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Imlifidase: streps to the rescue

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Table of contents

Original Research

Letter to the Editor

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TRANSPLANT TRIAL WATCH
published: 09 June 2023
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Transplant Trial Watch

Simon R. Knight[1,2]*

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Keywords: kidney transplant, simultaneous pancreas kidney transplantation, randomised controlled trial, systematic review, metabolic acidosis

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

Sodium Bicarbonate for Kidney Transplant Recipients With Metabolic Acidosis in Switzerland: A Multicentre, Randomised, Single-Blind, Placebo-Controlled, Phase 3 Trial.

by Mohebbi, N., et al. Lancet 2023; 401 (10376):557–567.

Aims

The aim of this study was to examine the effects of sodium bicarbonate treatment on graft function in renal transplant patients with metabolic acidosis.

Interventions

Participants were randomised to receive either oral sodium bicarbonate or matching placebo.

Participants

242 kidney transplant recipients with metabolic acidosis.

Outcomes

The primary outcome was the estimated glomerular filtration rate (GFR) slope over a treatment phase of 24 months. Secondary outcomes were serum bicarbonate and pH, albuminuria, and mean daytime systolic and diastolic blood pressure.

Follow-Up

24 months.

CET Conclusion

This multicentre study from Switzerland investigated the effect of using sodium bicarbonate to correct metabolic acidosis on the graft function of stable renal transplant recipients. Recipients with a serum bicarbonate level of <22 mmol/L were randomised to oral sodium bicarbonate or placebo for 2 years. Despite adequate correction of metabolic acidosis in the treatment group, there was no

Transplant Trial Watch

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difference in eGFR decline between groups, leading the authors to conclude that sodium bicarbonate supplementation to preserve GFR in renal transplant recipients is not recommended.

The methodology of the study is excellent, with centralised variable block randomisation and placebo-control. A modified ITT analysis is used including all patients who were randomised and attended a baseline visit. It should be noted that the mean serum bicarbonate level in both groups at baseline was only just below the lower limit of normal (~21 mmol/L), leaving the possibility that greater benefit may be seen in patients with a more profound acidosis. However, this was not supported by prespecified subgroup analysis (albeit with more limited statistical power).

Jadad Score
5.

Data Analysis
mITT.

Allocation Concealment
Yes.

Trial Registration
ClinicalTrials.gov—NCT03102996.

Funding Source
Non-Industry.

SYSTEMATIC REVIEW

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

by Ai Li, E., et al. Transplant International 2023; 36:10442.

Aims
This study aimed to assess the effect of heparin thromboprophylaxis in simultaneous pancreas-kidney (SPK) transplantation, pancreas after kidney (PAK) transplantation and pancreas transplant alone (PTA).

Interventions
A literature search was performed on PubMed, EMBASE, BIOSIS, MEDLINE, Cochrane Library and Web of Science. Two reviewers independently selected studies for inclusion and extracted the data. Risk of bias was assessed using the Methodological Index for Non-Randomized Studies (MINORS).

Participants
11 studies were included in the review.

Outcomes
Outcomes of interest were pancreas thrombosis during early post-transplant period, incidence of postoperative bleeding, pancreas graft loss due to thrombosis, acute return to the operating room, and units of packed red blood cells (pRBC) used.

Follow-Up
N/A.

CET Conclusion
This systematic review and meta-analysis investigated the role of heparin thromboprophylaxis in simultaneous pancreas-kidney (SPK) transplantation, pancreas after kidney (PAK) transplantation and pancreas transplant alone (PTA). Study selection and data extraction were performed in duplicate. Only 11 studies, all of which were retrospective, were included. However, all the included studies were considered high quality (MINORS score > 60%). The authors found that heparin thromboprophylaxis reduced early pancreas thrombosis and pancreas loss by over two-folds for SPK, PAK and PTA, without resulting in an increase in the incidence of bleeding or acute return to the operating room. Heterogeneity was high for some of the outcomes but was not explored. No adjustments for confounders were made in the analyses.

Registration
PROSPERO—CRD42021260585.

Funding Source
None.

