS-based nomogram was near the diagonal line (**Figure 4A**). The Hosmer-Lemeshow test yielded a χ^2 of 8.094 ($p = 0.424$). There was a good agreement between the predicted and observed probabilities. The C-index was 0.745 (95%CI: 0.672–0.817) (**Figure 4B**), indicating moderate to strong discrimination. The prediction model after the removal of VIS_{max} is shown in **Supplementary Table S2**. The C-index for the control model was 0.708 (95%CI: 0.629–0.786), which was inferior to that of the complete model (**Figure 4B**). The addition of VIS_{max} to the control model resulted in a positive categorical NRI of 0.136 ($p = 0.065$), a significantly positive continuous NRI of 0.398 ($p = 0.004$), and a significantly positive IDI of 0.0485 ($p = 0.006$), suggesting a significant improvement in the risk classification ability of the model. The decision curve showed that when the selected interference threshold was >10%, using the VIS-based nomogram to predict the severe adverse outcome created more net clinical benefit than using a treat-all, a treat-none, and the control models (**Figure 4C**).

DISCUSSION

In this study, we explored the relationships of the VIS_{max} with early outcomes and survival at different time scales after heart transplantation. Based on the relevant preoperative and intraoperative variables and VIS_{max}, a VIS-based nomogram was successfully developed with good performance in predicting the severe adverse outcome in heart transplant recipients.

The prognostic role of the VIS index on the early outcomes after heart transplantation in previous studies [17, 18] was confirmed in our study. Venema et al. divided 81 adult heart transplant recipients into three equal subgroups according to the mean VIS within 48 h postoperatively [18]. As a result, in-hospital outcomes such as ECMO, CRRT, and prolonged ICU and hospital stays were significantly associated with a high VIS index and the incidence of these outcomes was proportional to the VIS level. Our study confirmed the prognostic role of the VIS

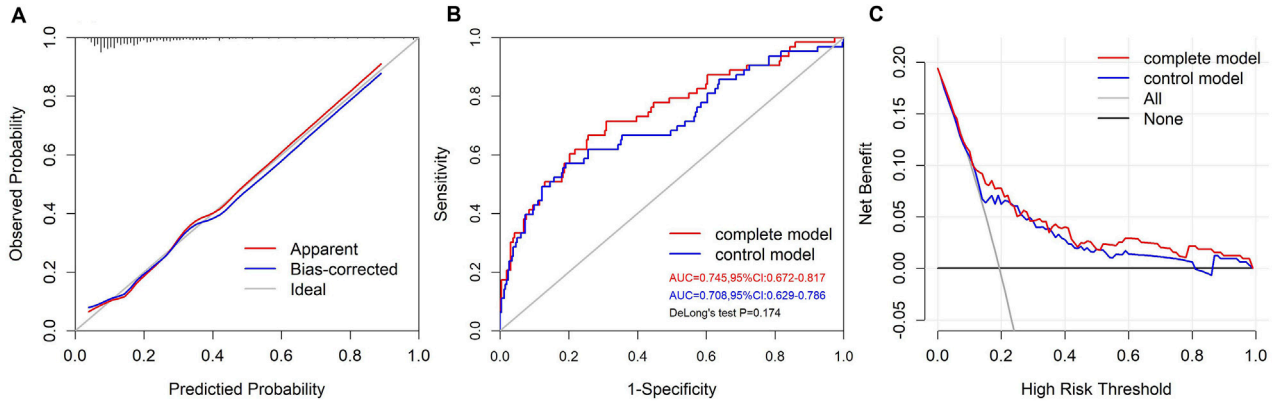


FIGURE 4 | The performance of the VIS-based nomogram. **(A)** The calibration plot of the VIS-based nomogram. **(B)** The ROC curves of the VIS-based and control models. **(C)** The decision curves of the VIS-based and control models.

index on various early outcomes and more clearly depicted a similar grade-dependent manner in these associations using 5 subgroups and a graphic presentation. As for the impact of VIS on post-transplant survival, there were only a few relevant studies. A previous study discovered a significant association between the VIS index and 5-year mortality after adult heart transplantation but this association was inconsistent with different statistical methods and needed further verification [18]. In contrast, the present study found that VIS_{max} is a useful predictor of short-term survival (90 days, 1-year) rather than long-term survival, which enriches the clinical value of the VIS index.

The complete model incorporates four reasonable predictors. A higher VIS_{max} represents a higher dose of vasoactive and inotropic drugs administered postoperatively, suggesting a worse recovery status of patients in the early post-transplant stage. Thus, VIS_{max} may serve as a predictor of the severe adverse outcome. Taegtmeyer et al. demonstrated that pre-transplant anemia was significantly associated with 1-year mortality after heart transplantation [19], indicating that a lower level of preoperative hemoglobin may predict a worse post-transplant outcome, in line with our discovery. Reduced baseline kidney function may increase 30-day [20] and 1-year mortality [3] after heart transplantation, which supports our finding that an increase in preoperative serum creatinine is associated with a higher risk of the the severe adverse outcome. Advanced heart failure treatment in the present study includes the preoperative administration of levosimendan or recombinant human brain natriuretic peptide. These two drugs are used in our center to treat heart failure patients who cannot be relieved by conventional therapy. Therefore, preoperative advanced heart failure treatment is related to a subset of patients with worse baseline cardiac function, which may lead to a worse early outcome after transplantation.

Current prediction models [6–11] in heart transplantation are mostly established for preoperative prediction rather than early postoperative stage prediction, with only a few focusing on early in-hospital outcomes or within 90 days of transplantation. Singh et al. derived and validated a risk prediction model for in-hospital mortality after heart transplantation from a large registry [11], which calibrated well (Hosmer-Lemeshow p = 0.48) and had moderate discrimination (C-index = 0.68). The Index for Mortality Prediction After Cardiac Transplantation (IMPACT) is a model developed by Weiss et al. to predict 1-year survival after heart transplantation [8]. Figueredo et al. used IMPACT in their cohort of heart transplant recipients to predict in-hospital death with moderate to strong discrimination (C-index = 0.742) [21]. A more recent study by Nair et al. derived a prediction model named "GIMVECH" to determine the risk of post-transplant stroke [10] and obtained moderate discrimination (C-index = 0.65). However, the limited number of relevant articles reveals a lack of models for early outcomes after heart transplantation, particularly for prediction in the early postoperative stage. In the present study, we developed a VIS-based nomogram as a prediction tool in the early postoperative stage for the subsequent early composite outcome with good calibration and moderate to strong discrimination (C-index = 0.745). Despite the difference in the predicted outcome, the performance of this model is comparable to the performance of previous models.

Despite the association between PGD based on high VIS and increased early mortality after heart transplantation [16, 22], the independent role of VIS in predicting outcomes of heart transplantation has rarely been studied. Whether the use of VIS aids in predicting early post-transplant outcomes has not been verified. Meanwhile, post-transplant factors can have a significant impact on subsequent survival and can be used to improve the performance of predictive models [6]. In our study, we show the role of VIS_{max} in significantly improving the performance of the control model that incorporates only preoperative predictors, providing evidence for the importance of introducing post-transplant variables into outcome prediction for heart transplantation. The introduction of the VIS index makes the model capture a key feature of early postoperative recovery and consequently improves its performance. Meanwhile, this introduction also creates a good prediction tool in the early postoperative stage that can be utilized at the end of the first postoperative day to help ICU clinicians better

identify high-risk recipients and formulate an individualized postoperative management plan.

There are several limitations to the present study. First, our cohort represents a cohort with high VIS and PGD rates, as evidenced by more than half of the recipients whose VIS_{max} was greater than 10 (actually 88.3% in the supplemental analysis) and 37.2% of the recipients using mechanical circulatory support postoperatively compared to the previously reported PGD rate (2.3%–28.2%) [15]. This fact may affect the generalizability of our nomogram to other centers. Second, the sample size of our cohort is relatively small compared to that of a large registry. A larger cohort is needed to confirm the prognostic role of VIS_{max}. Third, no independent internal or external validation set is available to validate the model's performance, which is needed in the future. Fourth, the VIS index has various forms in previous studies, but we only focused on the VIS_{max} within the first postoperative day based on 5 vasoactive-inotropic drugs. It remains to be studied whether other VIS indices can also predict the early post-transplant outcome.

CONCLUSION

The VIS_{max} is a valuable prognostic index that predicts various early outcomes and short-term survival after heart transplantation and reflects the early postoperative recovery of recipients. In the early post-transplant stage, this VIS-based nomogram can be easily used by ICU clinicians for individualized prediction of subsequent early outcomes and to better guide the postoperative management of heart transplant recipients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the Ethic Committee of Tongji Medical College. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because Our study is a retrospective study, so informed consent was waived.

AUTHOR CONTRIBUTIONS

Conceptualization, SC, TX, and WY; data curation, TX, WY, JC, HL, JZ, and YS; formal analysis, TX and WY; funding acquisition, YW, JS, and ND; investigation, TX, JC, HL, JZ, and YS; methodology, TX, WY, and SC; project administration, JS, ND, and SC; resources, YW, JS, ND, and SC; supervision, ND and SC; visualization, TX and WY; writing-original draft, TX; writing-review and editing, SC, TX, and WY. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.11354/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | The flow chart of patient selection and follow-up. A total of 346 patients have undergone heart transplantation between 1 January 2015 and 31 December 2018 in our center, 1 of them was excluded for re-transplantation or multiple organ transplantation, 2 were excluded for immediate death with the first postoperative day, 3 were excluded for extreme body weight (130 kg) and 15 were excluded for lack of vasoactive-inotropic data. Every patient was followed up until the occurrence of primary outcome or the 30th day after discharge (around 60th day after transplantation). After follow-up, 63 patients have developed primary outcome.

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COVID-19 Vaccine in Lung and Liver Transplant Recipients Exceeds Expectations: An Italian Real-Life Experience on Immunogenicity and Clinical Efficacy of BNT162b2 Vaccine

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This study assessed humoral and T cell-mediated immune responses to the BNT162b2 vaccine in orthotopic liver transplant (OLT) and lung transplant (LUT) recipients who received three doses of the vaccine from March 2021 at our institution. Serum samples were collected 60 days post-second and third dose to quantify antibodies against the spike region of SARS-CoV-2 while whole blood samples were collected to analyze the SARS-CoV-2-specific T-cell response using an IFN-γ ELISpot assay. We enrolled 244 OLT and 120 LUT recipients. The third dose increased antibody titres in OLT recipients (from a median value of 131 after the second dose to 5523 IU/mL, $p < 0.001$) and LUT recipients (from 14.8 to 1729 IU/mL, $p < 0.001$). T-cell response also increased in OLT recipients (from 8.5 to 23 IFN-γ SFU per 250,000 PBMC, $p < 0.001$) and LUT recipients (from 8 to 15 IFN-γ SFU per 250,000 PBMC, $p < 0.001$). A total of 128 breakthrough infections were observed: two (0.8%) OLT recipients were hospitalized due to COVID-19 and one died (0.4%); among LUT recipients, seven were hospitalized (5.8%) and two patients died (1.7%). In conclusion, the three-dose schedule of the BNT162b2 vaccine elicited both humoral and T cell-mediated responses in solid organ transplant recipients. The risk of severe COVID-19 post-vaccination was low in this population.

Keywords: COVID-19 vaccination, solid organ transplant recipient, vaccine immunogenicity, lung transplant recipients, liver transplant recipients, humoral response, cell mediated response

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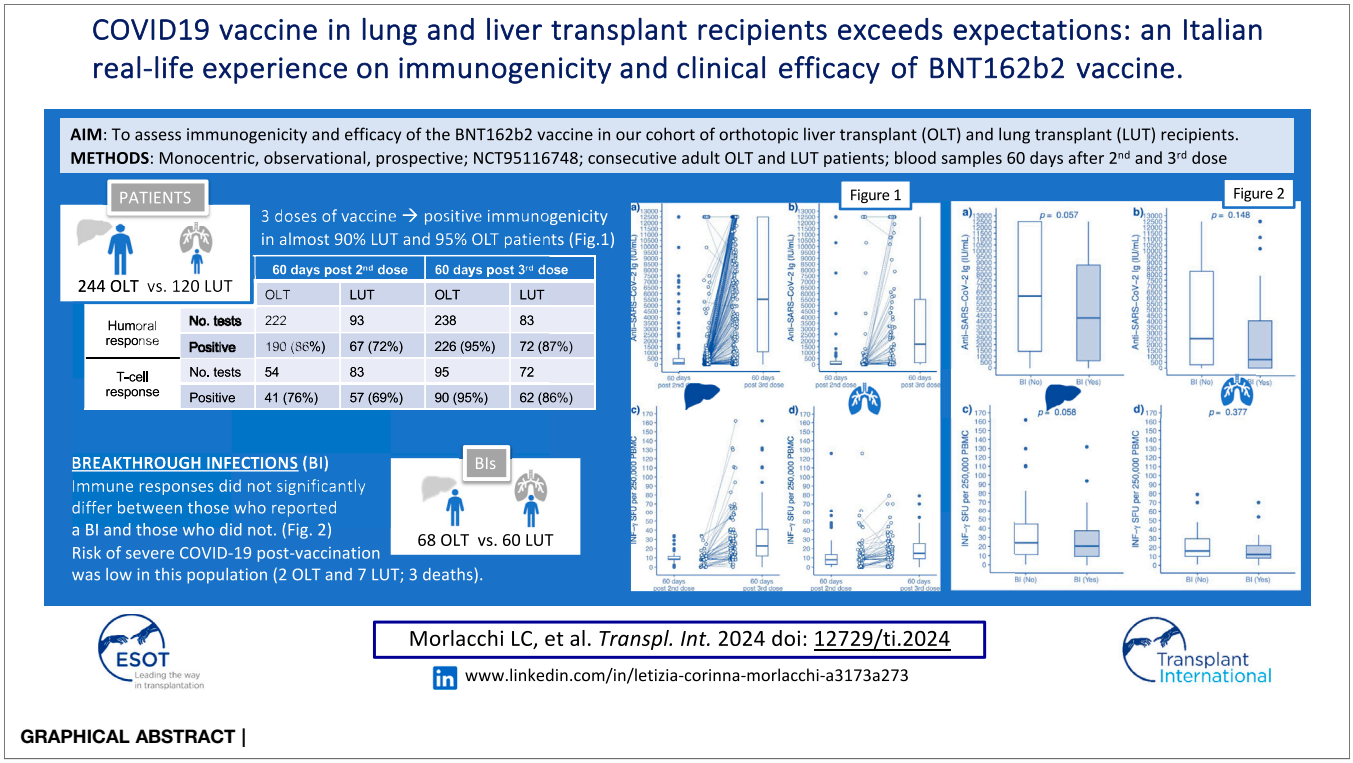
Abbreviations: BI, Breakthrough Infections; BMI, Body Mass Index; CLAD, Chronic Lung Allograft Dysfunction; COPD, Chronic Obstructive Pulmonary Disease; COVID-19, SARS-CoV-2 disease; ELISpot, Enzyme Linked ImmunoSpot; IFN-γ, Interferon-gamma; ISHLT, International Society of Heart and Lung Transplantation; LUT, Lung Transplant; MMF, Mycophenolate Mofetil; OLT, Orthotopic Liver Transplant; PBMC, Peripheral Blood Mononuclear Cells; SAE, Serious Adverse Event; SFU, Spot Forming Units; SOT, Solid Organ Transplant.



GRAPHICAL ABSTRACT |

INTRODUCTION

At present, no definitive data are available on the efficacy and the immunogenicity of anti-SARS-CoV-2 vaccines in solid organ transplant (SOT) recipients. The available evidence is inconsistent, and there is limited data on different type of SOT recipients and their cell mediated immune responses [1–4]. It is still unknown whether a specific level of serum antibodies may confer protection from infection or severe disease, and routinely measurements are not currently recommended. Conversely, mRNA vaccines appear to be safe in the transplanted population, and, at present, there are no concerns about the possible onset of rejection or other serious adverse events following their administration [5].

Aim

The primary aim of this study was to assess the immunogenicity of the BNT162b2 vaccine in our cohort of orthotopic liver transplant (OLT) and lung transplant (LUT) recipients. As a secondary aim, we evaluated the occurrence and the severity of breakthrough infections (BI) in this population after the completion of a three-dose vaccination course.

PATIENTS AND METHODS

Participants

We conducted a prospective, observational study enrolling consecutive OLT and LUT recipients attending our hospital. The study period extended from 1st March 2021, to 31st October 2022. We screened all patients living in Lombardy who received the vaccine at our hospital vaccination hub.

Patients were considered eligible for the vaccination if: time from transplantation was more than 3 months for OLT and 6 months for LUT; they had not recently received intensive treatment for rejection or any other clinical reason to wait for administering vaccinations; they had no history of allergy for any of the vaccine excipients. Patients were excluded if: they were under 18 years old; had COVID-19 between the administration of the second and the third dose of vaccination; lived outside Lombardy or refused to provide consent.

All patients received a three-dose schedule of the BNT162b2 vaccine (second dose was given 21 days after the first one; third dose was given 180 ± 30 days after the second dose); each dose was administered by intramuscular injection into the deltoid muscle.

Blood samples were collected 60 days after the second dose and again 60 days after receiving the third dose, in order to determine their anti-SARS-CoV-2 total Ig antibodies and T cell-mediated immune responses to vaccine. We were not able to perform T-cell mediated response analysis on the entire population due to high costs, complexity of the method and laboratory overloading activities at that time. Overall, 304 samples were analyzed for T-cell response. The first selection criterion was sampling time (specimens collected until the number of tests/kits available for analysis was exhausted, 10 kits after the second dose and 10 kits after receiving the third dose). The second selection criterion was to

discard samples with <6 mL of whole blood collected and consequently with an insufficient peripheral blood mononuclear cells PBMCs for analysis.

The following data were collected: date of birth and transplantation, body mass index (BMI) at enrollment, etiology and indication for transplantation, prior COVID-19, post-transplant comorbidities (including diabetes, chronic kidney disease and cancer), immunosuppression regimen and graft function. The latter was evaluated with liver stiffness (measured by Fibroscan®) for OLT recipients and with pulmonary function tests criteria for chronic lung allograft dysfunction (CLAD) for LUT patients, as defined in the ISHLT 2019 consensus document [6].

All patients received traditional follow up, with regular visits in our outpatient clinics (every two-three months for LUT recipients and every four-six months for OLT recipients); patients were also instructed to contact our center by means of email and/or phone call in case sentinel symptoms occurred in order to give them appropriate indication. No patient was lost to follow up.

The study was registered on Clinicaltrials.gov (NCT 05116748, COVID-19_VaxSOT).

Endpoints

The primary endpoints of the study included the anti-SARS-CoV-2 antibody titre and Interferon-γ (INF- γ)- secreting T cells measured 60 days after the second and third dose of the vaccine.

The laboratory procedures used for the quantification of humoral and T cell-mediated immune responses are reported in the **Supplementary Material**.

The secondary endpoints were the incidence of BI, including both asymptomatic/paucisymptomatic and severe forms. Additionally, the study collected unusual adverse events as well as serious adverse events (SAEs) and sentinel events.

Definitions of COVID-19 Outcomes

A BI was defined as an infection occurring 14 days or more after receiving the third dose [7], and it was documented by an RT-PCR test or antigenic test.

Severe COVID-19 was defined as SARS-COV2 infection requiring hospitalization and/or causing pneumonia, respiratory failure, sepsis, septic shock, acute respiratory distress syndrome or death.

COVID-19 related mortality was defined as a death with COVID-19 listed in the death certificate.

Statistical Analysis

Before the analysis, any antibody and IFN-γ SFU values that fell below the lower limit of quantification (LLOQ) were replaced with a value equal to 0.5 times the LLOQ. If any values exceeded the upper limit of quantification (ULOQ) and the actual values were not available, they were substituted with the ULOQ.

The data were presented as median and interquartile range (25th–75th percentile). Linear quantile mixed models with subject-specific random intercept were used to identify potential predictors of humoral and T cell-mediated responses [8]. We chose these models over other regression models because they do not assume a normal distribution of the response variable and are less sensitive to outliers. Separate models were fitted for OLT and LUT recipients, with anti-SARS-CoV-2 antibodies or INF-γ SFU per 250,000 PBMC as the response variables, and sex, age at vaccination, time from transplantation, prior SARS-CoV-2 infection, comorbidities potentially affecting immune responses, immunosuppressive therapy and vaccine dose (post third dose *vs* post second dose) as predictors.

Rates of BI were computed by dividing the number of BI by person-days and then multiplied by 1,000. Person-days were computed from 14 days after receiving the third dose until 31 October 2022 (the end of the study). The infection rate ratio (IRR) was computed with the rate observed among LUT recipients in the numerator and that observed among OLT recipients in the denominator, with the 95% confidence interval (95% CI) obtained using the Poisson distribution. Humoral and cell mediated responses measured after the administration of the third dose were compared between patients who reported a BI and those who did not within each SOT group using the Wilcoxon sum rank test. All statistical tests were two-sided and p-value<0.05 were considered statistically significant.

Ethics

This study received approval from the ethics committee of the I.R.C.C.S. Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani (Parere n. 422 del Registro delle Sperimentazioni 2020/2021). Written informed consent was obtained from all subjects.

RESULTS

This study included 244 OLT recipients and 120 LUT recipients, whose characteristics are summarized in **Table 1**.

A total of 636 measurements of anti-SARS-CoV-2 total Ig antibodies and 304 measurements of T-cell responses were obtained. Their distribution by group (OLT vs. LUT recipients) and time (60 days after the second dose vs. 60 days after the third dose of the vaccine) is presented in **Table 2**, along with the percentage of measurements above the positive response threshold.

Serum concentration of anti-SARS-CoV-2 antibodies and IFN-γ SFU measured at 60 days after the second or the third dose in OLT and LUT recipients are shown in **Figure 1**.

Prior SARS-CoV-2 infection was significantly associated with higher antibody titres in both OLT and LUT recipients and with higher INF-γ SFU among LUT recipients. Among OLT recipients, chronic renal failure was associated with lower INF-γ SFU (expected difference in median: −11.2 INF-γ SFU per 250,000 PBMC). Age was associated with lower INF-γ SFU only among LUT recipients, with an estimated difference in median INF-γ SFU of −0.2 per 1-year increment. The third dose was associated with higher immune responses in both OLT and LUT recipients. The expected median increase in antibody titre after the third dose was 4976 IU/mL among OLT recipients and 2345 IU/mL among LUT recipients. The expected median increase in INF-γ was 21.2 SFU per 250,000 PBMC among

TABLE 1 | Patient characteristics.