CLINICAL IMPACT SUMMARY

Graft thrombosis is a recognised and feared complication of pancreas transplantation, resulting from a thromboinflammatory response and relatively low flow through the graft [1]. It is more frequently seen in circulatory death (DCD) grafts and following pancreas transplant alone (PTA) compared to simultaneous pancreas kidney transplant (SPK) [1, 2]. Most centres employ some form of anticoagulation protocol in the peri-operative period to reduce the risk of thrombosis, although exact protocols vary considerably, and the evidence-base is limited. Use of anticoagulation is often monitored and adjusted using measures such as the activated partial thromboplastin clotting time (APTT) or thromboelastogram (TEG), with limited evidence that TEG monitoring may be beneficial [3, 4].

In their recent systematic review, Ai Li et al. attempt to summarise the literature regarding heparin thromboprophylaxis following pancreas transplantation [5]. They identified 11 studies investigating heparin use in SPK and PTA recipients, of which just four were comparative and none were prospective. They conclude that heparinization significantly decreases the risk of early pancreatic thrombosis and graft loss due to thrombosis, with no evidence of increased bleeding or reoperation risk.

Whilst the limited amount of observational data published in the literature does appear to support this conclusion overall, there are significant limitations to this study. There is no randomised controlled trial evidence available, and very limited comparative data meaning that the authors resort to comparing single-arm observational data to the control cohorts of other studies. Given the differences in protocols and surgical techniques between centres,

the validity of this is uncertain. Even in the four comparative studies, there is significant heterogeneity in treatment protocols and monitoring strategies, meaning that the optimum regimen is unclear.

The authors employ fixed effects methods in some of their meta-analysis. Given the heterogeneous and observational nature of the data, the assumptions of a fixed effects analysis are probably not met. Indeed, re-analysis using a random effects model increases uncertainty and loses the significant treatment effects seen in fixed effects analysis.

It is unlikely that there is enough equipoise to undertake a large RCT of heparin versus no heparin following pancreas transplantation as most centres now use some form of anticoagulation. However, there is scope for future studies to investigate the optimal protocol and monitoring strategy for anticoagulation, including the use of TEG monitoring.

Clinical Impact

2/5.

AUTHOR CONTRIBUTIONS

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

CONFLICT OF INTEREST

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

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FORUM
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Prime Time for HLA Desensitization: Imlifidase in the Spotlight

Oriol Bestard[1]*, *Francesc Moreso*[1] and *Anthony Dorling*[2]

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Keywords: transplant access, desensitization, rejection, survival benefit, HLA allosensitization

A Forum discussing:

Imlifidase for Kidney Transplantation of Highly Sensitized Patients With a Positive Crossmatch: The French Consensus Guidelines
by Couzi L, Malvezzi P, Amrouche L, Anglicheau D, Blancho G, Caillard S, Freist M, Guidicelli GL, Kamar N, Lefaucheur C, Mariat C, Koenig A, Noble J, Thaunat O, Thierry A, Taupin J-L and Bertrand D (2023). Transpl Int. 36:11244. doi: 10.3389/ti.2023.11244

An increasing number of highly human leukocyte antigen (HLA) sensitized patients are currently on kidney transplant waiting lists worldwide. The leading causes of sensitization are previously failed transplants, previous pregnancies, or blood transfusions. Because the HLAs to which patients have been previously exposed—or to which they have made an anti-HLA antibody—are listed with organ allocation bodies as "unacceptable," sensitized patients have a significantly reduced chance of finding an HLA compatible donor. Thus, they can wait for a very long time on chronic dialysis therapy, which has deleterious consequences in terms of mortality, quality of life, and healthcare costs.

In the last decade, different strategies have been developed and implemented in many countries, aimed at increasing the likelihood of finding HLA compatible organs for these patients, whilst maintaining a balanced equity access to kidney transplantation for all waitlisted patients. These include sliding-scale prioritization score programs, the acceptable mismatch program, and the expansion of kidney-pair exchange programs [1]. While all these strategies have been variously successful for many highly sensitized patients, a group of (very) highly sensitized individuals (>99.9% cPRA) have failed to benefit, and this expanding group still have an extremely low chance of finding a HLA compatible organ.

There are two approaches to improve the chances of these patients; "de-listing" which involves ignoring selected low-level HLA antibodies in the allocation process, and "desensitization" to reduce higher levels of HLA antibodies down to permissive levels that can then be ignored. These approaches offer no survival disadvantage [2] to waiting for a well-matched organ but are associated with higher rates of early and late aggressive rejection and a shortened graft half-life. Unfortunately, many patients have no delisting options, and current desensitization strategies have failed to demonstrate a consistent success, especially for those without a living donor. Therefore, there is an important unmet need for new drug development enabling access to transplantation to these (very) highly sensitized kidney transplant candidates [3].

Notably, a new drug Imlifidase, a recombinant cysteine protease with an unprecedented capacity to rapidly cleave all four IgG subclasses, both soluble and on the B-cell surface [4], has the potential to offer hope for these patients. By transiently depleting all circulating IgG, including HLA antibodies for several days, Imlifidase can uniquely create a window of opportunity for highly sensitized patients with high pathogenic anti-HLA antibody levels to undergo kidney transplantation [5]. Two phase I/II clinical trials [6] have demonstrated the capacity of Imlifidase to effectively convert a positive crossmatch to a negative one, leading to optimal 3- year graft and patient survival rates. Even though

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TABLE 1 | Main immunological, demographic and clinical variables influencing desicion-making regarding the use of Imlifidase for desensitization.

	Immunological characteristics	Clinical characteristics
Patient selection	**Delisting strategy** Step-wise strategy based on recent serum MFI Ab values > 6000 MFI (<5000 after 1:10 dilution) C1q/C3d negative Reduction of Ab titers after Serial dilutions High MFI values of Ab against repeated antigens in previous transplants[a]	**Recipient** Sensitized patients with urgent Tx needs Highly sensitized with long waiting time in proritization programs No vascular access Avoid frail candidates Increased IS burden (induction and rejection rescue theapies high risk of AR) For cause/surveillance bx Optimal Haemodynamics (BP)[a]
At Transplantation	**Prior to Imlifidase** Negative CDC-XM No FC-XM with positive virtual XM: DSA MFI >6000 in sera with <5000 MFI after 1:10 dilution (C1q/C3d negative) Availability of FC-XM[a]: Positives (pronase-treated) B/T-cell FC-XM **Post-Imlifidase**[a] Negatives FC and CDC-XM	**Donor** Minimize the risk of post-Transplant DGF Long CIT severe AKI Avoid (very) ECD

[a]*Recommendations not made by the French expert group but suggested by the authors of this editorial comment.*

high rates of antibody-mediated rejection (ABMR) were observed in these studies, occurrence of hyperacute or accelerated rejection was avoided. Based on these data, the EMEA recently provided a rapid marketing authorization approval throughout the European Union, conditioned to the outcomes of three on-going studies (17-HMedIdeS-14, 20-HMedIdeS-19, 17-HMedIdeS-20) and thus, it has become the first immunosuppressant approved for HLA desensitization in highly sensitized kidney transplant candidates of deceased donors.

Although an outstanding achievement, the use of Imlifidase has major implications for organ allocation systems, alters current algorithms for immune-risk stratification, and impacts on immunosuppression management. Most of these issues are directly related to the pharmacokinetic/pharmacodynamic nature of Imlifidase [4], which needs to be thoroughly understood; the transient effect of Imlifidase leads to a progressive IgG antibody repopulation in 3–5 days, the immunogenicity of the drug precludes repeated doses and importantly, the broad IgG cleavage effect also targets any (IgG) antibody-based therapy, thus precluding the use of most frequent induction therapies used in these patients. Furthermore, for patients to receive an Imlifidase-enabled deceased kidney organ offer, a thorough immunological evaluation must be conducted, including both an anti-HLA antibody de-listing strategy to reduce the virtual cPRA burden and also establish what is an acceptable positive cross-match (XM) (virtual and/or cell-based) to ultimately decide whether to undergo or abort kidney transplantation.

In this issue of *Transplant International* [7], a French expert transplant group endorsed by different French scientific societies (SFT, FNDT, SFHI), propose a set of clinical, immunological, and therapeutic recommendations on how to implement Imlifidase in clinical transplantation. The authors should be acknowledged for the thorough description of the different recommendations provided in this consensus report, especially considering the relatively low level of evidence currently available in this topic. Even though some recommendations are based on their national allocation policy, most of them may be perfectly generalized to any other transplant system worldwide.