Characteristic	OLT recipients, *N* = 244[a]	LUT recipients, *N* = 120[a]
Sex		
Females	70 (28.7%)	52 (43.3%)
Males	174 (71.3%)	68 (56.7%)
Age at vaccination (years)	66.5 (58.5, 71.3)	43.0 (34.8, 57.0)
Age at transplantation (years)	56.2 (48.5, 62.5)	36.0 (27.0, 51.3)
Time from transplantation (years)	8.3 (3.4, 15.9)	5.0 (4.0, 8.0)
BMI (kg/m^2)	25.0 (23.0, 27.0)	22.4 (20.5, 25.7)
BMI category		
Normal weight	98 (40.2%)	72 (60.0%)
Underweight	3 (1.2%)	11 (9.2%)
Overweight	103 (42.2%)	30 (25.0%)
Obesity	40 (16.4%)	7 (5.8%)
Indication for transplantation		
Decompensated cirrhosis	123 (50.4%)	—
Hepatocellular carcinoma	116 (47.5%)	—
Fulminant hepatic failure	5 (2.0%)	—
Cystic fibrosis	—	75 (62.5%)
COPD	—	10 (8.3%)
Idiopathic pulmonary fibrosis	—	14 (11.7%)
Other pulmonary diseases	—	21 (17.5%)
Cardiovascular disease	37 (15.2%)	23 (19.2%)
Diabetes	82 (33.6%)	94 (78.3%)
Cancer (excluding non-melanoma skin cancer)	42 (17%)	10 (8.3%)
Non-melanoma skin cancer	9 (3.7%)	16 (13.3%)
Chronic renal failure	54 (22.1%)	65 (54.2%)
Graft function		
FibroScan ≥8 kPa[b]	52 (21.6%)	—
Chronic lung allograft dysfunction	—	31 (25.8%)
Previous SARS-CoV-2 infection	12 (4.9%)	12 (10.0%)
Immunosuppression regimen		
Prednisone	33 (13.5%)	120 (100.0%)
Mycophenolate mofetil/Azathioprine	168 (68.9%)	55 (45.8%)
Tacrolimus/Cyclosporine	244 (100.0%)	120 (100.0%)
Triple immunosuppression	19 (7.8%)	91 (75.8%)

BMI, Body mass index; COPD, Chronic obstructive pulmonary disease; OLT, Orthotopic liver transplant; LUT, Lung transplant.
[a]*n (%); Median (IQR).*
[b]*Data not available in three patients.*

TABLE 2 | Number of measurements and positive humoral and T cell-mediated responses by organ transplant group (liver of lung) and time from COVID-19 vaccine doses.

Transplanted organ	Time	No. of antibody measurements	No. of T cell- mediated response measurements	Positive antibody responses (% of total measurements)	Positive T cell-mediated responses (% of total measurements)
Liver	60 days post 2nd dose	222	54	190 (85.6)	41 (75.9)
	60 days post 3rd dose	238	95	226 (95.0)	90 (94.7)
Lung	60 days post 2nd dose	93	83	67 (72.0)	57 (68.7)
	60 days post 3rd dose	83	72	72 (86.7)	62 (86.1)

OLT recipients and 5.8 SFU per 250,000 PBMC among LUT recipients. No significant associations were found for the remaining predictors considered in the models (**Table 3**).

After the administration of the third dose, there were 60 BI recorded among LUT recipients and 68 among OLT recipients, observed over a total follow-up time of 31,933 days and 77,811 days, respectively. This corresponded to infection rates of 1.88 per 1,000 patient-days among LUT recipients and 0.87 per 1,000 patient-days among OLT recipients (IRR among LUT recipients: 2.15, 95% CI: 1.49–3.09). The majority of BI occurred in 2022 (124/128, 96.9%) and did not require hospitalization. Two OLT and seven LUT recipients were hospitalized due to severe COVID-19, whilst two LUT recipients and one OLT recipient died due to COVID-19. Four LUT and four OLT recipients died during the study period due to non-COVID-19 causes.

Serum concentration of anti-SARS-CoV-2 antibodies and IFN-γ SFU after the third dose did not significantly differ among patients who reported a BI compared to those who did not (**Figure 2**).

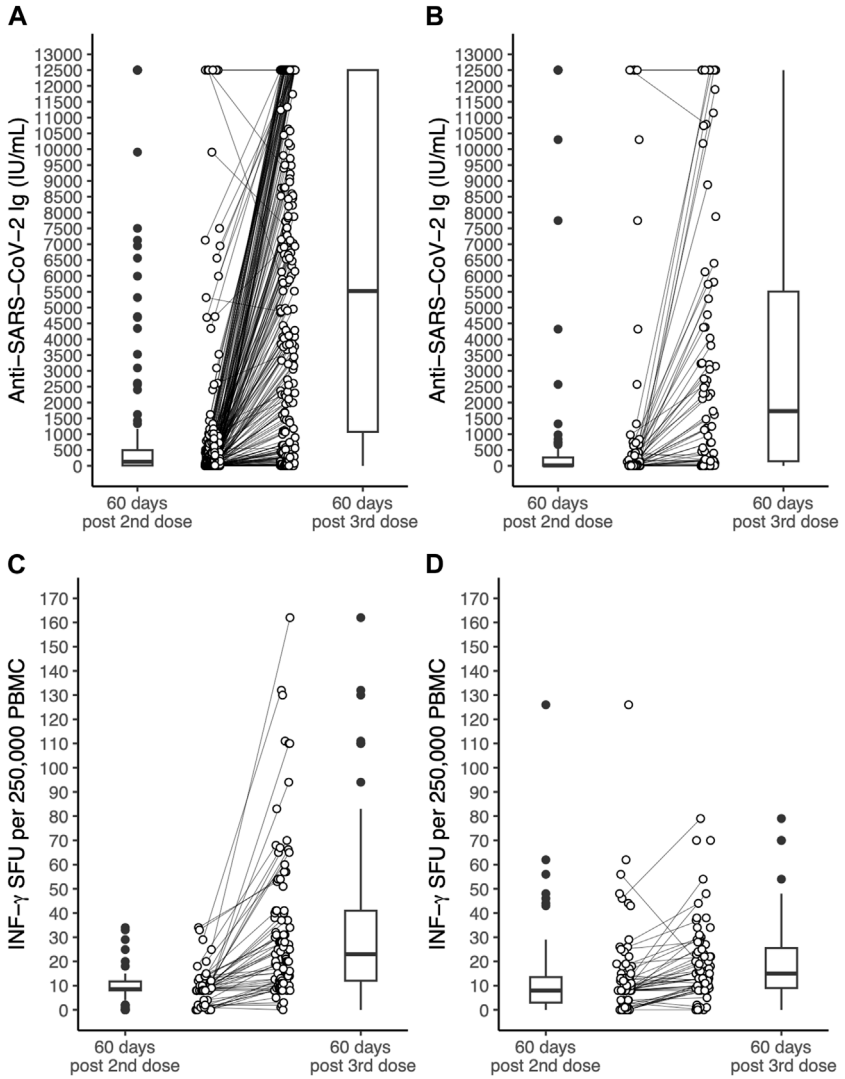


FIGURE 1 | Serum concentration of antibodies to the SARS-CoV-2 spike protein receptor binding domain and INF-γ SFU measured in OLT recipients and LUT recipients 60 days after the 2nd dose of the BNT162b2 vaccine and 60 days after the 3rd dose. **(A)**: anti-SARS-coV-2 antibodies in OLT recipients. **(B)**: anti-SARS-coV-2 antibodies in LUT recipients. **(C)**: INF-γ SFU in OLT recipients. **(D)**: INF-γ SFU in LUT recipients. The lines within the boxes indicate the median, the edges of the boxes are the lower and the upper quartiles (the interquartile range), the lines extending from the box (whiskers) indicate the adjacent values (the most extreme values that are still within a distance of 1.5 times the interquartile range from the nearest quartile) and the black dots beyond the whiskers are outliers. The white dots indicate individual values and the lines join the measurement on the same subject after the 2nd and the 3rd dose. INF, Interferon; LUT, Lung transplant; OLT, Orthotopic liver transplant OLT; PBMC, Peripheral blood mononuclear cells; SFU, Spot forming units.

No serious adverse event occurred; we report one case of transient leukopenia in a LUT patient after the administration of the second dose (spontaneous remission in the subsequent month).

DISCUSSION

In this large prospective study, we found that a two-dose course of the BNT162b2 vaccine elicited a positive immunogenicity in the majority (around 70%–80%) of OLT and LUT recipients included in our study. Humoral and T-cell mediated responses were higher among OLT recipients compared to LUT recipients. The administration of a third dose significantly enhanced both immune responses with positive response rates approaching 90% in LUT recipients and 95% in OLT recipients. No serious events were observed in both the transplanted cohorts.

Our findings may be somewhat unexpected. Long before the COVID-19 pandemic, several studies suggested that SOT recipients, given their immunocompromised state, tended to develop impaired immunogenicity to vaccination against viral pathogens and low overall response rates to other vaccines [9]. Following COVID-19 vaccination, lower serologic as well as T

TABLE 3 | Results of the quantile mixed models aiming at identifying predictors of antibody and T cell-mediated responses to BNT162b2 vaccine against SARS-CoV-2 in liver and lung transplant recipients.

Response variable	Predictors	OLT recipients		LUT recipients	
		β (95% CI)	*p*-value	β (95% CI)	*p*-value
Anti-SARS-CoV-2 Ig (IU/mL)	Intercept	564 (–589; 1,717)	0.330	1,372 (–924; 3,669)	0.236
	Male sex	152 (–670; 975)	0.711	222 (–817; 1,261)	0.670
	Age (years)[a]	–4 (–34; 27)	0.799	–13 (–42; 16)	0.364
	Time from transplantation	8 (–25; 42)	0.630	–6 (–191; 179)	0.951
	Prior SARS-CoV-2 infection	7,351 (5,704; 8,997)	<0.0001	6,874 (3,668; 10,080)	<0.0001
	Diabetes	–155 (–970; 659)	0.704	–674 (–2,248; 900)	0.394
	Chronic renal failure	–678 (–1,531; 174)	0.116	–917 (–2,091; 258)	0.123
	Cancer	301 (–562; 1,164)	0.487	1,129 (–1,476; 3,734)	0.388
	Mycophenolate Mofetil/Azathioprine	–562 (–1,403; 279)	0.186	975 (–27; 1,977)	0.056
	Triple immunosuppression	–645 (–2,302; 1,012)	0.438	–960 (–2,560; 641)	0.234
	Vaccine dose (post 3rd vs. 2nd dose)	4,976 (4,354; 5,597)	<0.0001	2,345 (1,380; 3,309)	<0.0001
INF-γ SFU (per 250,000 PBMC)	Intercept	7.4 (–3.8; 18.5)	0.192	7.6 (–7.9; 23.1)	0.331
	Male sex	2.9 (–7.6; 13.4)	0.580	2.6 (–2.8; 8)	0.333
	Age (years)[a]	–0.1 (–0.4; 0.2)	0.492	–0.2 (–0.4; 0)	0.020
	Time from transplantation	0.1 (–0.6; 0.8)	0.802	–0.1 (–1; 0.8)	0.812
	Prior SARS-CoV-2 infection			24.5 (13.4; 35.7)	<0.0001
	Diabetes	–0.1 (–10; 9.9)	0.988	0.5 (–10.6; 11.5)	0.933
	Chronic renal failure	–11.2 (–21.3; –1.1)	0.031	–3.3 (–11.4; 4.8)	0.419
	Cancer	6.6 (–4.6; 17.7)	0.245	6.1 (–7.1; 19.2)	0.359
	Mycophenolate Mofetil Azathioprine	–2.6 (–11.5; 6.4)	0.567	5.1 (–1.8; 12)	0.141
	Triple immunosuppression	–9 (–18.5; 0.5)	0.064	–1.7 (–7.5; 4.1)	0.550
	Vaccine dose (post 3rd vs. 2nd dose)	21.2 (14.4; 27.9)	<0.0001	5.8 (1.3; 10.4)	0.013

β, regression coefficients; CI, Confidence interval; OLT, Orthotopic liver transplant; LUT, lung transplant.

[a]*Centred to the mean. Mean ages among the OLT recipients were 63.9 years in the anti-SARS-CoV-2 Ig analysis and 63.5 years in the INF-γ analysis. Mean ages among the LUT recipients were 45.1 years in the anti-SARS-CoV-2 Ig analysis and 45.3 years in the INF-γ analysis.*

cell-mediated immune response were reported in SOT recipients when compared to the general population [5, 10].

In the ORCHESTRA cohort [11], OLT was associated with a significantly positive humoral response at 3 ± 1 months (79.1%), while lower rates of seroconversion were observed among LUT recipients (53.9%).

Limited data has been published regarding the role of T-cells in the protection against SARS-CoV-2 infection and this may be due to the complexity and cost of this technique if compared with serological analysis. In a study based on less than 40 transplanted patients, cellular immunity was more frequently found than humoral immunity (64.7%, vs. 35.3% for antibodies), suggesting that assessment of antibodies is probably insufficient to identify COVID-19-vaccine responders in SOT recipients [12]. Subsequent observations showed that this response could be enhanced after the administration of a booster dose. However, T cell response was still compromised when compared to that of healthy controls [13, 14].

Currently the two main methods used to conduct measurements of cellular immunity in vaccine studies are ELISpot and flow cytometry [15]. In our study, we used an INF-Y ELISpot assay, since its performance is maintained even in samples from patients with lymphopenia and immunosuppressed individuals [16]. ELISpot assay is already well known for its higher sensibility in cohorts of transplanted patients compared to other IGRAs [17, 18].

Our population is representative of general SOT recipients, as reflected in their demographic and clinical characteristics. However, the maintenance of a good graft function in the majority of our patients could partly explain the high rates of humoral and cellular responses in our population. Moreover, the better response in OLT recipients than LUT recipients may be linked also to the lower proportion of OLT recipients under a triple immunosuppressive treatment. These findings may reflect the pharmacodynamic effect of glucocorticoids regimen on INF-Y pathways secretion [19, 20]. Of note, in our cohort, chronic kidney disease was more frequent in LUT recipients than OLT recipients and low glomerular filtration rate was associated with lower antibody levels.

A daily dose of Mycophenolate Mofetil (MMF) > 1g/die could lead to a lower immunological response [12, 21]. In our study, we did not find a statistically significant association with the use of MMF, and this could be possibly explained by the low doses being used in our population.

To date, no definite conclusions can be drawn on the relationship between quantitative antibody measurement and protection from SARS-CoV-2 infection or COVID-19 disease.

Severe COVID-19 was uncommon in our cohort, with only a few patients requiring hospitalization (0.8% of OLT recipients and 5.8% of LUT recipients). The routine of both monoclonal antibodies and specific antiviral treatment early in the course of COVID-19 may have contributed to decrease the occurrence of severe and fatal cases [22, 23].

In the CONTRAST Study seroconversion occurred in the majority (78%) of 614 SOT patients, with an 18% incidence of BI. Levels of antibody response were associated with reduced risk of BI, while the burden of immunosuppressive drugs was not related with an increased risk of BI. OLT recipients were

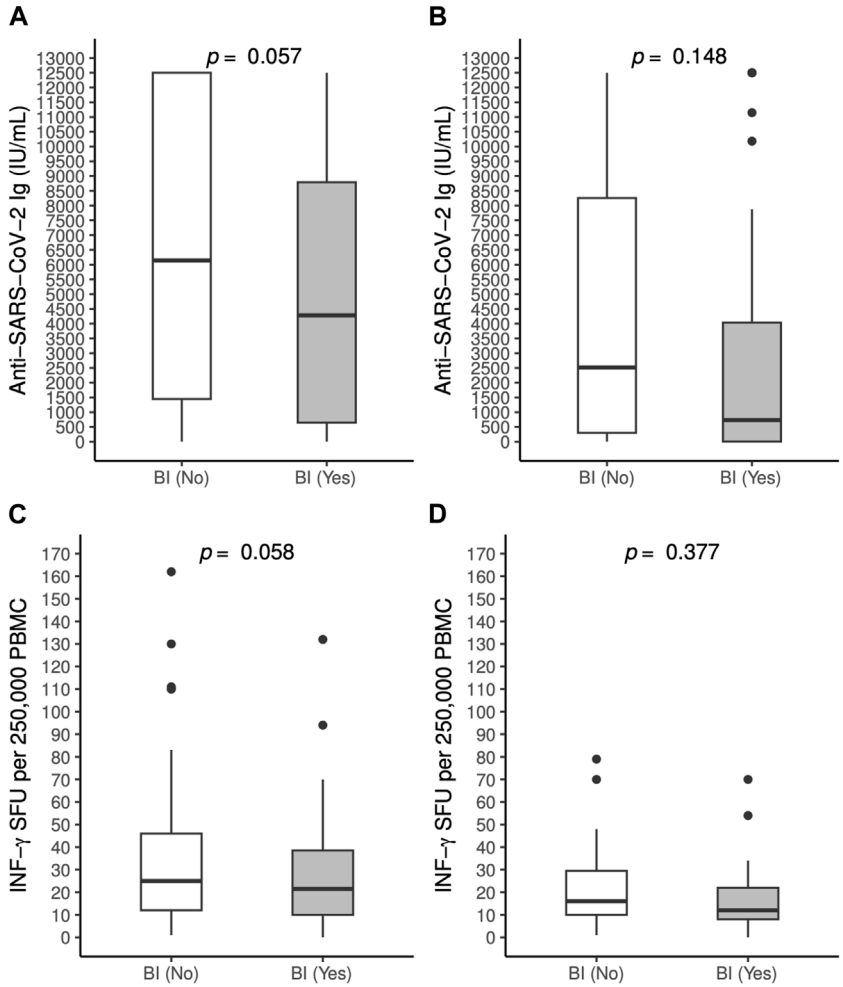


FIGURE 2 | Antibody [**(A)**: OLT recipients, **(B)**: LUT recipients] and T-cell mediated responses [**(C)**: OLT recipients, **(D)**: LUT recipients] to BNT162b2 vaccine against SARS-CoV-2 measured 60 days post 3rd dose in patients who experienced a breakthrough infection and those who did not, by solid organ transplantation group. BI, Breakthrough infection; INF, Interferon, LTU: Lung transplant; OLT, Orthotopic transplant; PBMC: Peripheral blood mononuclear cells; SFU: Spot forming units.

confirmed as being more likely to have a positive antibody response and a lower infection rate [24].

Data from the US Registry showed that SOT recipients have a higher risk of BI compared to the general population with the highest risk observed in LUT population (Hazard ratio, HR 2.11) and the lowest one in OLT (HR 1.39) recipients. The same study also showed a vaccine-related reduced mortality during BI for both general population and SOT recipients (HR 0.37 and 0.67, respectively) [25]. Our real-life data seems to confirm this trend as severe infections were uncommon in our cohort.

Overall, our findings highlight the importance of the third dose of COVID-19 vaccines as a booster. The benefits of booster doses are well established, both for COVID-19 [26, 27] but also for other vaccines, such as the inactivated polio vaccine [28].

Strengths and Limitations

The large sample size is certainly the main strength of our study. We also provided unique data on the T-cell mediated immune response elicited by one of the most used COVID-19 vaccine in SOT recipients, using a highly performant method in this particular setting.

However, we acknowledge some limitations, including its single centre nature and the absence of healthy controls. While currently available scientific evidence supports the administration of a fourth and a fifth dose of vaccine against COVID-19 worldwide in patients with impaired immune system, we did not collect further data on serum level of antibodies and T-cell mediated immune response after these additional doses.

Conclusion

The third dose of anti-COVID19 mRNA vaccine effectively enhanced both antibody and T-cell immune responses in SOT recipients. No significant risk factors related to lower responses were identified, and, more specifically, immunosuppressive therapy did not correlate to the grade of immune response elicited by the vaccination. Since it is well established that

antibody levels tend to decrease linearly with time from vaccination, a strategy of repeated booster doses as indicated by official Institutions could be a valuable option to prevent the development of severe COVID-19 disease in the transplanted population.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by Ethics committee of the I.R.C.C.S. Istituto Nazionale per le Malattie Infettive Lazzaro Spallanzani (Parere n. 422 del Registro delle Sperimentazioni 2020/2021). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LM, MD, GA, and SU conceived the study; SU and GG performed laboratory testing; LM, VR, GC, MD, CD, MS, and NZ performed patients' follow-up and collected follow-up data; GA, NZ, LM, and MD analyzed the data; LM, GA, MD, and MS drafted the original version of the manuscript; FB, PL, FC, LR, and BA revised the manuscript critically. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12729/full#supplementary-material

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ORIGINAL RESEARCH
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Donor Evaluation Tool: A New Technology Improves Donor Enrolment on ICU

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Uncertainties on the intensive care unit (ICU) regarding the eligibility of a patient to be a potential deceased organ donor may prevent their referral and enrolment in the pathway for organ donation. Healthcare staff may exclude potential donors for medical reasons, which are no longer applicable. Hence, Swisstransplant implemented a digital donor evaluation tool (DET) in 2021, which allows the local hospital's organ donation coordinator to send a direct request to medical advisors (MA) of the organ procurement organization before excluding potential donors. All 156 requests entered in 2022 were analyzed. 117 patients (75.0%) were primarily accepted by the MA as potential donors. Of those 60 patients (51.3%) became actual organ donors. Main reasons for using the DET were questions regarding malignancies (n = 33, 21.2%), infectious diseases (n = 35, 22.4%) and age/co-morbidities (n = 34, 21.8%). The average age of the actual "DET donor" compared to the regularly enrolled, actual "Non-DET donor" was 65.3 ± 15.8 vs. 56.8 ± 17.5 years, respectively (p = 0.008). On average 1.9 ± 1.1 organs compared to 3.2 ± 1.3 organs were retrieved from DET vs. Non-DET donors. In summary, this new digital donor evaluation tool supports reporting and facilitates eligibility decisions in uncertain, complex donor cases, potentially increasing the number of organ donations.

Keywords: digital, ICU, organ assessment, tool, organ evaluation

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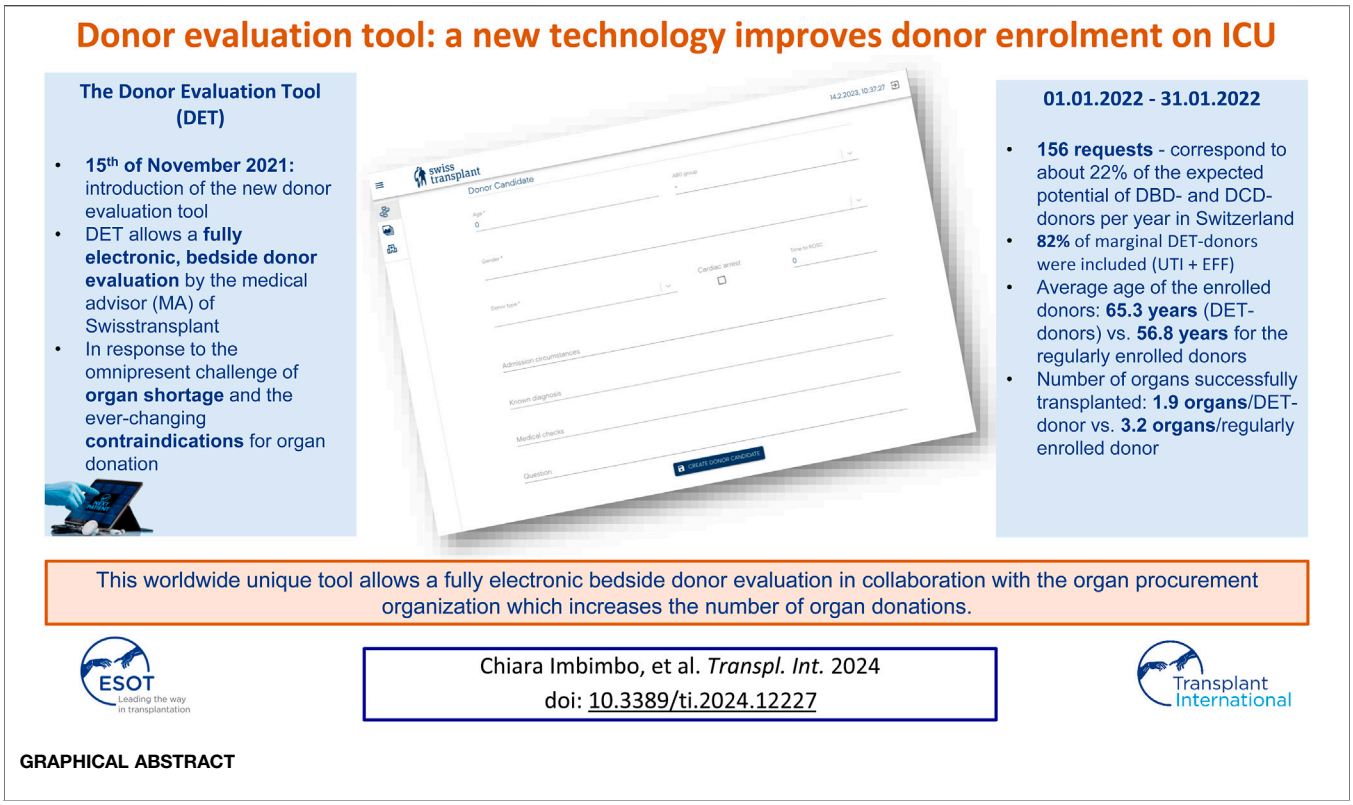
INTRODUCTION

Organ transplantation from deceased donors is a well-established medical treatment and very often the only curative therapeutical option in advanced organ failure. Organ shortage represents an omnipresent challenge in transplantation medicine worldwide. Switzerland is no exception with a post-mortem organ donation rate of 18.3 donors per million people (pmp) in 2019 [1]. In comparison, other European countries such as Spain or France register considerably higher numbers with post-mortem donor rates of 49.6 and 29.4 pmp, respectively [1]. Facing this issue Switzerland has implemented different approaches in the past. As in various other countries, donations after circulatory death (DCD) have been introduced and there has been an increased use of expanded-criteria donors over time [2–4].

In addition, national programs have been launched aiming to raise public awareness and improve structures, resources and processes at the hospital level [5]. Despite the great efforts undertaken, the demand for donor organs continues to exceed the current supply by far resulting



GRAPHICAL ABSTRACT

in extensive waiting periods and higher death rates on the waiting list [6]. These global realities mandate that novel approaches are urgently needed.

Over the years, the exchange between hospitals and Swisstransplant, the National Foundation for Organ Donation and Transplantation, has become more and more intensive, especially recently due to the COVID-19 pandemic. During this challenging time Switzerland followed a gradual shutdown-approach with the aim to prevent the transplantation activity from collapsing. A centralized evaluation of all potential organ donors was implemented and performed by medical advisors (MA) within Swisstransplant [7] with a special focus on factors such as availability of resources, organ quality and urgency status of the recipients on the National Waiting List. As a result, the number of transplantations performed remained almost unaffected despite the comparably high Covid-case load at that time [8].

This positive experience formed the basis for developing a digital platform to facilitate hospitals directly contacting Swisstransplant's MAs, in case of uncertainty regarding the suitability of a patient for organ donation and the further procedure. Eligibility criteria for organ donation are constantly updated and modified by new knowledge, so their application is not always easy to the multi-factorial cases of complex donors. Hence, guidelines for organ donation are regularly reviewed and adjusted. Most of the ICU staff is not involved in these discussions and potential donors may be excluded for reasons, which are no longer applicable. Moreover, enrollment of marginal donors may also depend on the medical urgency and profile of actual recipients on the National Waiting List. In critical urgent patients transplant centers are willing to take a higher risk in accepting marginal organs.

On the 15th of November 2021 Swisstransplant implemented a digital donor evaluation tool (DET), which allows the hospital's organ donation coordinator, informed by the ICU staff, to fill out a donor evaluation form and send a request to the medical advisors (MA) from Swisstransplant in any case of uncertainty regarding suitability for donation before excluding potential donors. Based on the medical condition of the potential donor and the situation on the National Waiting List the MA gives electronically written feedback to the requesting center.

The present article describes the effects of using the DET in the first year.

MATERIALS AND METHODS

This study analyzes all requests sent via the DET in 2022, the first calendar year after its introduction on 15th November 2021.

The provincial ethics committee (KEK) granted exemption for the underlying study (BASEC-no. Req-2024-00085). For this kind of retrospective study approval is not required according to the Swiss human research law (Humanforschungsgesetz, Art. 2, Abs. 1).

On behalf of Swisstransplant the company *isolutions AG, Berne, Switzerland*, programmed this application, which

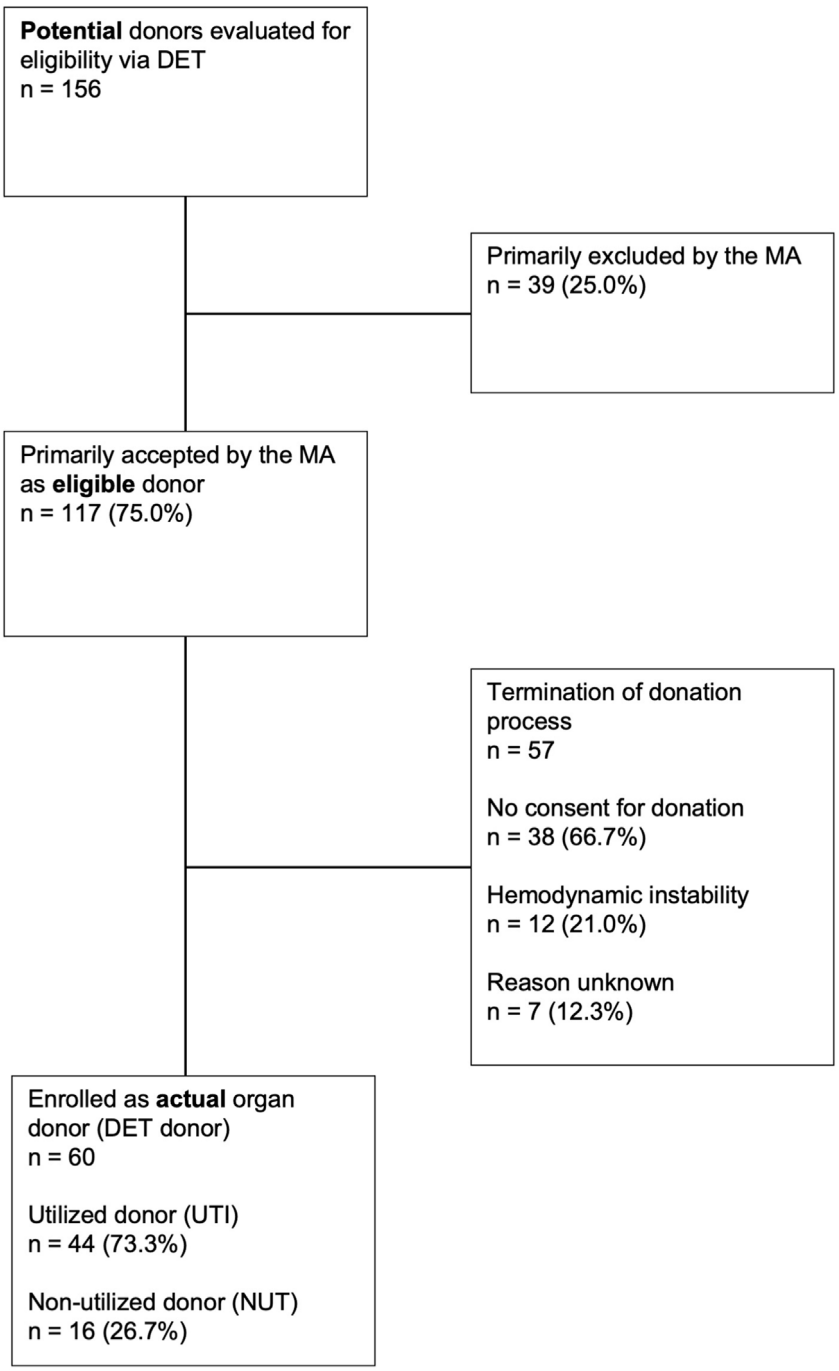


FIGURE 1 | Flowchart of all requests digitally submitted to the medical advisors via the donor evaluation tool (DET) Overview of course and final outcome of all 156 requests submitted in 2022 to the medical advisors (MAs) at Swisstransplant via the donor evaluation tool (DET). UTI, utilized donor; NUT, non-utilized donor.

enables a fast and digital exchange with the hospitals. In the supplement a link and QR-code is provided showing a video of the practical application of the DET. The aim of this work was to analyze the outcome of all digitally entered requests for evaluation. The decision process is shown in **Figure 1** and based on the nomenclature of the critical pathway for deceased donation [9, 10]. The assessment of the DET requests by the Swisstransplant MAs could lead to either direct exclusion or primary acceptance as medically suitable donor. The consecutive work-up could lead to either termination of the donation process or enrollment and registration as actual organ donor in the Swiss Organ

TABLE 1 | Comparison between donors enrolled with the donor evaluation tool (DET donors) and with the standard registration process (Non-DET donors).

		DET donors		Non-DET donors		*p*-value
		n	%	n	%	
Donors		60	32.3	126	67.7	0.004[b]
	UTI	44	73.3	113	89.7	
	NUT	16	26.7	13	10.3	
Age[a]		65.3	15.8	56.8	17.5	0.001[c]
Sex						0.87[b]
	Male	36	60	74	58.7	
	Female	24	40	52	41.3	
Type						0.41[b]
	DBD	29	48.3	69	54.8	
	DCD	31	51.7	57	45.2	
BMI[a]		28.8	4.6	26.9	5.3	0.013[c]
COD						0.19[d]
	Anoxia	27	45	48	38.1	
	Cerebral hemorrhage	19	31.7	50	39.7	
	Cerebral trauma	6	10	17	13.5	
	Cerebral disease	2	3.3	8	6.3	
	Cerebral tumor	1	1.7	0	0	
	Other	4	6.7	2	1.6	

DET, digital evaluation tool; UTI, utilized donor; NUT, non-utilized donor; DBD, donation after brain death; DCD, donation after cardiocirculatory death; BMI, body mass index; COD, cause of death.
[a]*mean (SD).*
[b]*Pearson's Chi-squared test.*
[c]*Two sample t-test.*
[d]*Fisher's exact test.*

Allocation System (SOAS), i.e., declared dead, eligible and consented for organ donation. These registered, actual donors were subcategorized into (a) "utilized donor" (UTI), i.e., at least one organ was transplanted and (b) "non-utilized donor" (NUT), i.e., no organ was recovered or transplanted.

The group of utilized donors enrolled through DET (DET donors) was then compared with the group of utilized donors enrolled through the standard, regular direct registration (Non-DET donors) in the Swiss Organ Allocation System (SOAS) during the same period of time. Donations of preceding years before installment of the DET system were not included due to annual variabilities in numbers, substantial effects of the Covid pandemic and changes in donor acceptance criteria over time.

The two groups of DET and Non-DET donors were compared with descriptive statistics using Fisher's exact test for categorical values (cause of death groups), two sample t-test for continuous variables (age and body mass index), and Pearson's Chi-squared test for categorical data (sex and donor categories).

RESULTS

Number of Requests Using DET

A total of 156 requests of individual patient cases were entered in the DET in 2022 (see also **Figure 1**, flowchart). This corresponds to approximately 22% of the estimated potential of DBD (donation after brain death) and DCD (donation after cardiocirculatory death) donors on ICUs per year in Switzerland according to the database of SwissPOD (Swiss Monitoring of Potential Donors). In this database all deaths on ICUs in Switzerland are recorded by Swisstransplant as required by law.

117 patients (75.0% of the total 156 requests) were primarily accepted as eligible donors on the initial assessment by the MA. Out of these 117 eligible donors 60 (51.3%) were ultimately enrolled as actual organ donors. In the remaining 57 (48.7%) patients no consent for organ donation was the main cause (n = 38, 66.7%) for stopping the donation process. This resulted in a refusal rate of around 33% of eligible donors within the DET group and is thus notably lower than the Swiss average of approximately 55% [11]. In another 12 cases (21.0%) donation was not possible due to the further clinical course with hemodynamic instability of the patient or findings in additional investigations. In the remaining 7 cases (12.3%), the reason for not donating could not be determined retrospectively.

Reason for Using DET

The analysis of the 156 enquiries identified three main concerns leading to the use of the digital evaluation tool: infectious diseases (n = 35, 22.4%), malignancies (n = 33, 21.2%) and old age and/or various co-morbidities (n = 34, 21.8%). Issues related to SARS-CoV-2 infections accounted for a large proportion of the infection-related queries. Out of the 35 infection-related requests 17 cases (49%) were related due to a diagnosis of SARS-CoV-2 infection.

Comparison of Donors Enrolled With DET vs. Standard Enrollment (Non-DET)

As shown in **Table 1** the evaluation of the patients' characteristics showed a significantly higher age of the actual DET donors compared to the actual Non-DET donors (65.3 ± 15.8 vs. 56.8 ± 17.5 years, resp., p = 0.001). In total 73.23% of the DET donors became utilized (UTI) donors, compared to 89.7% of the Non-DET donors (**Figure 2**).

Regarding gender distribution, both groups showed a largely equal distribution with a male share of around 60%. The main causes of death for both cohorts were anoxic brain damage (DET donors: 45%, Non-DET donors: 38%) and cerebral hemorrhage (DET donors: 32%, Non-DET donors: 40%).

In total, 98 (52.7%) DBD and 88 (47.3%) DCD donations were reported in 2022. The latter cold all be subclassified as controlled Maastricht III donors. Within the DET group 48.3% (n = 29) were DBD and 51.7% (n = 31) DCD donations, compared to the Non-DET donors, in whom the ratio of DBD to DCD donations was higher with 54.8% (n = 69) to 45.2% (n = 57).

On average, 1.9 ± 1.1 organs were transplanted per DET donor, compared to 3.2 ± 1.3 organs per Non-DET donor (**Figure 3**). Also the average number of offered organs were higher in the Non-DET compared to the DET donors (4.4 ± 1.4 vs. 3.0 ± 1.5, resp.).

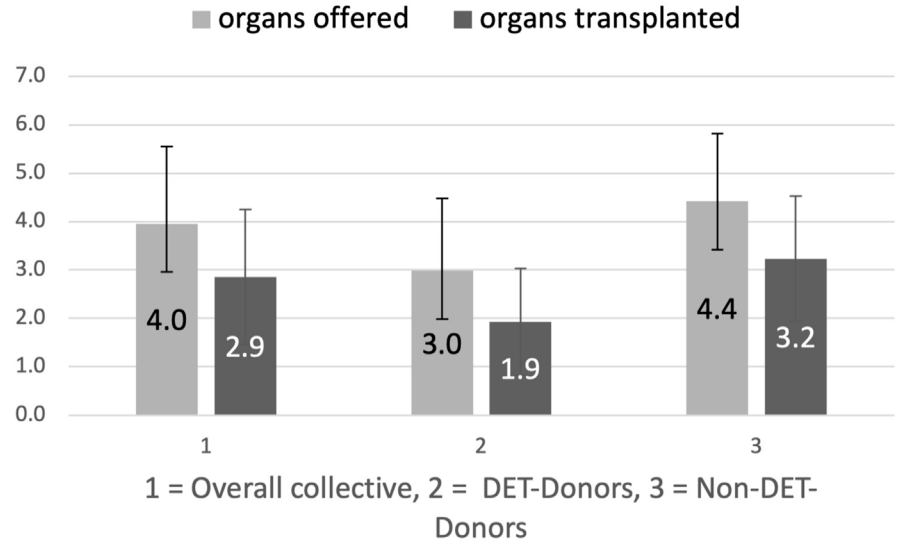


FIGURE 2 | Average number of organs offered (light grey bars) and transplanted (dark grey bars) of donors enrolled with the donor evaluation tool (DET donors) and enrolled with the standard registration process (Non-DET donors). Results in mean ± standard deviation (SD) organs per donor.

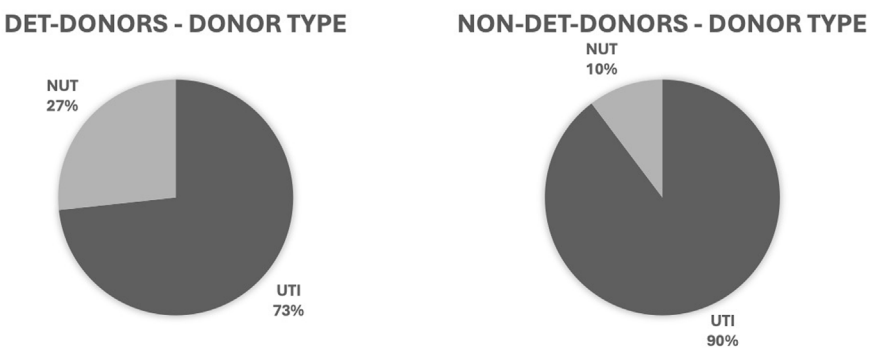


FIGURE 3 | Distribution of donor types enrolled with the donor evaluation tool (DET donors) and with the standard registration process (Non-DET donors). Results in relative frequency (%). UTI, utilized donor; NUT, non-utilized donor.

DISCUSSION

Key Factors of the Donor Evaluation Tool (DET)

For an organ to be successfully transplanted, a complex, time-consuming and labor-some process consisting of various steps must be completed beforehand. It starts with the identification of potential donors and the clarification of their suitability for donation. It includes a professional approach towards the relatives and specialized medical management to ensure the quality of the donation process. Each of these steps poses various challenges for the medical professionals involved and, if not handled properly, can lead to the loss of potential donors and ultimately of transplantable organs.

Based on the experience during the COVID-pandemic with a centralized evaluation, it became apparent that there is a potential to enroll more marginal donors for organ donation and also that knowledge of the current waiting list is of great importance in order to make decisions on donor eligibility.

The support of ICU staff in the assessment of medical suitability for donation is of increasing importance, in particular due to the progress of new findings justifying more liberal inclusion criteria for potential donors. Swisstransplant established the so-called "Donor Evaluation Tool" (DET) in November 2021 to offer hospitals a digital solution for quickly and directly contacting the specialist on duty in the event of uncertainty regarding the eligibility of a potential donor. Compared to the standard telephone contact, the tool offers

the advantage to upload important documents such as laboratory values or radiology reports from the patient's medical file in addition to the mandatory information such as age, gender, or suspected donation type (see also online video). This provides the MA directly with an information package facilitating the primary decision of principal eligibility of the patient as organ donor.

The analysis of the first year 2022 since the introduction of the DET suggests that direct accessibility of a specialist advisor, here the MA of Swisstransplant, and his or her consecutive expert evaluation especially regarding complex and marginal potential organ donors might be a resource to increase the number of organ donors to those regularly enrolled and registered.

The potential added value of the DET pathway is likely multifactorial. The MA is an expert in the field of organ donation and transplantation who is constantly learning about the latest international developments and findings. Thus, the MA supports the ICU staff with the highly specialized expertise in the assessment of complex cases regarding organ donation. Another decisive factor is the knowledge regarding the situation on the waiting list, which is then also considered by the MA in the evaluation process. In critical urgent patients transplant centers take a higher risk in accepting marginal organs and hence the referral rate by ICU specialists of cases that otherwise might be lost might be higher.

Main Concerns: Transmission of Malignancies and Infections

The key uncertainties on behalf of the ICU staff leading to the use of the DET were the potential risk that the donor might transmit an infectious or malignant disease.

Various studies have shown, that the risk of transmission of a malignant disease is always present, but overall classified as rather low. Studies from the United Kingdom (UK) indicated an overall risk of transmitting a malignant disease of about 0.05% [12], the risk of transmission by donors with a known history of malignancy of 1.1% [13]. A study in the US reached comparable results, 650 organs were transplanted from 257 deceased donors with a history of cancer. In the follow-up period of 45 months, none of the respective organ recipients developed a cancer of the original donor type [14].

Similar findings were published for the risk of transmission of infectious diseases. The "Ad Hoc Disease Transmission Advisory Committee (DTAC)" recorded 2,185 potential disease transmission events, of those only 15% (335 donors) were classified as proven/probable donor-derived diseases, including 244 transmitted infections and 70 malignant diseases. Despite overall rare, however, diseases transmitted by organ donation have a high morbidity and mortality and prevention strategies or approaches for early detection are necessary [15]. Overall, these studies indicate the need for specialist evaluation and reassurance of the treating physicians regarding infectious or malignant diseases in potential donors. They also explain why more than 40% of requests submitted via the DET to the specialist advisor were related to tumors and infections in potential donors.

Around 20% of requests were related towards age and/or comorbidities. This again indicates uncertainties of ICU staff regarding the eligibility of marginal donors, in particular as guidelines cannot provide clear-cut age-thresholds or disease exclusions. Here again the direct accessibility to an experienced MA overseeing the changing trends in donor/recipient criteria, special organ characteristics and waitlist demands is helpful [10].

Comparable Approaches in Other Countries

Experiences with a centralized evaluation of organ donors have so far only been described in the literature from Israel and Italy. Cohen and Ashkenazi analyzed the number and type of enquiries received over a period of 10 years since the introduction of a centralized "medical advisory service" (MAS) in Israel in 2007. Hospitals can call a specialist at any given time to discuss questions regarding the organ donation process. Concerns regarding the safety of organs for transplantation, especially in case of malignant or infectious donor diseases, were the main reason for enquiries to the MAS. The authors concluded that such a model would be a valuable tool to increase the number of donor organs as well as safety, quality and standardization of the donation process [16].