Assessing transplant candidates eligible for Imlifidase is considered in four main areas. First, the authors highlight the importance of selecting only those highly sensitized candidates who have extremely low chances of finding a HLA compatible transplant, according to the distinct national prioritization programs available (**Table 1**); in France this threshold is established by having a persistent cPRA ≥ 98% with a waitlist time of at least 3 years. While these thresholds may be relatively arbitrary, an objective calculation of the cPRA burden and time to receive an organ offer should be country/region-specific to maintain a transparent balance of access to transplantation between highly sensitized and non-sensitized transplant candidates. Sensitized patients with an urgent transplant need because of lack of vascular access could eventually be considered. Authors limit the use of Imlifidase to patients with no more than two previous transplants. While this may be understandable due to the scarcity of organs, the time onset of end-stage kidney disease should be considered, as this exclusion criteria may significantly hamper access to transplantation to pediatric patients who have a longer lifespan and thus, may need more than two transplants. Secondly, they recommend careful consideration of relevant recipient and donor characteristics: Avoiding recipients at higher risk of life-threatening opportunistic infections or cardiovascular-related events may minimize fatal outcomes and limit organ offers to those without severe acute kidney injury or long cold ischemia times (CIT), to help reduce the risk of delayed graft function and organ immunogenicity while maximizing the capacity of the organ to overcome allogenic insults. Thirdly, the de-listing strategy recommended by authors is designed to minimize the risk of

highly pathogenic antibody rebound after transplantation. They suggest avoiding de-listing antibodies with MFI values above those which highly correlate with both complement-fixing abilities, a strategy which may be further refined by using C1q or C3d assays. In addition, those antibodies were significantly reduced after a 1:10 dilution (<5000 MFI), and are also recommended for a first delisting. Importantly, since incompatible (donor specific antibody (DSA) positive) kidney transplantation with negative cell-based XM (both FCM and CDC-XM) may be feasible without Imlifidase [2], the authors describe a plausible MFI threshold (>6000 MFI) to infer the presence of a positive FCM but negative CDC-XM to accept with the use of Imlifidase. While this approach has high inter-laboratory variability due to the different type of SAFB used, it may simplify the logistics for those transplant programs not routinely performing cell-based XM, and ultimately reduce CIT. Notably, delisting antibodies may be performed in a stepwise approach, starting from less to more aggressive antibodies according to the reduction of the cPRA burden and thus, the likelihood of receiving a transplant offer. Finally, they consider immunosuppression management, and recommend pre-delisting rituximab, followed by T-cell depletion, high-dose IVIG, and rituximab after day 4 post-transplantation, in the context of tacrolimus, mycophenolate mofetil, and steroids beginning on day 0.

In summary, the advent of Imlifidase may revolutionize the field of HLA desensitization and opens a new window of opportunity for an increasing number of patients with extremely low chances of finding an HLA compatible organ. Although there is still more to be learned from ongoing, prospective, controlled trials it is imperative that clinical and immunological data is gathered from Imlifidase programs as they begin in multiple countries and it is highly recommended that Imlifidase should only be used in a rigorous and controlled manner as suggested by the authors here. There is an ideal opportunity, with the use of Imlifidase, to better comprehend the complex mechanism of alloimmune sensitization and allow a better understanding of the dynamics and pathogenicity of the humoral immune response. Importantly, all this effort would be highly advisable to be undertaken within international, cooperative scientific networks and endorsed by national and international transplant societies.

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

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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Enhancing Beta Cell Replacement Therapies: Exploring Calcineurin Inhibitor-Sparing Immunosuppressive Regimens

Rossana Caldara[1], *Valentina Tomajer*[2] *and Lorenzo Piemonti*[3,4]*

[1]Regenerative and Transplant Medicine Unit, IRCCS Ospedale San Raffaele, Milan, Italy, [2]Pancreatic Surgery, Pancreas Translational and Clinical Research Center, IRCCS Ospedale San Raffaele, Milan, Italy, [3]Diabetes Research Institute, IRCCS Ospedale San Raffaele, Milan, Italy, [4]Università Vita-Salute San Raffaele, Milan, Italy

Keywords: islet transplant, immunosuppression, calcineurin inhibitors, calcineurin inhibitor toxicity, tolerance induction

A Forum discussing:

A Multi-Modal Approach to Islet and Pancreas Transplantation With Calcineurin-Sparing Immunosuppression Maintains Long-Term Insulin Independence in Patients With Type I Diabetes
by Wisel SA, Posselt AM, Szot GL, Nunez M, Santos-Parker K, Gardner JM, Worner G, Roll GR, Syed S, Kelly Y, Ward C, Tavakol M, Johnson K, Masharani U and Stock PG (2023) Transpl Int. 36:11367. doi: 10.3389/ti.2023.11367