In 2003, Italy established a similar system on a national level with a continuously available expert task force. However, enquiries are limited to an evaluation of potential donors with a possible risk of transmission of an infectious or malignant disease. Nevertheless, the application of uniform guidelines and the expert evaluation and risk assessment also achieved a higher number of organs for transplantation [17].

These results from Israel and Italy are in line with the experiences made in Switzerland with the DET.

The Added Value of the DET Pathway to the Standard Process of Donor Enrolment

It is difficult to quantify the added effect of the newly implemented DET pathway in this retrospective study. In particular long-term data and comparable granularity of information for both pathways require prospective, future studies.

However, the requests put in the DET system indicate that the ICU staff needs help in the evaluation of marginal donors, i.e., complex patients with comorbidities and advanced age. In these cases, the central decision by a MA to proceed or not with the donation process is not only medically reassuring but likely also helpful for the staff to proceed with the laborious and emotionally demanding process of donor work-up, including family involvement, additional diagnostic tests, and extended stay on the ICU. In this context, it is very reassuring that out of the primarily accepted eligible donors 50% became actual donors. In addition, the positive impact of the DET process is underlined by the roughly 20% lower consent refusal rate compared to the Swiss national average rate. In addition, the digital submission of key data together with the question facilitates decision making for the MA.

As expected for a marginal donor population the DET patients were nearly 10 years older than those enrolled via the standard registration. However, despite the lower donor utilization rate of

74% vs. 90% and less organs transplanted of 1.9 vs. 3.2 for the DET vs. Non-DET donors, respectively, these numbers indicate the added value of the tool and justify the continuation of this service to the ICU staff. Future studies have to analyze the outcome data for DET vs. Non-DET donors and whether the total numbers of organs transplanted has significantly increased due to the DET pathway.

Limitations of the Study

Some limitations need to be mentioned. Robust numbers regarding a definite increase of donors due to DET cannot be given. The annual variability of donation rates due to effects such as initiation of DCD donation or COVID pandemics would require a longer period of observation. For example, in 2022 a substantial number of questions were related to COVID infections (11%). These inquiries might drop in the future. In addition, it is unclear whether all donors submitted and finally utilized via DET would ultimately have been missed without the digital tool. It is possible that the ICU-physician together with the responsible coordinator would have alternatively used the standard way of reporting for these potential donors or sought advice by other means and experts in the field.

Nevertheless, according to the current figures, the frequency of requests shows an increasing trend. This likely reflects the broad acceptance of the DET in the hospitals across the country.

Another limitation is the variability of information content given in the individual enquiries. For a comprehensive evaluation in regard to organ donation and for a better prospective analysis of the additional impact of the DET pathway, a further standardization of the mandatory electronic data input and a learning curve on both sides, ICU hospitals and organ procurement organization, will improve the performance of tool and its efficiency. In addition, another goal is to improve also information granularity and standardization in the Non-DET pathway which will allow then also a better comparison of both processes, in particular an in-depth analysis of phenotypes of DET vs. Non-DET donors. Addressing these early limitations will likely turn into additional strengths of the future applications of DET.

Overall, future prospective analyses will be necessary to further evaluate the impact of this new tool on donation rates as well as long-term postoperative transplant outcomes.

CONCLUSION

This retrospective study analyzes the first full calendar year (2022) of using a unique donor evaluation tool (DET). This electronic device allows direct and easy access for the ICU staff in case of uncertainty regarding eligibility of a potential donor. In addition the fully electronic donor evaluation facilitates also the decision making by the medical expert. This facilitated enquiry and evaluation process might increase deceased donor numbers but more long-term data and prospective studies are needed.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The provincial ethics committee (KEK) granted exemption for the underlying study (BASEC no. Req-2024-00085). For this kind of retrospective study approval is not required according to the Swiss human research law (Humanforschungsgesetz, Art. 2, Abs. 1).

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: Study conception and design: FI, CI, NK, and AE. Data collection: CI, FB, MN, and TC. Analysis and interpretation of the results: CI, TM, FI, and AE. Draft manuscript preparation: CI, FI, and TM. All authors reviewed and 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.

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We would like to thank all our colleagues at Swisstransplant who have played a key role in the development and implementation of the donor evaluation tool.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12227/full#supplementary-material

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ORIGINAL RESEARCH
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Qualitative Content and Discourse Analysis Comparing the Current Consent Systems for Deceased Organ Donation in Spain and England

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England switched to an opt-out system of consent in 2020 aiming to increase the number of organs available. Spain also operates an opt-out system yet has almost twice the organ donations per million population compared with England. We aimed to identify both differences and similarities in the consent policies, documents and procedures in deceased donation between the two countries using comparative qualitative content and discourse analysis. Spain had simpler, locally tailored documents, the time taken for families to review and process information may be shorter, there were more pathways leading to organ donation in Spain, and more robust legal protections for the decisions individuals made in life. The language in the Spanish documents was one of support and reassurance. Documents in England by comparison appeared confusing, since additions were designed to protect the NHS against risk and made to previous document versions to reflect the law change rather than being entirely recast. If England's ambition is to achieve consent rates similar to Spain this analysis has highlighted opportunities that could strengthen the English system-by giving individuals' decisions recorded on the organ donor register legal weight, alongside unifying and simplifying consent policies and procedures to support families and healthcare professionals.

Keywords: organ donation, consent, England, Spain, opt-out

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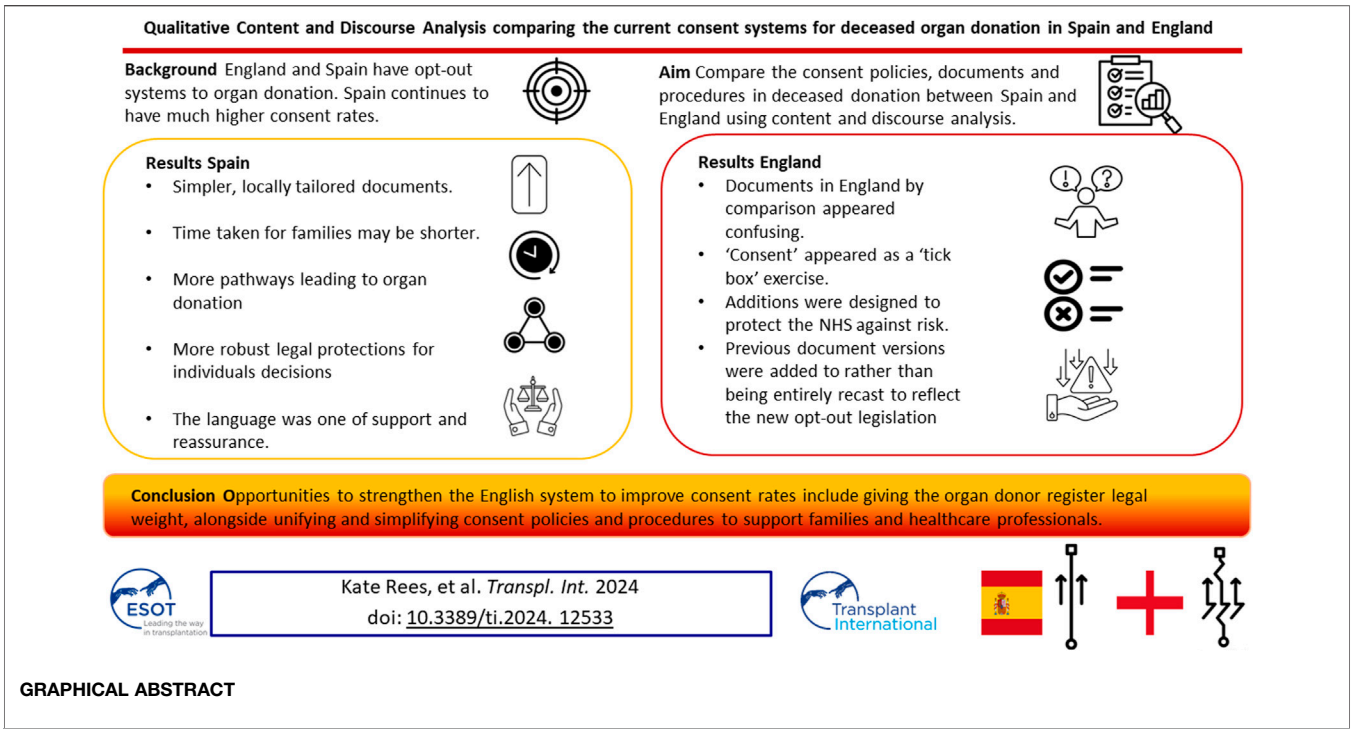
INTRODUCTION

Since England switched to an opt-out consent system in May 2020, with the aim of making more organs available for transplant through the introduction of deemed consent, consent rates for deceased organ donation have not increased [1]. Despite high ambitions [2] England still appears to be falling short of the number of organs available achieved by Spain which has consistently had much higher organ donation rates (46.7 per million population in 2022) despite having a similar legal framework for deceased organ donation consent [3].

Presumed consent (sometimes referred to as deemed consent) means that a person is considered to have no objection to donating their organs after death unless they have registered or informed someone close to them that they do not wish to do so. There have been many studies that have



GRAPHICAL ABSTRACT

concluded that presumed consent alone does not explain the fluctuation in donation rates between countries [4]. Legislation, public knowledge and awareness of organ donation, donor availability and characteristics, religious beliefs, transplant service infrastructure and healthcare system capacity (e.g., in intensive care), all play a part in making more organs available for transplant, but their relative importance is unclear [5–8].

England implemented their opt-out system to increase the consent rate for deceased organ donation, the assumption being that more organs would become available for transplant. However the opt-out legislation was nested within the existing opt-in system, and despite the addition of the new deemed consent pathway, the failure to secure consent for deceased organ donation retrieval from those involved in end-of-life discussions is still widely regarded as the single most important obstacle to making more organs available for transplant in England [9].

The purpose of this analysis was to identify differences and similarities in consent policies and associated documents between England and Spain and to consider whether there are opportunities to further increase consent rates for deceased organ donation and improve current practice in England.

Overall Context and Scope

This analysis was undertaken as part of a broader evaluation of the impact of opt-out in England on the organ donation system [10]. During the study it became clear that the processes involved in consent, in particular were lengthy, excessive and negatively impacted families in England [11]. Specialist staff involved in consent also felt the process to be excessive and burdensome [12]. The research team was aware of extensive research into gold standards in terms of pathways to organ donation, i.e., what should happen and by whom to achieve the desired outcomes (the so-called Spanish model) [13] but was unable to find examples (or research) of the consent documents used in practice in countries with opt-out systems, which are considered leaders in organ donation. We felt that as the main study was specifically commissioned to examine a policy that (in theory) shifts from a model of informed consent to a model of presumed consent it would be a worthwhile and interesting analysis to look more closely at the consent documents and associated policies and guidelines of the world's leading country with an opt-out system and compare them.

MATERIALS AND METHODS

Research Question

What are the differences in roles, processes, consent forms and practices between the Spanish and English systems of organ donation and how do any identified differences begin to explain the higher consent rates in Spain?

Data Collection

We identified and obtained key policy and procedure documents and consent forms from the websites of the "Organizacion Nacional de Transplantes" (ONT) in Spain and NHS Blood and Transplant (NHSBT) in England. Documents published in Spanish were translated into English for analysis using the "TransPerfect" computer software. **Table 1** lists the documents included in the analysis [14–22]. The documents were read, reread, compared and coded.

TABLE 1 | Documents included in the analysis.

Document	Description	Page length
From Spain ONT:		
Private Sector Donation [14]	Framework Protocol for organ and tissue donation in the private sector	93
Exchange SS1 2396 [15]	The basis of the Quality and Safety Framework Program for the procurement and transplantation of human organs and exchange with other countries	9
National Consensus Document 2012 [16]	Describes the situation in 2012 of asystole donation in Spain and other countries and provides a number of recommendations for the development of new these features and/or to improve the effectiveness of existing programs	205
Quality Improvement Programme [17]	This report shows the results of an evaluation of the current organ donation and transplant process (year 2019)	27
Royal Decree 1723–2012 [18]	Regulates the activities of obtaining, clinical use and territorial coordination of human organs intended for transplantation and establishes quality and safety requirements. (The first Legal document)	34
Barcelona University Hospital Consent Form	The current consent Form used for donation at the Barcelona University Hospital	1
Catalonia Regional Consent Form	The current consent form used for donation in the Catalonia region	1
Virgin Del Rocio University Hospital Consent form	The current consent form used for donation in the Virgin Del Rocio University Hospital	2 (page 1 consent, page 2 revoking of consent)
Emergency Professionals and the process of Donation [19]	Recommendations/Guidelines for Emergency Clinicians with respect to organ donation at presentation to hospital	27
From England NHSBT:		
Organ and/or Tissue Donation Manual (SOP5818/2) [20]	The guidelines governing organ and tissue donation within the United Kingdom	33
Code F: Donation of Solid Organs and Tissue for Transplantation. Human Tissue Authority (HTA) [21]	Human tissue authority Codes of Practice	44
Consent Form for Organ and/or Tissue Donation [22]	The United Kingdom wide consent form for organ and tissue donation	7

We worked with a Spanish intensive care doctor (co-author) via email and two online team sessions to clarify the correct interpretation of the documents and the donation system. This enabled us to verify that the current practice was in line with the written protocols. We engaged stakeholders through meetings with academics and cliniciansorganized by the European Society for Organ Transplantation (ESOT) to help establish the context of the English and Spanish organ donation systems within which the documents for analysis were produced. We consulted with a United Kingdom senior nurse (co-author) involved in the English NHSBT education program and United Kingdom legislation. A summary flowchart of the Spanish and English organ donation structures and processes was made for comparison (**Figures 1**, **2**).

These processes helped to build a better understanding of broad cultural factors, such as religious beliefs, ethnic diversity, family dynamics, the reaction of families to the system and whether they had ever challenged the law, and how these might be underpinning any differences observed in the documents analyzed in detail.

Data Analysis

Qualitative content analysis was used to code, analyze, compare and interpret the textual data and diagrams in the included documents to gain insight into the meaning and context of the policy, and the links between content, process and outcome [23].

Coding involved assigning attributes to words, sentences, or paragraphs to compare and contrast content, process and meaning. Consent forms were compared for structure, content, and length [24].

Principles of critical discourse analysis were used to make additional interpretations of the text, complemented by engagement with experts in the Spanish and English systems. This was done to systematically explore the often-opaque relationships between what is written (i.e., policies, guidelines) and what happens in practice, with multiple stakeholders, many with different objectives. This process helped to examine, for example, who or what the subjects and objects are in the respective structures, discourses and processes, and how and why the two systems manage to generate and maintain different forms of language (rhetoric) [25]. The flowcharts constructed (**Figures 1**, **2**) helped to show where objects in relation to consent such as the Organ Donor Register, the roles of the staff, e.g., clinicians, transplant coordinators, nursing staff and the role and hierarchy of the family, etc. fit together in a complex system. The rhetorical analysis specifically searched for opportunities to give or decline consent within the process. This enabled us to understand more about the mechanisms underpinning the Spanish consent pathway, and thus extrapolate findings that may be applicable, with adaptation, to England [26].

Author Reflexivity

Co-authors LM and JN were already working with colleagues in the English system and were connected via multiple professional networks to clinical and academic colleagues in Spain. Once the lead investigators had a good understanding of the two systems, we had further discussions with the Spanish

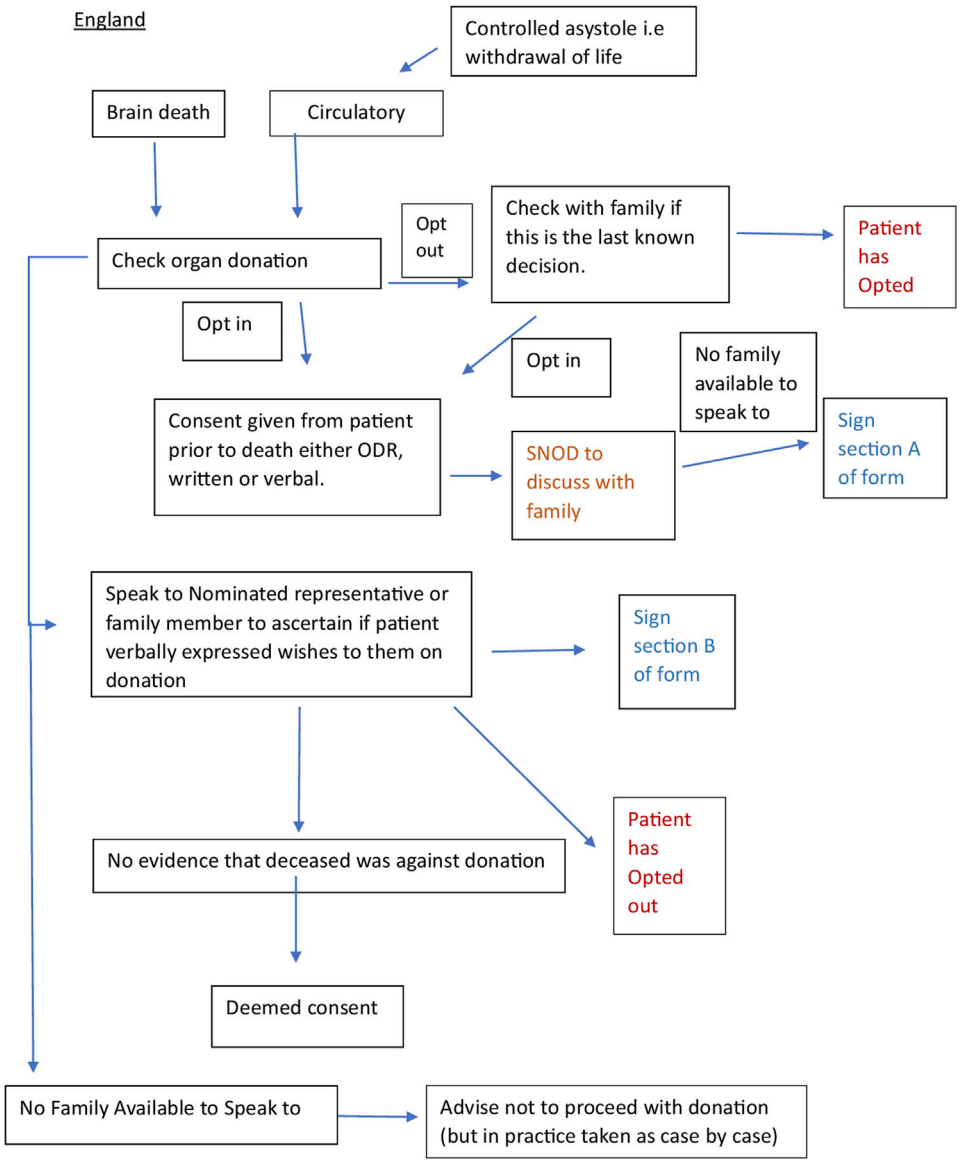


FIGURE 1 | Flow chart of the English and Spanish process constructed from documents (20–22) and stakeholder engagement.

consultant and Senior United Kingdom nurse co-authors to validate the interpretation of the two systems. We presented this work at several multi-disciplinary meetings and events including the Deconstructing Donation Special Interest Group for additional critique and insight. We adapted the recommendations for rigor, transparency, evidence, and representation to present the results [27].

BOX 1 | Comparison of key performance indicators between Spain and England 2022.

Key performance indicator	England	Spain
Number of Transplants performed	51.3 per million population (pmp)	122.1 pmp
Deceased donor rate	20.1 pmp	46.7 pmp
Deceased donor consent rate	65%	84%

RESULTS

Box 1 provides a comparison of some key performance indicators in England and Spain in the year 2022.

A direct comparison of the systems, processes, and cultural and linguistic styles between Spain and England in relation to consent for deceased organ donation is described below.

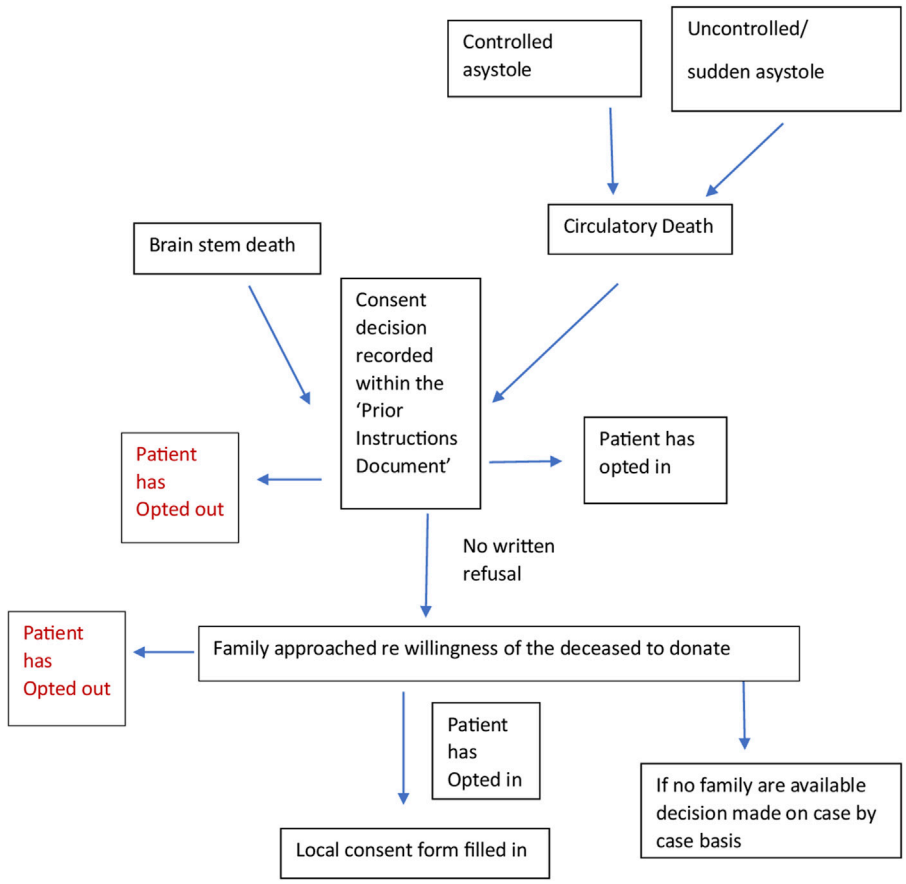


FIGURE 2 | Flow chart of the Spanish process constructed from documents (14–19).

Table 2 highlights similarities and differences within the systems with specific reference to consent (**Table 2**). The mechanisms that may or may not be a factor in achieving the desired outcomes in relation to consent are further unpacked and described in **Table 3**.

Overall System

England has a diverse population with deep-rooted Christian traditions and multi-faith communities. England switched to an opt-out system of consent to deceased organ donation in May 2020. The organ donation system is run by the NHSBT, which is publicly funded and not privately available. Deceased organ donation is considered for those who die from brain stem or controlled circulatory death. Donation is therefore only possible for those who are admitted to an intensive care unit (ICU), but ICU admission is for treatment and prognostic purposes, not for organ donation [28–30].

England has an intensive care bed capacity of approximately 6.6 per 100,000 people [31]. Organ donation is possible in every acute NHS hospital. When the patient is identified as a potential donor the clinical team caring for the patient will refer the patient via a national referral number. The regional NHSBT team will assess the patient and mobilize a Specialist Requester (SR) or Specialist Nurse in Organ Donation (SNOD) – depending on who is available. After checking the national Organ Donor Register (ODR), the SNOD/SR will visit the unit and approach the family about donation – this is a nurse-led process and care pathway. The ODR has various options (e.g., opt-in, opt-out, nominate a representative and the ability to specify a small number of organs/tissues that people do or do not want to donate after death) but it has no legal status and family members have the ability to override it in practice and even register a decision on behalf of their loved one. Hospitals are reimbursed a small sum for facilitating organ donation, (approximately 1,000 pounds per donor) but this figure has not increased substantially over time and complex and bureaucratic finance systems often make it difficult to spend and save money to promote organ donation.

Spain is a predominantly Catholic country and has had an organ donation system for 44 years [32]. The organ donation system is overseen by the ONT. It is possible to be an organ donor while being treated privately, by being transferred to the public health system for donation purposes only. In addition to the pathways in England, deceased organ donation can be obtained from sudden unexpected

TABLE 2 | Similarities and differences within the Spain and England systems with specific reference to consent.

	England	Spain
Consent system	"Soft" opt-out, opt-in and family consent for organs and tissues. Scheduled purposes and research not covered by the Act	"Hard" opt-out, opt-in and family consent based on the will of the deceased for scientific and therapeutic purposes
Eligibility criteria to apply opt-out system	Over 18, ordinarily (12 months prior to death) and voluntarily resident in England, dies in England, with full mental capacity	Over 18, has full mental capacity and be in adequate health
Age of consent for adults	18	18
Organs and Tissues included in opt-out system in place	Only organs and tissues "routinely collected and used for life saving/improvement treatments"	Includes both organs and tissues routinely collected for life saving/improvement treatments, scheduled purposes and research
Family made aware prior to admission to ICU to consider organ donation	no	yes
Family spoken to regarding withdrawal of treatment in DCD death and tests for DBD	yes	yes
Organ Donor Register	Yes – but has no legal status	No ODR in Spain
Prior Instructions Document	No	Yes – and has legal status
Determine the last known decision of the deceased	Yes	Yes
Nominated representative	Yes	No
Family hierarchy	Yes	Yes
Key hierarchical family member identified and spoken to as a priority	No (it is in the guidelines but rarely done in practice as a priority)	Yes
Witness to the conversation between SNOD and relatives/TC and relatives	Yes	Yes
Mandatory/legal requirement that family member signs donation form	No	Yes
Leaflets given	Yes – content and context varies	No
Details of all organs and Tissues taken explained	Yes	Simply
Details of body appearance following donation described to the family	Yes	Yes
Family continued to be supported by TC or SNOD if consent declined	No	Yes
Family follow-up	If signed consent given	No
Family informed of those whom donation helped	If signed consent given	No-can receive a thankyou letter if they sign for this
Can be contacted by those receiving donation	If signed consent given	No

circulatory deaths and those undergoing euthanasia. Spain has an intensive care bed capacity of approximately 9.7 beds per 100,000 people [33]. In Spain, patients admitted to the Emergency Department with catastrophic brain or cardiac damage where treatment is considered futile, can be intubated, and admitted to the ICU for the purpose of organ donation [34]. Also, those who are suspected of developing brain death or have already been declared brain dead in private institutions or the Emergency Department, can be admitted to the ICU solely for the purpose of organ donation, unlike in England.

Spain has dedicated hospitals where deceased organ retrieval can occur, with designated transplant coordinators (TC) in each of these hospitals (approximately 70% being physicians and 30% nurses). Often in hospitals with no TC, there will be proactive ICU staff who can identify donors. They can request support from a dedicated hospital which will usually send a TC to aid in speaking with the family. Any healthcare professional can contact the TC regarding a potential donor. Once alerted to a potential donor the TC will visit the potential case, review the medical records, and

TABLE 3 | Mechanisms which may be a factor in bringing about the desired outcomes, or not, in relation to consent.

Healthcare Professionals	England Only SNODS/SRs are allowed to approach family members about organ donation. Anyone else is actively discouraged from mentioning organ donation. This is because it is thought that NHS staff may create a context where organ donation is not presented in an appropriate way leading to reduced opportunities to gain consent. During the family discussion the SNOD/SR guidance document suggests that SNODs should remain impartial but often the advice and legislation is open to interpretation, often misleading, with arguments for and against ways to act. Therefore interpretation of this depends on the individual SNOD/SR involved Spain Although TCs are encouraged to speak to families about organ donation, other health professionals are able to offer encouragement for donation should it be mentioned earlier [19]. Organ donation is thought of by health professionals outside of ICU and thus a lot earlier in the care pathway of the patient, even extending to community and emergency services	Although all "*families are encouraged to support the decision their relative made in life*." In England 43% of families said no in 2022–23 whereas around 10% of families still refuse in Spain (outcome). The Spanish system therefore contains more factors that create supportive contexts that bring about higher consent rates (mechanisms) Having a more unified and bespoke approach for the TCs and this being reflected in a wider culture of support appears to be a factor that creates a mechanism for achieving higher consent rates (outcome) In the Spanish system, the potential for organ donation can create a context that subsequently influences the decision as to which hospital the patient is brought, enabling discussions to occur about admittance to ICU purely for donation, rather than recovery (mechanism). By empowering those outside of ICU to consider organ donation, creates a context which helps highlight potential donors to the TC and potentially aid conversations to patients prior to their death (mechanism)
System configuration	England ICU beds remain a scarce and precious resource to treat patients who are alive. There are no specialist organ donation centres in England. Every acute hospital is able to offer/honour organ donation on site as it is the organ retrieval team and SNOD/SRs who travel to the hospital Spain Patients in Spain can be admitted to ICU purely for the purpose of donation. Spain has specialist organ donation hospitals which have designated TCs	The lack of ability to admit potential organ donors to ICU purely for organ donation reflects unequal End of Life care policies between England and Spain (comparative context) and could help explain the differences in consent rates (outcomes) but also potentially indicates a discrepancy in priorities between countries (contexts and mechanism) that also impacts negatively on consent rates (outcome) In Spain organ donation is more visible and acceptable – due to capacity to host more potential organ donors without adding strain or worry to the ICU service. This creates a context and mechanism that makes organ donation easier. The NHS would however need to increase ICU capacity to adopt this approach to create a similar context and mechanism leading to better consent outcomes In both countries it is specialist teams that provide the care (context), but in England the more complex process can take hours to days (context). This means that the family may have to wait a length of time before being able to speak to the SNOD/SR and go through the longer processes (mechanism) and this can often influence their decision to decline donation (outcome) The length of time can also give families more time to revoke consent (mechanism leading to outcome) if it is given and they may decline consent straight away feeling that their loved one has already suffered enough or to be able to start making funeral arrangements (mechanism and outcome)
Faith and Beliefs	England Throughout the English guidelines faith/beliefs are mentioned frequently and there are documents dedicated to this. There is also the option of recording this when someone registers a decision on the organ donor register Spain Although faith and beliefs are important they are rarely specifically mentioned in the documents or given a huge amount of coverage	While there are detailed guidance on faith/beliefs (context) the guidance in the documents for healthcare professionals and options on the ODR are not translating into practice - vast inequalities remain in organ donation in the United Kingdom. (outcome)
The organ donor register	England The organ donor register enables people to record a decision about organ donation prior to death. It enables people to choose which organs and tissues they would like to donate or not. However there are many avenues to recording a decision, the forms are not universal and they do not reflect what the family is asked after death. Therefore despite people making these decisions the family will still be approached and questioned to ensure that the decisions made by the potential donor have not changed In England the HTA states that although "*consent has been obtained, it is not mandatory that organ donation proceeds"especially if "the family do not support it."* The SNOD/SRs are left to determine each situation on their own best judgements as the current guidelines are not clear Ironically if the nominated representative cannot be contacted in time, consent can be deemed yet if no family are available and there is nothing recorded on the ODR it is advised that consent does not go ahead	In England, although the organ donor register gives the opportunity for people to record a decision prior to death it does not have any legal status (context). This means that family members can easily override their relative's decision to donate their organs made in life (unintended mechanism) resulting in lowered consent rates than anticipated (outcome) In England, the consent process for the bereaved family is more burdensome (context), potentially contributing to revoking of consent or reluctancy to give consent (mechanism and outcome). It can also be a surprise to the family that a decision has been recorded by the potential donor as a decision can be made effortlessly when applying for a boots advantage card or drivers licences, for example, but these are kept separate and independent from medical notes (context and mechanism). This could help explain why the numbers of people opting-in to donation have increased somewhat, but overall consent to deceased organ donation has not (outcome)

(Continued on following page)

TABLE 3 | (*Continued*) Mechanisms which may be a factor in bringing about the desired outcomes, or not, in relation to consent.

	Spain The patient will be required to get a form from their GP which has to be signed by a witness. This decision is then shared on their health record. Due to the increased effort in Spain to register an opt-out decision which is witnessed, this may explain why there are higher numbers of organ donation but also that families are more likely to discuss the decision with their relatives or friends and have more trust in the system that it is an integral part of end of life care In practice an opt-in decision is always discussed with the family, and the guidance advises that even opt-out decisions should be discussed to ensure this was the last known decision	In England, deemed consent is not properly or always understood by family members yet as a positive decision that supports organ donation and perhaps why families are continuing to override the deemed consent
Opportunities to say no	England There are further opportunities for consent to be declined as highlighted in **Figure 1**. The potential donor can opt-out/in via the ODR or by expressing it verbally to family and friends. The nominated representative may also decline donation. By further checking if an opt-in decision on the ODR was the final decision offers a further opportunity. If the family disagree with the potential donors decision in life, sometimes donation does not occur out of fear of upsetting the family and risk to what messages would be interpreted by the wider public. The family are frequently reminded that they can decline at any point until the retrieval has commenced, *"Withdrawal of consent should be discussed at the outset when consent is being sought."* This is also a regulatory requirement written into the procedural documents before deemed consent was introduced This suggests that no is the default answer expected, which is the opposite of a deemed consent system Spain Consent to donation can be declined either by writing in the prior instructions document or declared to the family who can then continue to decline on the potential donors behalf after their death. In Spain the TC will strive to understand the reasons why donation is declined and they are encouraged to give the family time to ensure this is the final decision before accepting it	Despite a law which switched the default to one where organ donation is presumed, documents and guidance appear to support the opposite in the England (context). Tailoring this part of the process to the family and being allowed to speak more simply about the organs, tissues and processes may make this process easier and shorter. Therefore easier to say yes, and easier for everyone involved to go through (mechanism) and give their consent to organ donation (outcome)
The family and language	England When families are approached, they are asked what the potential donor's last decision would have been and whether the deceased expressed any thoughts on becoming a donor. The current policy suggests that the family are approached according to the highest qualifying relationship. This does not always happen in practice as the SNOD/SR tries to navigate the family dynamics while at the same time tries to gain evidence to support a '*final decision on donation'* that can be from any family member Spain The family are asked what would have been the willingness of the deceased to donate their organs and the key family member is identified In both countries the family are made aware that donation can be declined (if no decision has already been made by the deceased). In both cases the decision is respected and the TC/SNOD seeks to understand why However, in Spain the TC gives the family time to further think about their decision before accepting it as the final decision. The TC can also bring up ethical arguments for organ donation and also use the argument that it is likely that they will need an organ in their lifetime, which could influence the family decision and use arguments for courage, generosity and proximity, e.g., *"you are likely to need an organ at some point in your life*"	Rhetorically, this language possibly evokes feelings and thoughts of being brave and confident in testing times (context and mechanism). In England, the language appears to be less emotive by asking about the last known decision of the potential donor, which may not be as impactful as the rhetorical language typically used and encouraged in Spain Willingness itself evokes feelings or thoughts about the inclination or desire to help others if it is needed (context and mechanism). It appears to be almost a leading question. Nonetheless family dynamics can often be difficult to grasp and work with, particularly at times of acute grief. Families are complex and not all respond in the same ways to simple linguistic interventions and again this mechanism does not always work in practice (outcome)
Consent forms	England The 7 pages consist of yes/no tick box answers for a list of organ, tissues and processes involved in donation. For every donation (even for opt-in decisions on the ODR) the family will go through the same process. This is done to conform with the human tissue act 2004, although the forms have no legal status and are not mandatory to sign	The length and detail of the consent process and form (context) could become overwhelming for a family and dissuade them from supporting (mechanism) the current donation (outcome) or what they perceive might be involved in the future in terms of retrieval (mechanism). The consent form may also leave SNODs/SRs feeling vulnerable given it is not mandatory for the authoriser to sign especially if there has been some conflict on the final decision between the family (context and

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TABLE 3 | (*Continued*) Mechanisms which may be a factor in bringing about the desired outcomes, or not, in relation to consent.

	Spain Consent forms are created by local hospitals using the ONT template. They are short (one/two pages) and it is mandatory for the authorising family member to sign. Some forms have space for the family to write which organs they believe donor would or would not wish to donate; in others this decision is written within the medical notes instead. The form has legal status	mechanism), and the SNOD/SR more likely to stand down (outcome). The SNOD/SR may also be more likely to accept a decline in consent (outcome) given the mixed messages in the legislation and guidance, and if the family are divided, or especially traumatised, or the donor is borderline given the additional time and burden of the consent and retrieval processes (mechanism)

determine whether or not there is a "*prior instructions document.*" This document has legal status.

System Processes Concerning Deceased Organ Donation Consent

In England "*the individual leading the family approach for organ donation must be suitably trained and qualified with sufficient knowledge and skills to sensitively answer any questions and have the time to support the family,*" [21, pg 9]. In practice, this is always the SNOD/SR, anybody outside of this role is actively discouraged from discussing organ donation [12].

As illustrated in **Figure 1**, the English system has many pathways to consent. If the deceased opted for organ donation during their lifetime, this is discussed with the family to ensure that this was the last known decision. If the deceased had opted out of the ODR "*providing work load allows, the SNOD should also discuss with the family if this was the last known decision.*" [SIC] [20, pg 11]. If this is not possible due to workload, the SNOD/SR will "*coach the clinician in the discussion to have with the family and agree actions.*" If the clinician feels unable to do this, the family will have to wait for the arrival of the SNOD/SR. In practice, detailed discussions with the family when the deceased has opted out rarely happen due to limited resources and concerns about NHSBT being seen as pushing for organ donation when the deceased has opted out.

Another pathway, although rare, is the "nominated representative," where a person nominates someone else to make a decision on their behalf before they die. "*If despite all reasonable efforts the nominated representative cannot be contacted in time or to make a decision, then consent may be deemed.*" [SIC] [21, pg 19] Nonetheless, the donation can only take place after the family has also been consulted.

Only after the SNOD/SR has established that none of the above pathways apply, can they check whether consent can be assumed. If the family cannot agree, despite being given time and further information, then "*the hierarchy of consent, i.e., highest qualifying relationship," applies but the final decision to proceed lies with the [SNOD/SR].*' [SIC] In reality, it is the family member with the strongest voice (either for or against donation) whose wishes are followed [11, 35]. In addition, the SNOD/SR cannot proceed with the donation unless they have the full support (and permission) of the treating clinical team(s). If the family cannot be contacted and there is no prior expression of a decision, then although *"consent could be deemed it is advised that donation must not proceed."*"[SIC] [21, pg 17].

To override a decision, families need only provide a "*level of information that would lead a reasonable person to conclude that they [i.e., the deceased] did not want to be a donor.*" [21, pg 24]. This may be verbal or written. Any evidence from any family member at this point can be taken into account. [21, pg 18] The SNOD/SR will make a judgment about the reliability of the information and whether it is right for the donation to proceed. "*Sometimes clinical staff will reach the judgement that although there is a legal basis to proceed with the donation, the human considerations involved mean that it should not go ahead. While the presence of appropriate consent permits organ and tissue donation to take place, it does not mandate that it must. . ..(and) where the risks to public confidence might outweigh the benefits of donation proceeding, donation should not proceed even though the law permits it.*" [SIC] [21, pg 7].

In Spain, there is no organ donor register but a prior instructions document is available from the patient's GP. Patients can register their consent or refusal to be an organ donor in the document which will be made available in the local Advance Directives Registry. Their families will be approached and informed of the recorded decision. If a "No" to the donation has been recorded, the family will still be asked if there has been any recent change to this decision. However, there would have to be substantial evidence to overturn this notion since the prior instructions document has legal value and is signed by a witness.

It is recommended that the healthcare professional who mentions organ donation be different from the professional who has discussed the likelihood of the patient dying to avoid a conflict of interest for the TC who may also have a role as an intensivist, etc. It is mandatory in some hospitals that the TC be contacted before withdrawal of treatment in the ICU, a condition introduced by some hospital medical directors.

The Consent Forms

The English consent form is seven pages long, with all organs, tissues and retrieval processes listed as yes/no checkboxes, including options for additional information. The family will need to answer "Yes" or "No" to everything irrespective of what the deceased had registered about what organs they wanted to donate while they were alive and this will include organ donation for research (not just therapeutic purposes). The family will be made aware that the decision can be revoked until *"knife to skin."* [20, pg 24]. The family members "*are encouraged to sign the consent form*" although there is no legal obligation to do so. The process may take hours to days.

The SNOD/SR will document the conversation in the patient's notes and on the NHSBT's national digital system, also verified by a witness. If the family were to override the decision or revoke consent this will be respected and the reasons would be acknowledged and recorded by the SNOD/SR.

Each Spanish region has its own form based on examples from the ONT protocols. Often they are a single page requesting the name and relationship of the relative and the date. Some do have a free text space for the family to write what organs or tissues they believe the deceased would not have wished to donate. In other cases, these wishes are documented in the medical notes instead. Once a decision is reached after discussion with the family, it is mandatory that the consent form be signed by the dissenting family member(s).

Approach and Language for Consent

In England, when families are approached, they are asked, "*what the potential donor's last decision would have been and whether the deceased expressed any thoughts on becoming a donor" [SIC].* The guidelines suggest that SNODs/SRs should establish who is the next of kin (in accordance with the established highest qualifying relationship guidance) and approach that relative to organ donation. Although the opportunity to help others is often mentioned, the overall guidelines suggest that the SNOD/SR should remain impartial [21].

In Spain, if there is no recorded decision made while alive, the family is generally asked: "*what would have been the willingness of the deceased to donate their organs to help other people?" [SIC] [*16, pg 197]. *"If the family are in doubt, the TC can assist in decision-making, reinforcing positive verbalisations to donation and courage in those moments, and conveying ideas of generosity and proximity and enquiring whether the deceased gave to charity or donated blood during their lifetime, etc." [SIC]* [16, pg 126].

In the case of large families, the TC seeks to speak to the "key family" member. The key family member is identified through discussion with the family and the knowledge of the staff caring for the patient. Should a family be divided over the issue of donation, the TC will not proceed. If there is no family present, the TC *"strive(s) through links with social services and the police to find a family member"[16, pg 120]* but may still consider organ donation if no family can be found.

Should the family decline the donation, "*it is important to make it clear that the decision is respected and understood but that, however, it is advisable to think about the matter more slowly without the presence of a TC."[16, pg 126]* The TC also explores the reasoning behind the refusal and corrects misunderstandings. The TC may approach the family as often as necessary.

During the consent process, the family is usually asked which organs they believe the deceased would not want to donate. The conversation aims to combine "*speed and effectiveness in communicating with families, with respect for ethical principles and transparency that must preside over the process*." [SIC] [16, pg 116] On average, the process of gaining consent takes 30 min.

DISCUSSION

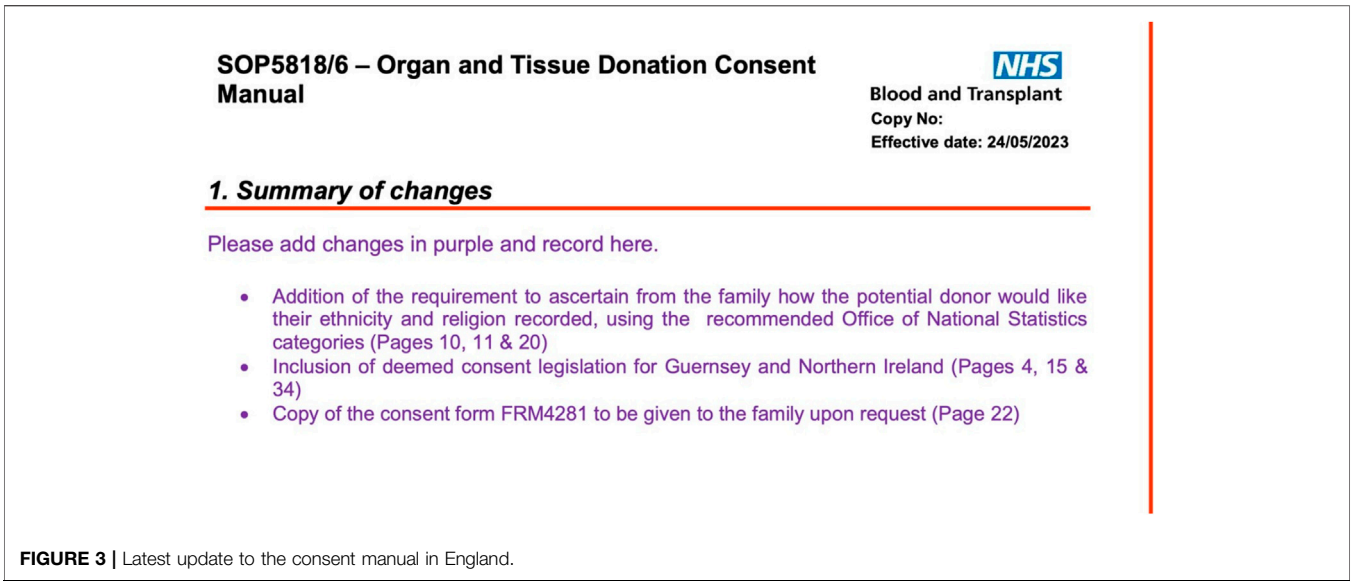
This is the first detailed documentary comparison between the Spanish and English opt-out systems of consent to organ donation. The biggest differences observed were that the Spanish system was less complex in terms of consent, evidently pro-donation with a willingness to take some risks, likely to take less time, better resourced, with better access to ICU beds and a more locally tailored opt-out system with some legal protection for the potential organ donor's life choices. England in contrast has a more complex centralized system with risk-adverse protocols, an itemized approach to consent, implemented in a country where there are fewer ICU beds, and no legal protection for the potential organ donor.