The study conducted by Wisel et al. [1] offers valuable insights into the long-term outcomes of beta cell replacement therapies and the use of immunosuppression in managing Type 1 diabetes (T1D). This 10-year follow-up study examined ten consecutive non-uremic patients with T1D who underwent islet transplantation. The patients were treated with calcineurin inhibitor (CNI)-sparing immunosuppressive regimens using either belatacept (BELA) or efalizumab (EFA). Out of the 10 patients, four achieved long-term insulin independence for an average duration of 13 years following a single islet infusion. On the other hand, six patients experienced failure of their initial islet transplant, with an average time to failure of 19 months. Four of these patients received a second islet infusion, one patient declined a second infusion and returned to insulin use, and one patient proceeded to undergo pancreas-after-islet (PAI) transplantation. Among the patients who received a second islet transplant, the average duration of insulin independence was 45.5 months. However, all four patients eventually reverted to insulin use. Two of them subsequently underwent PAI transplantation, while the remaining two continued to rely on exogenous insulin. At the time of publication, six out of ten patients still maintained insulin independence, including the three patients who underwent PAI transplantation.

The achievement of 40% insulin independence at 10 years following a single islet infusion is to be considered one of the most remarkable results ever reported. Comparatively, recent retrospective analyses using different immunosuppression regimens and multiple infusions have reported insulin independence rates of 4.8% [2], 20% [3], and 28% [4] at 10 years. Moreover, it significantly exceeds the prevalence of insulin independence reported in the Collaborative Islet Transplant Registry (CITR) 11th allograft report, where the value of 40% is achieved approximately 2 years after the last infusion [5]. Remarkably, this rate is closely comparable to the best outcome achieved after 10 years in solitary pancreas transplantation [6].

Forum

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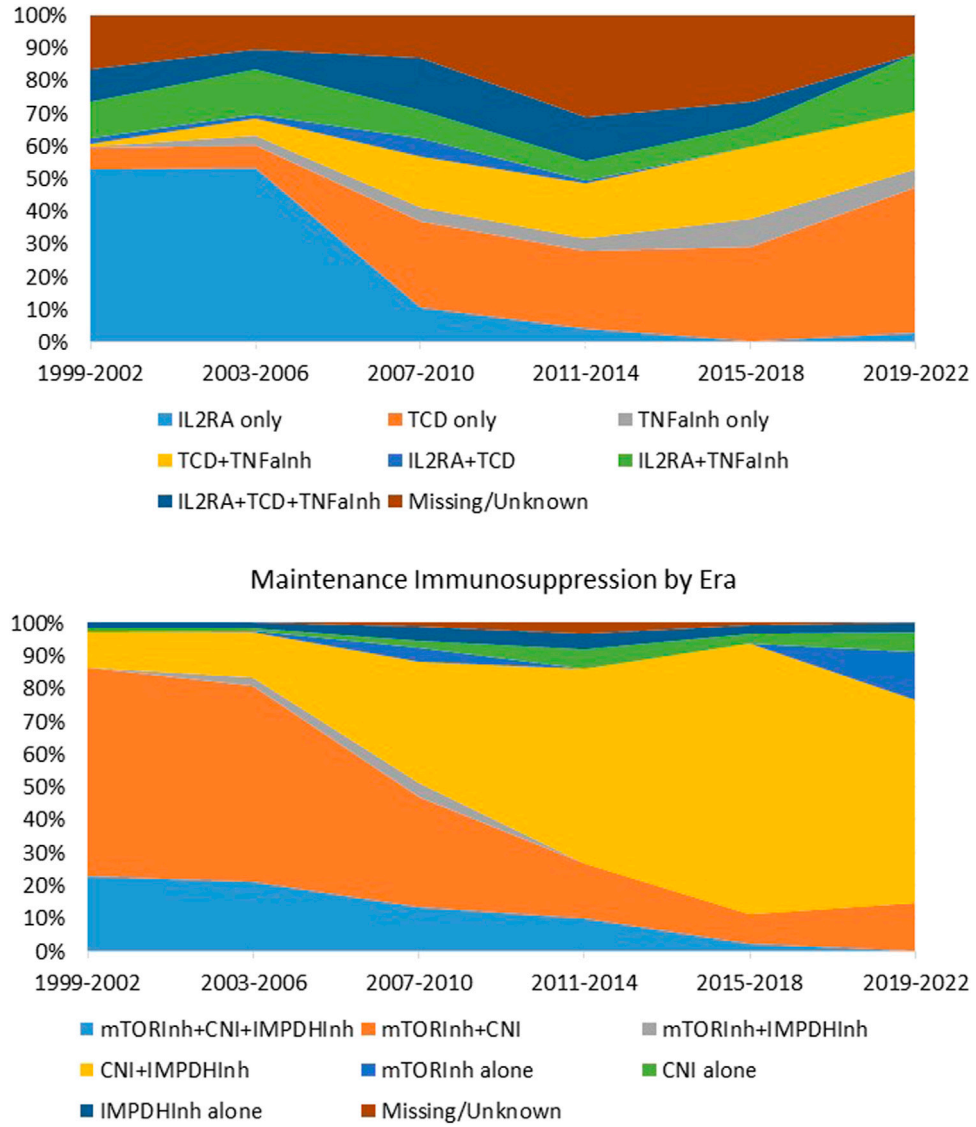

FIGURE 1 | Induction and maintenance immunosuppression in islet transplantation by era. Immunosuppression regimen of 1,108 individuals with T1D who received Islet Transplant Alone ($n = 992$) or Islet after kidney ($n = 186$) between 1999 and 2022 and were followed by the CITR. Data source: Collaborative Islet Transplant Registry Coordinating Center: Eleventh allograft report 2022. TCD, T cell depleting agents; Inh, inhibitor; CNI, calcineurin Inhibitor; IMPDH, Inosine-5′-monophosphate dehydrogenase; IL1RA, IL1 receptor antagonist.

The authors aimed also to minimize the risk of nephrotoxicity associated with CNIs. Hence, an important question addressed in this paper is whether there was an advantage in preserving renal function. During the observational period of the study, there was a slight reduction in the estimated glomerular filtration rate (eGFR) from the initial value of 76.5 ± 23.1 mL/min, indicating an annual decline of 1.93 mL/min (1.76 and 2.1 mL/min in patients receiving BELA or EFA, respectively). This decline was minor among subjects with functional islet grafts (1.19 mL/min), higher in patients undergoing PAI transplantation with CNIs (3.45 mL/min). To assess whether these values indicate an advantage in preserving kidney function, we can compare them to two reference populations. The first population consists of an age-unadjusted cohort of 1,141 individuals with T1D who were followed in the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) study. This population started with a mean eGFR level of 126 mL/min. Over the 22-year duration of the study, an average decrease in eGFR of 1.2 and 1.56 mL/min per year was reported for the intensive therapy and conventional therapy groups, respectively [7]. The second reference population consists of an age-unadjusted cohort of 1,108 individuals with T1D who received islet transplantation alone and were followed by the CITR. This population started

with a mean eGFR level of 91 mL/min. Over the 5-year period following the last infusion, they experienced a mean decline in eGFR of 2.4 mL/min per year [5]. Based on these comparisons, while the preservation of renal function in the study's patients is not as favorable as that seen in the DCCT/EDIC cohorts, it does show an advantage over the decline observed in the CITR cohort. Further analysis and comparisons with larger matched reference populations would be beneficial for a more comprehensive assessment of the advantages in preserving kidney function with CNIs-free immunosuppression.

The involvement of CNIs in the NFAT signaling pathway, crucial for Treg differentiation, maintenance, and suppressive abilities, can have significant consequences [8]. It undermines immune tolerance and raises the risk of immune-related complications. Additionally, it diminishes the effectiveness of adoptive therapy that employs tolerogenic donor-specific Tregs. Hence, there is a requirement for research to examine the safety and feasibility of immunosuppressive regimens that minimize the utilization of CNIs [9, 10]. The study by Steven A. Wisel et al. highlights two significant findings: 1) patients treated with BELA showed stable levels of Tregs compared to circulating T cells in the first year after islet transplantation; 2) patients who received EFA exhibited increased levels of circulating Tregs, including a remarkable case with a substantial expansion of Tregs following islet transplantation. What's truly remarkable is that even after discontinuing EFA treatment, this particular patient maintained insulin independence for a 10-year period without any notable immune response towards the transplanted islets, indicating the presence of operational tolerance.