The Spanish system covers both public and private hospitals and has dedicated resources for organ donation, such as stand-alone centers and in-hospital beds. In England, for deceased organ donation, the NHSBT only covers NHS hospitals so some potential donors in the private sector are lost. There are no dedicated resources in England organ donation takes place when the system has the capacity to manage it which can potentially lead to frustration and disengagement of non-specialist staff. Euthanasia and organ donation are legal in Spain (illegal in England) and although the pathway is relatively recent it has created an additional platform to embed organ donation as a routine end-of-life process–the initial requests for this pathway have come from people who had requested euthanasia and not in the originaleuthanasia protocols. Potential organ donors with neurodegenerative conditions requesting euthanasia also tend to be younger without underlying co-morbidities and a single donor could potentially decide to donate all of their organs and tissues to help others, again increasing the visibility of organ donation in the system.

Families are as involved in decision-making in Spain as they are in England, but the consent process is shorter in Spain. The language used with family members and staff was also observed to be different in tone and meaning. The English system focuses on establishing the "*last known decision of the deceased*"whereas the Spanish system aims to establish "*the will of the potential organ donor to donate their organs as well as the will to help others."* In England, current guidelines and codes of conduct reflect the human tissue authority's position on consent to organ and tissue retrieval. This appears to be more in line with the old "opt-in" system and thus encourages unnecessary risk aversion which is contrary to the spirit of the opt-out legislation and appears confusing and neutral.

Organ donation appears to be more embedded within the Spanish healthcare system as an integral part of end-of-life care, with many healthcare professionals being aware of it and being encouraged to be involved with it. As such, it may be more likely to be discussed by families as there may be a healthcare worker in the family or someone they know who has been through the process before.

The legally binding prior instructions document is also available from the GP or local hospital and is signed with a witness present. Therefore, the witness, i.e., an accompanying family member is likely to be able to verify the document. Once completed, it is part of the person's local medical record, meaning that there is a more complex process if family members want to challenge their loved one's organ donation decision in life. There


SOP5818/6 – Organ and Tissue Donation Consent Manual

NHS
Blood and Transplant
Copy No:
Effective date: 24/05/2023

1. Summary of changes

Please add changes in purple and record here.

- Addition of the requirement to ascertain from the family how the potential donor would like their ethnicity and religion recorded, using the recommended Office of National Statistics categories (Pages 10, 11 & 20)
- Inclusion of deemed consent legislation for Guernsey and Northern Ireland (Pages 4, 15 & 34)
- Copy of the consent form FRM4281 to be given to the family upon request (Page 22)

FIGURE 3 | Latest update to the consent manual in England.

is a significant risk to donor decisions in England as anybody can go onto the ODR and register - SNODs/SRs continue to find cases where the opt-out decision was registered at a time when the person was being ventilated in the ICU [9].

The structure of the hospitals - i.e., that specific hospitals manage deceased organ donation, that patients can be admitted to the ICU purely for the need to ventilate organs and drug infusion in preparation for donation - is also very different from England. Matching the Spanish approach would undoubtedly cost the NHS more at the expense of another area of the health service. However, Spain states that "*the social value of organ donation justifies staff efforts and the economic cost involved*" [SIC] [16, pg 195], indicating an overall difference in priority in terms of deceased organ donation between the two countries.

In addition to the marked differences in the provision of ICU beds required for organ donation to proceed, in 2019, Spain had 3 hospital beds per 1,000 people whereas England had 2.5 beds per 1,000 people. In 2019, Spain had a bed occupancy rate of 76%, whereas in England the same rate was 92% for general and acute overnight beds [33]. Given the relentless pressure on NHS staff to continuously manage such a high bed occupancy rate, it becomes clearer why a centralized system of organ donation was implemented via a separate NHS body (NHSBT) with its own governance and management structures [36]. The NHSBT was created in 2008 and to a certain extent, its centralized opt-in system was successful in that consent rates steadily increased over the following decade before the law was changed. Nonetheless, the NHSBT has not been able to replicate the success of Spain. In 2020, a "soft" opt-out was implemented within the existing centralized national system alongside the existing opt-in system, and the two systems have been operating together in a complex way ever since. Although Spain does also offer the ability for patients to opt-in through their decision on the prior instructions document, this is rarely seen since the Spanish public trusts the organ donation system and knows that their families will always be consulted so they do not see it as important to record their life decisions. This makes the law appear more consistent and in line with a system of presumed consent, unlike in England [37].

Recommendations for Policy and Practice in England and the United Kingdom

The NHS is built on the ethos that "if it is not written down it did not happen." This has been generally applied to mitigate any potential legal action against staff or the NHS in the future. This is partially why consent documents and protocols tend to reflect ambiguity and risk aversion when compared with Spain which appears more comfortable with the spirit of presumed consent. However this is potentially creating a context where SNODs/SRs are not able to openly and proactively emphasize the benefits of organ donation or feel fully supported to do so with families. We suggest, given a change in legislation that has changed the default for nearly 60 million citizens to support the donation of their organs after they die, unless they say otherwise, that documents and standard operating procedures, particularly in relation to consent, reflect this and are revised with a view to simplification and presumption.

The ODR also lacks legal status. Approximately 10% of families override their relatives' opt-in decision but the same rates are not observed for opt-out decisions. Despite having an ODR, it is not mandatory to follow the organ donation decision on the register. If the ODR was given greater legal status and the decisions in it were used as a basis for the conversation with the family after death (preferably by simplifying the latter to bring it more in line with the Spanish approach to consent after death), this could make it easier for the family to support the potential donor's decision. It may also create a context in which people are more likely to discuss what they want in terms of organ donation. Aligning language, processes and guidelines with the legislation on presumed consent may generate a more positive initial response to organ donation and help address doubts or concerns that are common in these complex end-of-life discussions.

The linking of the ODR to a patient's medical record can also make it easier for healthcare professionals to discuss with the patient if they still stand by their recorded decision should anything life-threatening happen during their admission, similar to a "Do Not Attempt Resuscitation" form.

Although organ donation has expanded in Spain, the underlying principles of legal standing, guidelines and protocols have not changed substantially. Since 2021 the latest NHSBT consent manual has had six updates. The most recent updates are included in **Figure 3** for reference. The consent form has undergone multiple revisions in recent years, with each iteration adding further layers of complexity and processes. This is wasteful and prevents opportunities for innovation that would benefit patients at an individual level. Any revisions to the documents need to be more mindful of the users (e.g., SNODs/SRs and acutely bereaved families) and provide a more personalized and sensitive approach to consent that is aligned with the ambitions of opt-out legislation.

Limitations

Due to resource constraints, we were not able to back-translate very long policy documents from English into Spanish. We relied on software to translate Spanish documents and then verified key concepts and processes with a small number of Spanish experts.

Policy documents alone do not entirely reflect actual practice and there is of course variation in the implementation of processes within each health system. We acknowledge this limitation and mitigated it by engaging with organ donation practitioners in Spain and England as co-authors to complement our documentary analysis with their perceptions, experiences and knowledge. There are also significant differences within and between countries that are not reflected in a discourse analysis focused on consent such as detailed public attitudes to organ donation [37, 38] as well as potential donor characteristics and methods of optimizing organ donor potential which vary widely.

Finally England has a much higher number of live donors than Spain, emphasizing the complexity of organ donation and the fact that there are more ways to increase the number of organs available, reflecting that deceased donor consent rates are not the only measure of a successful organ donation system.

CONCLUSION

The Spanish system has a simpler and more streamlined approach to family consent to organ donation and the documents very proactively encourage donation. If England's ambition is to achieve the consent rates consistently seen in Spain, there may be opportunities to do so by giving greater legal protection and status to the ODR and also by changing the culture from being impartial and risk-averse toward the promotion of organ donation. Significant investment in staff and resources would also be required to match the availability of ICU beds seen in Spain as well as dedicated resources, including specialist sites, which were previously deemed too expensive to invest in. However, there are potentially modifiable issues that appear to work better in Spain such as a shorter and simpler consent process and much more positive language throughout the process, which would improve the experience of staff and acutely bereaved families. In parallel, research is needed (ideally in a controlled context [39]) to understand more about what works, for whom and why in order to maintain the supply of organs to meet the increasing demand.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The main study received favorable ethics opinions from the LSHTM Research Ethics Committee (Ref. 26427 – 20/07/2021) and from the Health Research Authority (HRA) Research Ethics Committee (Ref. 21/NW/0151 – 03/06/2021). Research approvals were received from the HRA and Health and Care Research (HCRW) Wales (IRAS project ID 297313) on 3/06/2021, and from the Research, Innovation and Novel Technologies Advisory Group (RINTAG) (ODT Study no 113; NHSBT Change Control ref: cc/11164) on 11/06/2021. This particular analysis involved comparing publicly accessible protocol and policy documents and engagement with experts by opinion, specific ethical approval for this work was not required. At the time of publication, a revised 2-page consent form has received approval from the HTA and educational research is in progress (Miller) following the implementation of opt-out.

AUTHOR CONTRIBUTIONS

KR - undertook first draft of protocol, translation and analysis of documents, prepared the initial report and contributed to preparing the manuscript for submission. JN – conceptualised the study, reviewed the protocol, contributed to primary analysis, reviewing the draft and preparing the manuscript for submission. LM – conceptualised the study, reviewed the protocol, undertook stakeholder engagement, contributed to primary analysis, reviewing the draft and preparing the manuscript for submission. NM -Reviewed the protocol and contributed to preparing the manuscript for submission. DP-Z – contributed to data analysis, stakeholder engagement and reviewed the manuscript for submission. CM – contributed to data analysis, stakeholder engagement, contextual understanding and reviewed the manuscript for submission. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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BRIEF RESEARCH REPORT
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Time Course of High-Energy Phosphate Depletion During Cold Storage of Human Heart Grafts Using the Celsior Solution

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The aim of this study was to provide insight into high-energy phosphate compound concentration dynamics under realistic clinical cold-storage conditions using the Celsior solution in seven heart grafts discarded from transplantation. The hearts of seven local donors (three males, four females, age 37 ± 17 years, height 175 ± 5 cm, weight 75 ± 9 kg) initially considered for transplantation and eventually discarded were submitted to a Magnetic Resonance Spectroscopy observation in a clinical Magnetic Resonance Imaging scanner over at least 9 h. The grafts remained in their sterile container at 4°C during the entire examination. Hence, Phosphocreatine (PCr), adenosine triphosphate (ATP), inorganic phosphate (Pi) and intracellular pH were recorded non-destructively at a 30-minute interval. With the ischemic time Ti, the concentration ratios decreased at PCr/ATP = 1.68–0.0028·Tis, Pi/ATP = 1.38 + 0.0029·Tis, and intracellular pH at 7.43–0.0012·Tis. ATP concentration remained stable for at least 9 h and did not decrease as long as phosphocreatine was detectable. Acidosis remained moderate. In addition to the standard parameters assessed at the time of retrieval, Magnetic Resonance Spectroscopy can provide an assesment of the metabolic status of heart grafts before transplantation. These results show how HEPC metabolites deplete during cold storage. Although many parameters determine graft quality during cold storage, the dynamics of HEPC and intracellular pH may be helpful in the development of strategies aiming at extending the ischemic time.

Keywords: magnetic resonance spectroscopy, phosphorus, heart transplant, high-energy metabolism, cold storage

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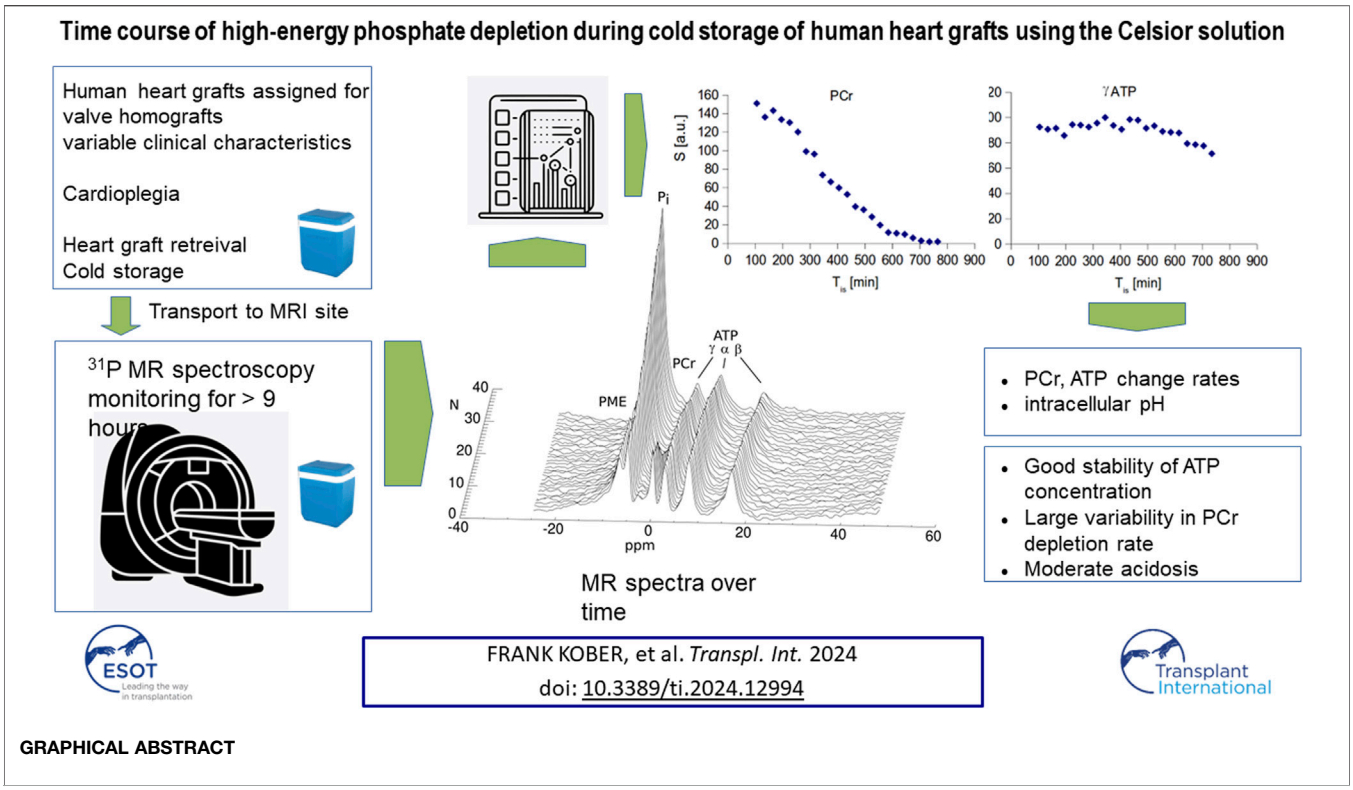
INTRODUCTION

Evaluation of heart grafts using non-invasive techniques before transplantation into the recipient appears as a useful addition to the current standard clinical practice, in which no objective examination of the graft is performed immediately before transplantation. A large variety of destructive and non-destructive biomarkers have been proposed in both clinical and preclinical settings [1]. Despite emerging machine-perfusion beating-heart storage devices [2–4], cardioplegic arrest followed by cold storage of the graft currently remains the most widely used method. In both warm and cold storage situations, an objective evaluation before transplantation could contribute to increasing transplant safety on the one hand and to widening the donor pool on the other. One



GRAPHICAL ABSTRACT

important indicator of graft quality is given by high-energy phosphate compound (HEPC) concentrations [5, 6] that decrease in the absence of nutriment and oxygen supply via perfusion, and it is one purpose of preservation solutions to prevent their rapid depletion. These metabolite concentrations can be assessed in a completely non-destructive way using phosphorus-31 Magnetic Resonance Spectroscopy (^{31}P MRS). Using MRS, the quality of graft preservation with different cardioplegic solutions has been assessed in animal hearts [3, 7–10], and metabolite preservation before graft implantation has been analyzed in human hearts [5, 6] during cold storage and, more recently, during warm storage under machine perfusion [4]. However, little is known about the influence of storage time on the metabolic preservation quality of human heart grafts in a clinical context, where an estimation of metabolic depletion rates in human grafts could help strengthen safety. The purpose of this work was to assess the time course of HEPC alterations during cold storage of human heart grafts. This was possible using hearts that were discarded from transplantation based on clinical donor criteria but that underwent the same retrieval and cold storage procedure as transplanted grafts for the purpose of this study.

MATERIALS AND METHODS

No rupture of sterility or low temperature was caused to the grafts by this non-destructive assessment. The study was approved by the University Hospital's ethics committee "Comité consultatif de protection des personnes dans la recherche biomédicale—Marseille 2" (Authorization #99/16) and conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The donors' next of kin gave their written informed consent to the heart graft retrieval. This retrospective dynamic data analysis was carried out in 2024 on Magnetic Resonance data acquired from 1999 to 2001. According to current rules confirmed by the French Agence de Biomédecine, this analysis is therefore exempted from additional specific ethical approvals.

Donors

The hearts of seven local donors (three males, four females, age 37 ± 17 years, height 175 ± 5 cm, weight 75 ± 9 kg) initially considered for transplantation were clinically evaluated before retrieval. Based on echocardiography data (Left-Ventricular Ejection Fraction < 50%) and/or the level of administered inotropes (Adrenalin > 2 mg/h) administered to the donor, all grafts used in this study were eventually qualified unsuitable for heart transplantation, but they were retrieved for valve graft excision.

Retrieval and Transport

All hearts were arrested and preserved with Celsior® [11] cardioplegic solution at 4°C. After excision, the grafts were placed in sterile plastic bags filled with Celsior® solution. The bags were sealed and inserted in plastic jars containing physiologic NaCl solution. The jars were put on ice in an

insulated container and transported to the MRI/MRS facility. The average ischemic time at arrival on the MR site was 124 ± 71 min.

MR Protocol

The ice container was positioned on a commercially available $^{31}P/^{1}H$ surface radiofrequency coil in the magnet of a Siemens 1.5 T clinical MR system. This way, sterility and stable temperature conditions could be ensured at any time of the examination. Automated localized map shimming was performed. A stack of multi-slice T1-weighted proton MR images was acquired to determine the position of the graft within the container. A single-pulse free induction decay spectroscopy sequence (TR = 10 s, 32 averages, duration 5 min) was used to obtain global full-relaxed ^{31}P-MR spectra at 25.9 MHz. The acquisition was repeated every 30 min as long as the scanner was available. Since the majority of grafts arrived at week nights, the time was limited to 9 h, except for one examination that could be left running for 13 h. The time between cardioplegic arrest and the beginning of the MR examination was recorded.

Data Processing

All spectra were quantified without user interaction by the AMARES time-domain fitting routine part of the MRUI software package [12]. The signal ratios of phosphocreatine (PCr)/adenosine triphosphate (ATP), PCr/P_i, P_i/ATP and phosphomonoesters (PME)/ATP were calculated. The γATP resonance was used to represent ATP. The intracellular pH value (pH_i) was obtained by Kost's formula [13] using the chemical shift difference between the PCr and the P_i resonances. Linear regression was used as a simple approach to characterize dynamic alterations of metabolite signals (PCr, P_i) and pH_i over time. The slopes obtained with linear regression were expressed as percent decrease with respect to the extrapolated signal value at cardiac arrest. No absolute quantification of metabolite levels could be performed, since the position, size and shape of the grafts varied across the exams.

RESULTS

An example stack plot of spectra from the graft observed during 13 h as a function of time is displayed in **Figure 1**. **Figure 2** shows the signal amplitudes of PCr, P_i, γATP and phosphomonoesters (PME) as a function of time after cardioplegic arrest for all examined grafts. The graphs account for the variations in arrival time on site. For visualization, three data points from misfitted spectral resonances of graft number 2 were removed. **Figure 2** also shows the intracellular pH as long as PCr was measurable.

In the seven grafts, PCr signal, normalized to that at the time of retrieval, decreased by 10.3% ± 1.9%/hour and P_i increased by 12.6% ± 5.0%/hour. From the graft followed for 13 h (shown in **Figure 1**) it can further be seen that ATP levels were stable until PCr was depleted, and PCr depletion did not occur in the other observed grafts, i.e., before 9 h of storage. PCr decrease and P_i increase were well represented by the linear regression over 9 h. The concentration ratios PCr/ATP and Pi/ATP can, therefore, be expressed as a linear function of the ischemic time T_{is} [min]: PCr/ATP = $1.68-0.0028\cdot T_{is}$, Pi/ATP = $1.38 + 0.0029\cdot T_{is}$. Intracellular pH obtained from the chemical spectral shift of Pi also showed a shallow linear decrease over time: pH = $7.43–0.0012\cdot T_{is}$, indicating good stability of the acidic level.

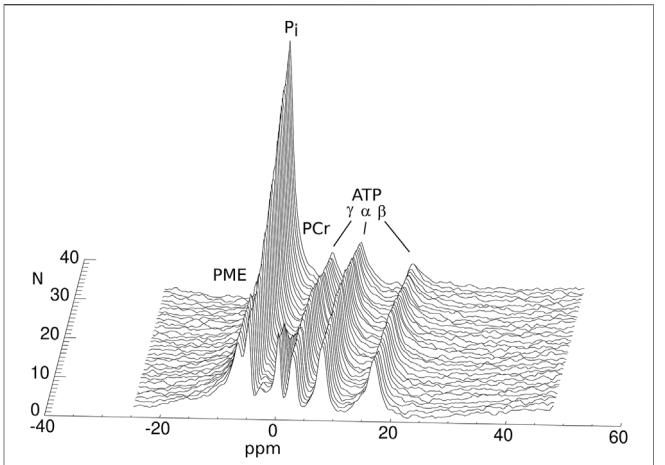


FIGURE 1 | Phosphorus MRS spectra of a human heart graft discarded from transplantation during cold storage. The spectra were acquired with a 30-minute interval between them (N: number of the acquisition). The annotated resonances in each spectrum represent the concentrations of the different HEPC.

DISCUSSION

We have measured the time course of changes in HEPC metabolite concentrations in seven human heart grafts during cold storage. HEPC metabolites are known contributors to the graft quality during transplantation. Depletion of high energy phosphates during ischemia is linked with harmful injury in the ischemic myocardium [6], and levels of high energy phosphate have been correlated to the recovery of function after transplantation both in animal models and human studies [6, 14–16]. In a more recent study by Föll et al. [17], the ischemic storage time was also shown to correlate with regional wall motion after transplantation.

Signal variations show that on a timescale typical for cold storage duration in heart transplantation (maximum 4 h), HEPC metabolites in human heart grafts are well preserved. Although PCr was decreasing rapidly, the ATP levels in all six example heart grafts were stable for at least 9 h following cardioplegic arrest and storage with Celsior. This indicates that in current clinical practice using these conditions of preservation, the level of ATP is well preserved over a long cold storage duration. ATP is indeed related to numerous cell structure mechanisms and to contractility, and its depletion associated with irreversible loss of precursors. These results also show a moderate acidosis of the grafts at pH 6.8 after 9 h of ischemia. Earlier studies carried out in animal hearts during ischemia with cardioplegia have shown that the intracellular pH is dependent on the pH and on the buffering capacity of the preservation solution [10]. While the heart is able

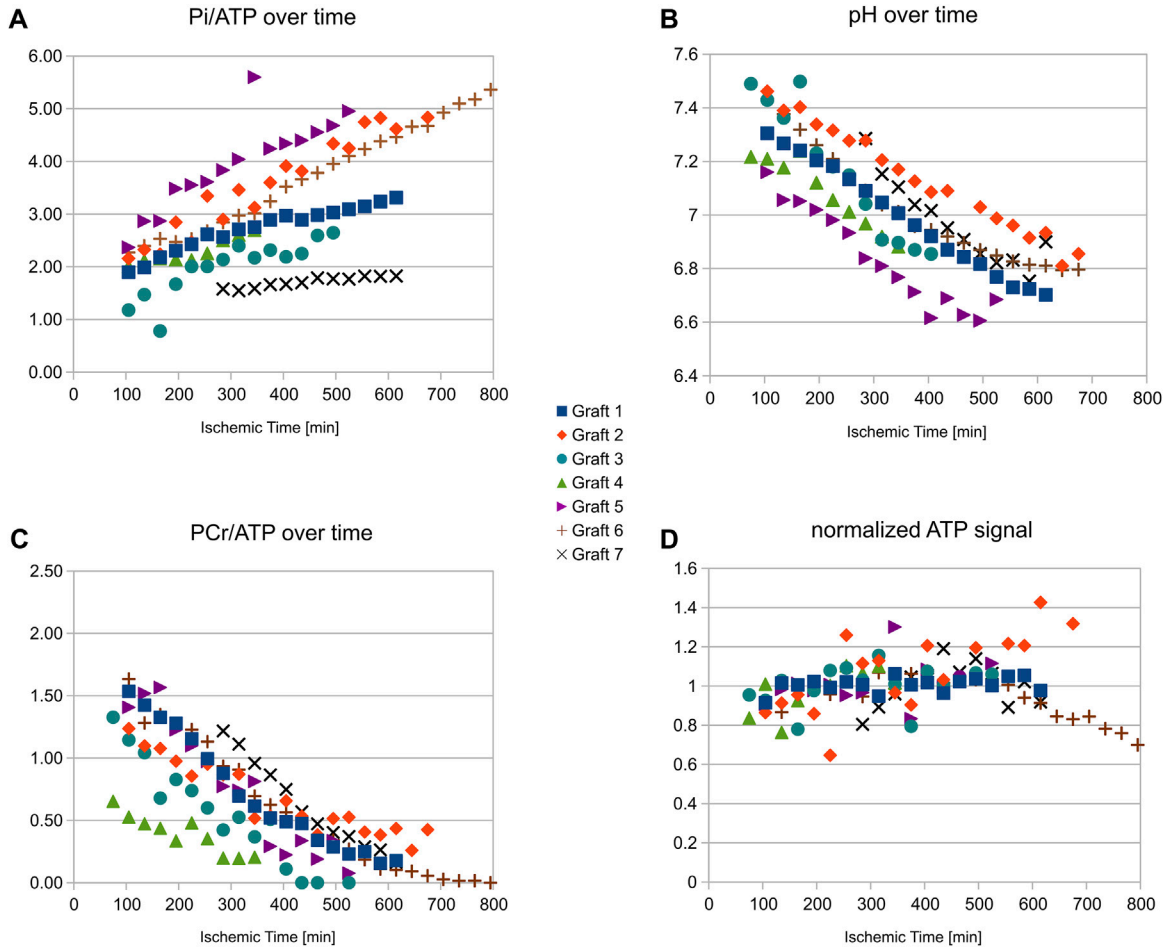


FIGURE 2 | Resonance amplitudes for different HEPC and intracellular pH versus ischemic time during cold storage for all grafts studied. Note that the grafts arrived with different transport times on site. Despite a relatively large variability in the concentration ratios (especially for P_i **(A)**), the measurements show good stability of the ATP **(D)** concentration as long as PCr **(C)** was not fully depleted (always more than 9 h in this study) as well as moderate acidosis **(B)**. The stronger concentration variability in some grafts was caused by variable distances of the grafts in their container from the MR radiofrequency antenna.

to recover from mild acidosis, severe acidosis would induce cell necrosis.

We have used ^{31}P Magnetic Resonance Spectroscopy for this analysis. This tool proved to be particularly useful, since the entire exam could be accomplished with preserved sterility and temperature with *in situ* determination of metabolites and intracellular pH. The dynamic measurement allowed us to derive change rates for the major compounds ATP, PCr and P_i and for the intracellular pH. These rates can be considered as characteristic for the particular group of grafts assessed here. As a difference with earlier work by our group and others [5, 6], all grafts studied here were discarded from transplantation based on their clinical score. This leaves some uncertainty on the initial metabolic status of the grafts before retrieval, since grafts with lower clinical scores were shown earlier to have lower PCr/P_i concentration ratios [5]. This likely explains the relatively low initial PCr/ATP and high Pi/ATP ratios found even when linearly extrapolating them back to the moment of retrieval.

The influence of ischemic time on HEPC metabolism in heart grafts has been evaluated earlier by van Dobbenburgh and coworkers [6] in a study correlating functional performance and metabolic status before transplantation in 25 heart grafts arrested with St Thomas cardioplegic solution. One has to keep in mind that van Dobbenburgh et al. assessed grafts that were actually transplanted at relatively short ischemic times (<2 h), allowing them to acquire only one time point per graft. The authors also reported a correlation of metabolism and the variable ischemic time (T_{is} [min]) of each graft, although only one time point per graft was available. Their constructed decreases over time were PCr/ATP = 1.31–0.0039·T_{is}, Pi/ATP = 0.26 + 0.0064·T_{is} and pH = 7.66-0.0040 ·T_{is} and can be compared with our regressions: PCr/ATP = 1.68–0.0028·T_{is}, Pi/ATP = 1.38 + 0.0029·T_{is}, and pH = 7.43–0.0012·T_{is}. During our nine-hour observation period, PCr, Pi and pH, therefore, decreased slower than in the <2 h period observed in van Dobbenburgh's mult-organ study. Initial Pi, as calculated by linear extrapolation to the moment of explantation, was, however, found much higher in our study. These differences may be related to the low clinical scores

reported for the grafts used here. In our study, time courses were obtained from each graft individually over time periods of 9 h. The change rates may also vary as a function of the storage solution (here: Celsior), which was shown earlier to have an influence on metabolite levels during reperfusion in rat hearts [14].

Similarly to observations reported by van Dobbenburgh, we observed a relatively large variation in both PCr/ATP and, especially, Pi/ATP ratios of the donor hearts as well their change rates. These differences may be caused by the condition of the donor, the use of pharmacological therapy, and the quality of cardioplegic arrest and hypothermic preservation before arrival on site. The low number of grafts available for this study, however, did not allow us to evaluate statistical correlations with these parameters.

LIMITATIONS

For obvious reasons, the number of grafts at our disposal was limited by the availability, since only a single center contributed to this study. The standard deviation of metabolite change rates was therefore relatively high (in the 20% range). We report these results to give an estimate of the order of magnitude of metabolic changes during cold storage. Further observations will be needed to strengthen these results and to generalize the conclusions with sufficient confidence.

CONCLUSION

We have non-destructively analyzed the long-term behavior of high-energy phosphate compounds in human heart grafts. Phosphorus MRS proved to be a useful tool for such a non-destructive analysis. Although many parameters affect graft quality during cold storage, the dynamics of HEPC and intracellular pH may be helpful in the development of strategies aiming at extending the ischemic time. Beyond cold storage, MRS may also provide interesting additional information for evaluating grafts during or after machine-perfused graft storage in comparison.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study was approved by the University Hospital's ethics committee "Comité consultatif de protection des personnes dans la recherche biomédicale—Marseille 2" (Authorization #99/16) and conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The donors' next of kin gave their written informed consent to the heart graft retrieval. This retrospective dynamic data analysis was carried out in 2024 on Magnetic Resonance data acquired from 1999 to 2001. According to current rules confirmed by the French Agence de Biomédecine, this analysis is therefore exempted from additional specific ethical approvals.

AUTHOR CONTRIBUTIONS

Authors TC and AR enabled the insertion of the MRI observation in the transplantation process, and Authors FK and YL wrote dedicated spectroscopy post-processing software. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

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BRIEF RESEARCH REPORT
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Is Night Surgery a Nightmare for Lung Transplantation?

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Night work is frequently associated with sleep deprivation and is associated with greater surgical and medical complications. Lung transplantation (LT) is carried out both at night and during the day and involves many medical healthcare workers. The goal of the study was to compare morbidity and mortality between LT recipients according to LT operative time. We performed a retrospective, observational, single-center study. When the procedure started between 6 AM and 6 PM, the patient was allocated to the Daytime group. If the procedure started between 6 PM and 6 AM, the patient was allocated to the Nighttime group. Between January 2015 and December 2020, 253 patients were included. A total of 168 (66%) patients were classified into the Day group, and 85 (34%) patients were classified into the Night group. Lung Donors' general characteristics were similar between the groups. The 90-day and one-year mortality rates were similar between the groups (90-days: n = 13 (15%) vs. n = 26 (15%), $p = 0.970$; 1 year: n = 18 (21%) vs. n = 42 (25%), $p = 0.499$). Daytime LT was associated with more one-year airway dehiscence (n = 36 (21%) vs. n = 6 (7.1%), $p = 0.004$). In conclusion, among patients who underwent LT, there was no significant association between operative time and survival.

Keywords: mortality, lung transplantation, outcome, morbidity, operative time

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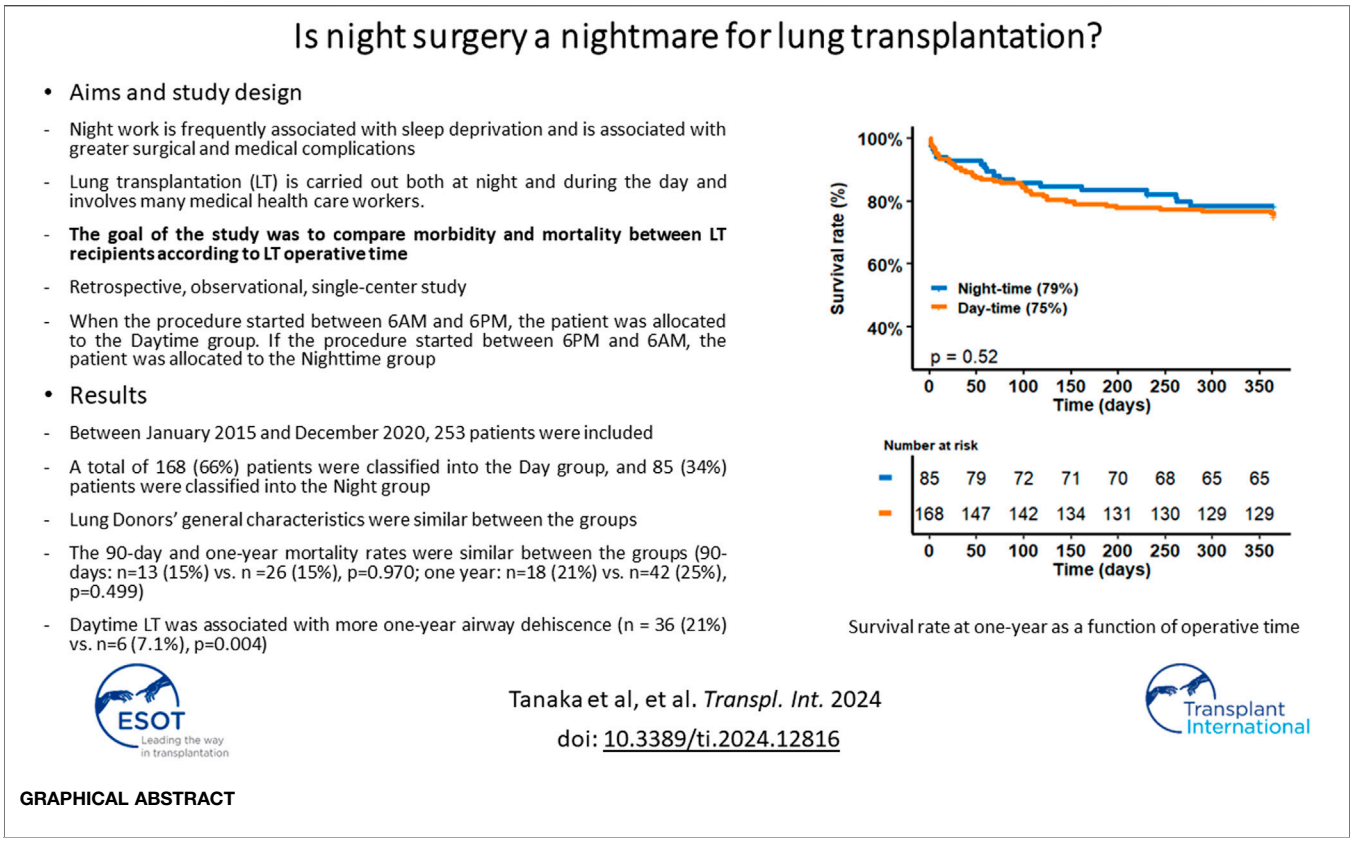
INTRODUCTION

Sleep deprivation is a major public health issue that concerns the entire population but also healthcare professionals. Sleep deprivation is known to have major consequences for attention and medical reasoning and can lead to serious medical errors [1–3]. Night work is frequently associated with sleep deprivation and circadian disruption and is also associated with greater surgical and medical complications [4–7].

For more than 30 years, lung transplantation (LT) procedures have been performed worldwide; these procedures involve both night and day care and involve many medical and nonmedical healthcare workers, especially surgeons, anesthesiologists and intensivists [8, 9]. A large retrospective study in North America



GRAPHICAL ABSTRACT

including more than 27,000 lung and heart transplant recipients did not reveal any difference in survival according to the procedure schedule (night, 7 PM-7 AM versus day, 7 AM-7 PM) [10]. Interestingly, among lung transplant recipients, there was a slightly greater rate of airway dehiscence associated with nighttime transplants.

Given that transplantation organizational procedures and caregivers working time legislation differ from one country to another [11, 12], the goal of the present study was to compare lung transplant characteristics and outcomes performed in a French LT center according to the operative time.

MATERIALS AND METHODS

Study Population

All consecutive patients who underwent LT at Bichat-Claude Bernard Hospital, Paris, France, from January 2015 to December 2020 were retrospectively included in this observational, single-center analysis. The data were collected prospectively. The Paris North Hospital Institutional Review Board (Paris Diderot University, Assistance Publique Hôpitaux de Paris No. 0007477) reviewed and approved the study.

OBJECTIVES

- The main objective of this study was to compare one-year mortality between LT recipients according to LT operative time.
- The secondary objective was to assess the associations between operative time and donor characteristics, recipient general characteristics, perioperative data, postoperative outcomes and complications.

Operative Time

Total operative time was defined as the complete time from arrival to discharge from the operating room, including the time of anesthetic management and surgical procedure.

Patients were stratified by operative time. When the procedure started between 6 AM and 6 PM, the patient was allocated to the daytime group. If the procedure started between 6 PM and 6 AM, the patient was allocated to the nighttime group.

Perioperative Management

Perioperative care was standardized for all patients according to current practices [13–15]. After the surgical procedure, all patients were admitted to our surgical intensive care unit (ICU). Postoperative ECMO was required in case of PGD3, severe pulmonary arterial hypertension, perioperative cardiac dysfunction and in the case of ARDS in the context of early pneumonia. ECMO was also required if intraoperative bleeding and transfusion have been consistent and could lead to pulmonary edema and ARDS.

The LT team was composed of a senior anaesthetist and a resident, and the surgical staff of a senior surgeon, a junior surgeon and a resident. There were five senior surgeons with at least 5 years' experience as junior surgeons.

TABLE 1 | General characteristics of the patients in the whole population and in the different groups.

General characteristics	Overall population (n = 253)	Night (n = 85)	Day (n = 168)	*p*
Age, years, median [IQR]	57 [50–62]	57 [51–63]	57 [50–62]	0.525
Male sex, *n* (%)	162 (64)	55 (65)	107 (64)	0.874
BMI (kg/m^2), median [IQR]	24.0 [20.0–27.0]	24.6 [20.0–27.0]	24.0 [20.0–27.0]	0.699
Diabetes mellitus, n (%)	26 (10)	11 (13)	15 (8.9)	0.321
Chronic coronary disease n (%)	10 (4.0)	3 (3.5)	7 (4.2)	>0.999
Mean pulmonary artery pressure (mmHg), median [IQR]	25 [20–30]	24 [20–28]	26 [21–30]	0.146
Need of preoperative ECMO, n (%)	19 (7.5)	9 (11)	10 (6.0)	0.186
Diagnosis leading to LT				
COPD/emphysema, n (%)	91 (36)	38 (45)	53 (32)	0.039
Pulmonary fibrosis, n (%)	122 (48)	34 (40)	88 (52)	0.063
Other pathologies, n (%)	41 (16)	13 (15)	28 (17)	0.780
Double LT, n (%)	173 (68)	57 (67)	116 (69)	0.748

Continuous variables are expressed as medians and interquartile ranges (IQRs) and were compared using the Mann–Whitney U test. Categorical variables are expressed as n (%) and were compared with Fisher's exact test. BMI, body mass index; COPD, chronic obstructive pulmonary disease; LT, lung transplantation.

Data Collection

- General demographic data of the donors, including age, sex, cigarette use, best PaO_2/FiO ratio and length of mechanical ventilation, were collected.
- Demographic characteristics, underlying disease, and medication use during the pretransplant assessment period were prospectively recorded.
- Perioperative data, including length of total operative time, duration of surgery, procedure start time (i.e., arrival in the operating room), need for transfusion and need for ECMO support, were collected.
- Postoperative complications and variables during ICU hospitalization following LT were also recorded, such as postoperative ECMO support, primary graft dysfunction (PGD) defined according to the ISHLT revised definition [16], acute kidney injury, need for renal replacement therapy, episode of pneumonia during the ICU stay, duration of vasopressor agent administration, duration of mechanical ventilation and length of stay in the ICU. Airway dehiscence, acute cellular and humoral rejection during the first postoperative year, and mortality at 90 days and 1 year were also prospectively collected.

Statistical Analysis

Continuous variables are expressed as medians with interquartile ranges (IQRs) and were compared with the Mann–Whitney *U* test. Categorical variables are expressed as counts and percentages and were compared with Fisher's exact test or the chi-square test, as appropriate. Time-to-event analyses were estimated with Kaplan-Meier analyses, and survival differences were analyzed using a log rank test. Multivariate associations were computed with binary logistic regression models. For all the models, variables with nominal 2-tailed *p* values less than 0.1 were entered into the multivariate model, except for variables with obvious collinearity. All the statistical analyses were performed using R statistical software[1]. And RStudio (version 1.3.1056, © 2009–2020 RStudio, PBC). A p-value <0.05 was considered to indicate statistical significance.

RESULTS

Population

Between January 2015 and December 2020, 269 patients underwent LT at our institution. Patients who underwent liver and lung transplantation were excluded from the analysis (n = 3). Thirteen patients were excluded from the analysis because perioperative data were incomplete. A total of 253 patients were ultimately included in this study.

A total of 168 (66%) patients were classified into the Day group, and 85 (34%) patients were classified into the Night group.

The median LT procedure time was 440 [390, 530] minutes, and the median surgical time was 320 [270, 390] minutes. The median procedure start times were 10 am [8 am–12 am] and 2 am [10 pm-4 am] for patients in the day and night groups, respectively.

The general characteristics of the patients in the whole population and according to the LT schedule are presented in **Table 1**. Interestingly, LT in COPD/emphysema patients were statistically more often performed at night (Nighttime, n = 38 (45%) vs. Daytime, n = 53 (32%), p = 0.039).

Donors' Characteristics

The donors' characteristics were similar between the two groups and are described in **Supplementary Table S1**.

Pre- and Postoperative Variables

The length of total operative time was statistically longer during the day than at night (7h40 [6h40-8h50] vs. 6h50 [5h50-8h30], p = 0.002).

The distribution of surgeries was fairly homogeneous between surgeons. With the exception of one surgeon, all surgeons performed the majority of their grafts during the day, and their proportion of daytime grafts was identical to their proportion of night-time grafts (**Supplementary Figure S2**).

[1]http://www.r-project.org/

TABLE 2 | Pre- and postoperative variables.

Per/postoperative and outcome variables	Overall population (n = 253)	Night (n = 85)	Day (n = 168)	*p*
length of total operative time, hours, median [IQR]	7h20 [6h30-8h50]	6h50 [5h50-8h30]	7h40 [6h40-8h50]	0.002
Length of surgery, hours, median [IQR]	5h20 [4h30-6h30]	5h12 [4h20-6h30]	5h30 [4h30-6h30]	0.414
Peroperative transfusion ≥ 2RBC, n (%)	122 (48)	43 (51)	79 (47)	0.592
Need of peroperative ECMO, n (%)	178 (70)	57 (67)	121 (72)	0.414
SAPSII score on ICU admission, median [IQR]	43 [38, 52]	43 [40, 48]	45 [38, 54]	0.491
SOFA on ICU admission, median [IQR]	7.0 [6.0–9.0]	7.0 [5.0–9.0]	7.0 [6.0–9.0]	0.170
Duration of vasopressor agent administration, days, median [IQR]	2.0 [1.0–4.0]	2.0 [1.0–4.0]	2.0 [1.0–4.0]	0.702
Need of ECMO support during ICU stay, n (%)	73 (29)	24 (28)	49 (29)	0.877
Stage III PGD, n (%)	48 (19)	16 (19)	32 (19)	0.966
Acute kidney injury, KDIGO stage 3, n (%)	36 (14)	11 (13)	25 (15)	0.677
Renal replacement therapy, n (%)	29 (11)	8 (9.4)	21 (12)	0.466
Postoperative pneumonia, n (%)	219 (87)	75 (88)	144 (86)	0.579
Duration of MV, days, median [IQR]	3 [1, 20]	3 [1, 11]	4 [1, 21]	0.405
ICU length of stay, median [IQR]	17 [10–33]	16 [11–28]	17 [10–33]	0.618
Acute humoral rejection during the first year, n (%)	44 (18)	15 (18)	29 (18)	0.957
Acute cellular rejection during the first year, n (%)	49 (20)	18 (22)	31 (19)	0.653
Airway dehiscence during the first year, n (%)	42 (17)	6 (7.1)	36 (21)	0.004
90-days mortality, n (%)	39 (15)	13 (15)	26 (15)	0.970
One-year mortality, n (%)	60 (24)	18 (21)	42 (25)	0.499

Continuous variables are expressed as medians and interquartile ranges (IQRs) and were compared using the Mann–Whitney U test. Categorical variables are expressed as n (%) and were compared with Fisher's exact test. ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; KDIGO, kidney disease-improving global outcomes; MV, mechanical ventilation; PGD, primary graft dysfunction; RBC, red blood cell; SAPS-II, Simplified Acute Physiology Score II; SOFA, sepsis-related organ failure assessment; ICU, intensive care unit.

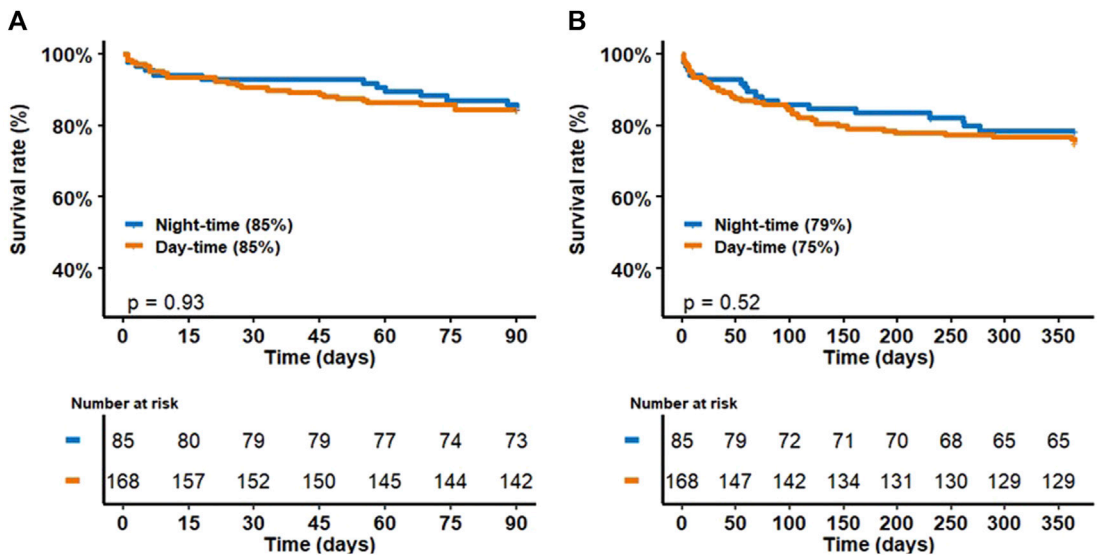


FIGURE 1 | Mortality at 90 days **(A)** and 1 year **(B)** as a function of operative time.

Interestingly, airway dehiscence was more frequently associated with daytime transplantations (n = 36 (21%) vs. n = 6 (7.1%), p = 0.004). The pre- and postoperative variables are expressed in **Table 2**.

Mortality at 90 Days and One Year According to Day or Night LT

There was no difference in mortality at 90 days and 1 year between patients transplanted during the day and those transplanted at night. **Figure 1** shows mortality at 90 days and 1 year as a function of operative time.

Airway Dehiscence

Given that airway dehiscence is more frequently associated with daytime transplantations, we explored the factors associated with the occurrence of airway dehiscence in our center. These data are presented in **Table 3**.

DISCUSSION

We showed in this study that LT schedules had no influence on patient mortality at 90 days or 1 year. Nevertheless, patients who

TABLE 3 | Relationships between general characteristics, perioperative and postoperative variables and airway dehiscence during the first year after LT.

Variables	Univariate analysis				Multivariate analysis		
	Overall population (n = 253)	No airway dehiscence (n = 211)	Airway dehiscence (n = 42)	*p*-value	Odd-ratio	95% CI	*p*-value
Age, years, median [IQR]	57 [50, 62]	57 [50, 62]	58 [52, 62]	0.670			
Male sex, *n* (%)	162 (64)	129 (61)	33 (79)	0.032	2.03	[0.90–4.97]	0.102
BMI (kg/m^2), median [IQR]	24.0 [20.0, 27.0]	24.0 [20.0, 27.0]	25.0 [23.0, 28.0]	0.066	0.14	[0.01–0.76]	0.067
Diabetes mellitus, n (%)	26 (10)	22 (10)	4 (9.5)	>0.999			
Chronic coronary disease, n (%)	10 (4.0)	9 (4.3)	1 (2.4)	>0.999			
Mean pulmonary artery pressure (mmHg), median [IQR]	25 [20, 30]	25 [21, 30]	25 [19, 35]	0.514			
Need of preoperative ECMO, n (%)	19 (7.5)	16 (7.6)	3 (7.1)	>0.999			
COPD/emphysema, n (%)	91 (36)	75 (36)	16 (38)	0.753			
Pulmonary fibrosis, n (%)	122 (48)	99 (47)	23 (55)	0.353			
Double LT, n (%)	173 (68)	142 (67)	31 (74)	0.407			
Need of peroperative ECMO, n (%)	178 (70)	145 (69)	33 (79)	0.202			
Peroperative transfusion ≥2 RBC, n (%)	122 (48)	103 (49)	19 (45)	0.672			
Daytime LT, n (%)	168 (66)	132 (62)	36 (86)	0.004	4.65	[1.77–15.13]	**0.004**
length of total operative time, hours, median [IQR]	7.20 [6h30, 8h50]	7h30 [6h30, 8h50]	7h05 [6h22, 8h10]	0.393			
SOFA on ICU admission, median [IQR]	7.0 [6.0, 9.0]	7.0 [6.0, 9.0]	7.5 [6.0, 9.75]	0.278			
Duration of vasopressor agent administration, days, median [IQR]	2.0 [1.0, 4.0]	1.0 [1.0, 3.0]	3.0 [2.0, 9.5]	<0.001	1.10	[1.03–1.19]	**0.007**
Need of ECMO support during ICU stay, n (%)	73 (29)	56 (27)	17 (40)	0.069			
Stage III PGD, n (%)	48 (19)	33 (16)	15 (36)	0.002			
Acute kidney injury, KDIGO stage 3, n (%)	36 (14)	29 (14)	7 (17)	0.621			

Continuous variables are expressed as medians and interquartile ranges (IQRs) and were compared using the Mann–Whitney U test. Categorical variables are expressed as n (%) and were compared with Fisher's exact test. BMI, body mass index; ECMO, extracorporeal membrane oxygenation; KDIGO, kidney disease improving global outcomes; MV, mechanical ventilation; PGD, primary graft dysfunction; LT, lung transplantation; SOFA, sepsis-related organ failure assessment; ICU, intensive care unit. The bold values are statistically significant.

underwent transplantation during the day had a greater incidence of airway dehiscence. According to our multivariate analysis, day-time LT and prolonged administration of vasopressors were associated with increased airway dehiscence.

Given that quality of life at work for caregivers is a crucial issue, the aim of this study was to complete the analysis and determine whether night work had an influence on patient outcomes in our center. Unexpectedly, we found no difference in mortality between patients who underwent surgery during the day and those who underwent surgery at night. George et al. demonstrated that night-time transplantation had no influence on prognosis in a large retrospective study [10]. However, these North American data are difficult to translate to France, where, for example, the working hours of anesthetists and surgeons are limited to 24 h in a row, compared with 12 h in most Anglo-Saxon countries. Despite these differences, our study showed no difference. As the outcome of patients after LT is associated with a wide variety of factors [9, 17, 18], the variable operating time, which is a determinant of caregiver fatigue and concentration, did not seem to play a predominant role in our study. Nevertheless, to our knowledge, no high-powered study has taken into account many factors, including donor data, recipient data, preoperative variables and both early and late postoperative variables. The absence of any difference in mortality according to the time of the procedure was confirmed in a meta-analysis grouping together different types of transplantation, but given the heterogeneity of the transplantation and the different definitions used to define night or day work, the authors concluded that it was impossible to reach an objective conclusion [19].

Interestingly, our study pinpointed an important complication for patient outcome, airway dehiscence. Even though the associated factors are poorly described and have a rather complex pathophysiology, our study seems to show that, in our center, the occurrence of airway dehiscence seems more important during transplants occurring during the day. We propose several hypotheses, the first of which is a longer procedure duration with a prolonged duration of vasopressor use during day-time surgery. Second, the trend toward more pulmonary fibrosis occurring during the day may also be an explanation [20]. Nevertheless, univariate and multivariate analyses did not reveal this factor in the occurrence of airway dehiscence. Finally, the increased presence of residents and junior surgeons performing anastomoses during the day may be an explanation that deserves further analysis.

It is very reassuring to note that there is no difference in mortality between patients transplanted at night and during the day, which highlights the unfailing professionalism of the transplant team. Nevertheless, although little studied in the transplant context, burnout among professionals is a reality that can directly affect both the physical and mental health of the professional [21–23]. At a time when there is a shortage of healthcare professionals and growing awareness of the importance of quality of life at work, it seems important to limit night work and try to transplant more during the day.

This would also make it possible to better plan the relief of surgeons and anaesthetists, and to concentrate caregivers of all transplant staff during the day for greater efficiency and to reduce burnout. To achieve this, there are a certain number of ways of improvement to recommend: In the case of donors after brain death, it seems reasonable to optimize organ removal schedules as much as possible so as to be able to transplant during the day. This strategy is increasingly used in transplant centers. *Ex-vivo* lung perfusion (EVLP) is a strategy that is also increasingly used worldwide to both increase the graft pool and optimize grafts prior to transplantation [24]. Although this strategy still needs a great deal of evaluation, EVLP could reasonably be used to optimize grafts at night so that they can be transplanted during the day. Centralized organ recovery and reconditioning centers are already implemented in some countries, and reducing the number of nighttime transplants is already one of the objectives of these structures [25]. Even if it sounds seductive, it deserves further investigation.

Our work has several limitations. First, it was a single-center study with a small sample size. Second, the choice of schedules (6–6) was chosen purely locally. Considering the pulmonary artery unclamping schedule could be a more rational choice. Third, a crude analysis of schedules without stratifying according to surgeon or anesthesiologist may lead to bias. However, the analysis based on the operator does not seem ethical. Fourth, more LT in COPD/emphysema patients were performed at nighttime, which is a source of bias because patients with fibrosis are often more fragile, and surgery in these specific patients is classically more difficult, which can have repercussions on the post-operative period. In our cohort, the longer operative time during the day and the higher proportion of dehiscence may potentially be secondary to this selection bias. Unfortunately, we do not have a rational explanation to highlight these population differences between daytime and nighttime. Finally, a longer-term analysis (i.e., 5 years) could be more relevant.

Our work has several strengths. To our knowledge, this work is the first to integrate the anesthetic time before the surgical incision rather than just the surgical duration. LTs involve many stakeholders, and anesthetic care is essential and can greatly interfere with patient outcomes. The monocentric character is obviously a limitation but can also be compared to a strength because it allows local constraints to be understood in a precise manner, particularly on the choice of schedules (6–6) with considerations of change of time for nurses or even availability of operating theaters.

In conclusion, we did not observe any difference in mortality between patients who underwent transplantation at our center at night and those who underwent transplantation during the day. A French multicenter analysis seems necessary in the future. Furthermore, the criteria for unclamping could be rational in this multicenter analysis. Finally, a measure of stress or fatigue of surgeons and anesthetists could also be integrated into the analysis. A more detailed analysis of airway dehiscence seems necessary.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving humans were approved by the Paris Diderot University, Assistance Publique Hôpitaux de Paris No. 0007477. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because the study was retrospective.

AUTHOR CONTRIBUTIONS

ST, CdT, ED, EA, AT-D and PM contributed to study concept and design. ST, CdT performed statistical analysis. ST, CdT, AT-D, ED, C-ZT, BL-J, SJ-B, YC, HM, PiM, EA and PhM were involved in data analysis and interpretation. ST, CdT, ED, AT-D, BL-J, AH, SJ-B, SB, C-ZT, MS, YC, HM, PiM, AG, EA and PhM performed critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12816/full#supplementary-material

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LETTER TO THE EDITOR
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In Vitro and *In Vivo* Neutralizing Efficacy of Monoclonal Antibodies Against Sars-Cov-2 Variants in Kidney Transplant Recipients

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Dear Editors,

Solid organ transplant recipients continue to face a heightened risk of severe COVID-19, despite a decrease in virus virulence since the emergence of Omicron [1]. Managing preventive and therapeutic strategies in this population poses challenges due to their reduced vaccine response, potential drug-drug interactions with nirmatrelvir-ritonavir, and the ability of variants to escape neutralizing monoclonal antibodies (mAbs) [2, 3]. Neutralization is a surrogate marker of protection for both active (from previous infection or vaccination) and passive immunity (from monoclonal antibodies), and it is utilized for immunobridging of newly available therapeutic antibodies [3, 4]. However, its use in optimizing care for immunocompromised patients is rare, partly due to the absence of a well-defined protective threshold. The emergence of SARS-CoV-2 variants that evade neutralization necessitates ongoing evaluation of therapeutic mAbs and provides an opportunity to explore the relationship between neutralization activity and clinical outcomes. Here, we evaluated the *in vitro* neutralizing activity of sotrovimab and other therapeutic mAbs against XBB.1.5, XBB.1.16.1, and XBB.1.9.1 variants. We also retrospectively investigated the neutralization against these variants of sera from kidney transplant recipients (KTR) who received sotrovimab.

Our initial focus was to assess the *in vitro* neutralizing activity of mAbs that had been utilized since the end of 2021 (namely sotrovimab, cilgavimab-tixagevimab, and imdevimab-casirivimab). As controls, we analyzed the neutralizing activity against the ancestral D614G strain. Neutralization of authentic SARS-CoV-2 isolates were performed with the S-Fuse assay as described in **Supplementary Material** and previously [5]. Sotrovimab exhibited neutralizing activity against the XBB.1.5, XBB.1.16.1, and XBB.1.9.1 variants, albeit at low levels (with ED50 titers of 0.70 μg/mL, 1.18 μg/mL, and 1.41 μg/mL, respectively, as opposed to 0.04 μg/mL against the D614G variant,

Abbreviations: BAU, binding arbitrary units; COVID-19, coronavirus disease 2019; KTRs, kidney transplant recipients; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; mAbs, monoclonal antibodies.

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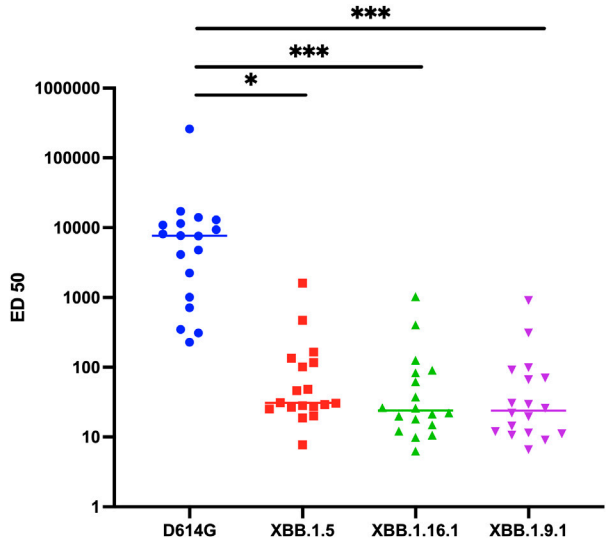

FIGURE 1 | Neutralization activity against D614G, XBB.1.5, XBB.1.16.1, and XBB.1.9.1 variants in sera of COVID-19 kidney transplant recipients (n = 18) receiving Sotrovimab infusion. Results are effective dilution 50% (ED50; titers) as calculated with the S-Fuse assay. Each dot is an individual. Lines indicate medians. The dashed lines indicate the limits of detection. *p = 0.04 according to Friedman test with Dunn's multiple comparison correction and Spearman non-parametric correlation test. ***$p < 0.0001$ according to Friedman test with Dunn's multiple comparison correction and Spearman non-parametric correlation test.

Supplementary Figure S1). The cilgavimab-tixagevimab combination and imdevimab-casirivimab displayed no discernible neutralizing activity.

Given this weak but consistent *in vitro* activity of sotrovimab against these variants, our subsequent investigation delved into its *in vivo* neutralization, using the same assay and sera retrieved from 18 KTR followed at Strasbourg University Hospital. These patients had received sotrovimab treatment for confirmed COVID-19, and had accessible post-sotrovimab serum samples during BA.1 and BA.2 breakthrough period spanning from January to March 2022. The administration of sotrovimab was conducted intravenously at a dose of 500 mg. The median age of this cohort was 60.5 years (interquartile range [IQR] 45.2–70.2 years). The median time from transplantation to COVID-19 diagnosis was 2.47 years (IQR 0.34–8.54 years). All but one patient had been vaccinated against SARS-CoV-2, but only two of them demonstrated an effective vaccine response with an anti-spike antibody titer above 264 BAU/mL (**Supplementary Table S1**). The measurement of *in vivo* neutralizing activity was conducted after a median of 34 days (IQR 18–51.5 days) following sotrovimab administration. All patients' sera displayed significant serum neutralization against the D614G variant, with a median ED50 titer of 7,641 (IQR 934–11,859). In contrast, although a majority of sera exhibited neutralizing activity above the threshold against XBB.1.5. (n = 17/18), XBB.1.16.1 (n = 16/18), and XBB.1.9.1 (n = 17/18) variants, the titers were low and significantly reduced compared to the neutralization titers against D614G, with median ED50 titers of 31 (IQR 26–121, $p = 0.04$), 24 (IQR 14–85, $p < 0.0001$), and 24 (IQR 11–75, $p < 0.0001$) for XBB.1.5, XBB.1.16.1, and XBB.1.9.1 variants, respectively (**Figure 1**). The neutralizing titers for XBB.1.5, XBB.1.16.1, and XBB.1.9.1 were reduced by a median of 87-fold, 115-fold, and 154-fold, respectively, compared to D614G.

In a subgroup of 10 patients, sera neutralization was assessed before and after sotrovimab administration. After administration of sotrovimab, neutralization activity increased slightly against XBB.1.5 (from a median of 15.61–38.72, $p = 0.01$), XBB.1.16.1 (from a median of 10–26.19, $p = 0.004$), and XBB.1.9.1 (from a median of 10–25.66, $p = 0.004$), **Supplementary Figure S2**.

Notably, non-hospitalized patients exhibited higher median titers compared to hospitalized patients for each variant: 74.7 (IQR 30.2–142) vs. 26 (IQR 20.4–27.2, $p < 0.01$) for XBB.1.5, 48.4 (IQR 24.2–93.2) vs. 11.3 (IQR 10.2–11.8, $p < 0.01$) for XBB.1.9.1, and 49.8 (IQR 21.6–117.1) vs. 11.4 (IQR 9.0–20.0, $p < 0.01$) for XBB.1.16.1 (**Supplementary Figure S3**). Advanced age also correlated with lower neutralizing titers against XBB.1.5 and XBB.1.9.1 (Spearman correlation coefficients: −0.49, $p = 0.04$ and −0.54, $p = 0.02$ respectively). None of the other clinical and demographic characteristics were found to be associated with neutralizing titers.

Collectively, the data presented here indicate a persistent *in vitro* and *in vivo* neutralization of sotrovimab against XBB.1.5, XBB.1.16.1, and XBB.1.9.1 variants. These variants are no longer circulating and the current dominant variants (JN.1 and derivatives) fully evades sotrovimab [6]. Nevertheless, our study raises interesting observations on the therapeutic use of mAbs, as it shows a residual antiviral activity of sotrovimab even after its discontinuation from the clinical setting. The minimum dose of mAb necessary to achieve adequate protection has not been established. Data on adintrevimab have shown that a low level of neutralization may be sufficient to provide clinical effectiveness against omicron BA.1 and BA.1.1 [7]. Conversely, during the BA.2 wave, increasing the dosage of tixagevimab-cilgavimab led to a rise in serum neutralizing activity and a decreased risk of COVID-19 breakthrough infections [8]. As higher doses of mAbs administration were found to be safe [9], it may be interesting to consider an increase in the dosage of therapeutic mAbs to boost efficacy against variants harboring partial escape. Indeed, the neutralizing activity against circulating variants is correlated with protection against COVID-19 infection, whether the immunity is passive or active [4]. Furthermore, sotrovimab exhibits an antibody-dependent cellular cytotoxicity (ADCC) activity against the XBB.1.5 variant [5]. Whether such non-neutralizing activities of antibodies contribute to the clinical efficacy of mAbs deserves further investigations. Altogether, our data and the literature suggest that a better mechanistical characterization of antibody activities against variants is needed to optimize patient care.

It is essential to acknowledge the limitations of our study. Being a retrospective, single-center study with a relatively limited sample size and lacking a control group, the findings should be interpreted with caution. We must also consider the potential impact of natural anti-COVID-19 immunity in this population infected with the BA.1 or BA.2 variant. However, it is important to note that the XBB.1.5 variant has the ability to evade antibodies generated after infection by these variant [10].

Providing data on the link between serum neutralization and mAbs efficacy (in our case sotrovimab and XBB.1.5, XBB.1.16.1, and XBB.1.9.1 variants) enables to create a framework to associate neutralization to clinical efficacy over the course of SARS-CoV-2 evolution and to help predict the efficacy of future therapies against future variants.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study protocol was approved by the local ethics committees (identifier: DC-2013-1990 and DC-2021-4460), and written informed consent was obtained from all participants.

AUTHOR CONTRIBUTIONS

Concept and design: IB and SC. Experimental strategy and design: TB and OS. Laboratory experiments: MJ-G and IS. Cohort management and clinical research: IB and SC. Viral strains and key reagents: AB, OD, and ES-L. Statistical analysis: IB and SC. Manuscript writing and editing: IB and SC. Critical revision of the manuscript for important intellectual content: All authors. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

TB and OS have a pending patent application for an anti-RBD mAb not used in this study (PCT/FR2021/070522). IB received travel grant and payment or honoraria for lectures from Astra Zeneca. SC has received consultancy fees and has served on advisory boards for Astra Zeneca, Alexion, Chiesi, Pierre Fabre, and Pfizer.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13272/full#supplementary-material

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