Regrettably, there is currently no available guidance or formal consensus on the optimal or standard immunosuppressive strategy for human islet transplantation. Over the years, a significant shift in immunosuppression approaches has occurred in the absence of evidence-based practices (**Figure 1**). Several studies conducted on small cohorts have proposed various combinations of immunosuppressive agents [11–14]. These include T and B cells depleting agents (alemtuzumab, teplizumab, antithymocyte/lymphocyte globulin, rituximab), inhibitors of T-cell activation (IL2R antagonists daclizumab and basiliximab), replication inhibitors (azathioprine and mycophenolate mofetil/mycophenolic acid), mTor inhibitors (sirolimus and everolimus), lymphocyte tracking inhibitors (EFA), desensitizing agents (intravenous immunoglobulin), co-stimulation inhibitors (monoclonal antiCD28 belatacept/abatacept), CNIs (cyclosporine and tacrolimus), and anti-inflammatory agents (corticosteroids, IL1 receptor antagonist, and TNF-alpha inhibitors). It is important to note, however, that most of these studies were observational, predominantly retrospective or prospective single-center single-arm studies. Only one recently reported randomized controlled trial study focused on CXCR1/2 inhibitors stands out as an exception [15]. It is crucial to draw attention to the notable gap in consistent studies regarding the use of immunosuppression in the field of beta cell replacement, particularly considering the potential emergence of new sources of insulin-producing cells in the future. In this context, conducting research on immunosuppressive regimens that minimize the use of CNIs will greatly advance beta cell replacement therapies.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://citregistry.org/system/files/11th%20Allograft%20report%20May%2031%202022.pdf.

AUTHOR CONTRIBUTIONS

LP, VT, and RC contributed to conceptualisation and study design, contributed to data interpretation. LP wrote the original draft of the report. VT and RC reviewed and edited the report. LP is responsible for final submission of the manuscript for publication and all authors approved the final version before submission. LP accessed and verified the underlying study data. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

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CONSENSUS REPORT
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Imlifidase for Kidney Transplantation of Highly Sensitized Patients With a Positive Crossmatch: The French Consensus Guidelines

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Imlifidase recently received early access authorization for highly sensitized adult kidney transplant candidates with a positive crossmatch against an ABO-compatible deceased donor. These French consensus guidelines have been generated by an expert working group, in order to homogenize patient selection, associated treatments and follow-up. This initiative is part of an international effort to analyze properly the benefits and tolerance of this new costly treatment in real-life. Eligible patients must meet the following screening criteria: cPRA ≥ 98%, ≤ 65-year of age, ≥ 3 years on the waiting list, and a low risk of biopsy-related complications. The final decision to use Imlifidase will be based on the two following criteria. First, the results of a virtual crossmatch on recent serum, which shall show a MFI for the immunodominant donor-specific antibodies (DSA) > 6,000 but the value of which does not exceed 5,000 after 1:10 dilution. Second, the post-Imlifidase complement-dependent cytotoxicity crossmatch must be negative. Patients treated with Imlifidase will receive an immunosuppressive regimen based on steroids, rATG, high dose IVIg, rituximab, tacrolimus and mycophenolic acid. Frequent post-transplant testing for DSA and systematic surveillance kidney biopsies are highly recommended to monitor post-transplant DSA rebound and subclinical rejection.

Keywords: kidney transplantation, desensitization, imlifidase, highly sensitized patients, positive crossmatch

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BACKGROUND ON IMLIFIDASE

Imlifidase is a recombinant cysteine protease derived from *Streptococcus pyogenes* and produced in *Escherichia coli*, which has the ability to cleave and degrade all human IgGs [1]. Four to 6 hours after Imlifidase infusion, the entire IgG pool is degraded into F(ab')2 and Fc fragments [2]. *In vitro*, Imlifidase inhibits HLA antibody-mediated NK cell activation and antibody-dependent cell-mediated cytotoxicity [3]. Imlifidase degrades also the IgG of the B cell Receptor (BCR),

inhibiting BCR-mediated cell signal, transiently preventing memory B cell response to antigenic stimulation and their transition into antibody-producing cells [4].

Two clinical studies have been designed to determine whether Imlifidase could inactivate IgG donor-specific antibodies as a desensitization strategy in highly sensitized candidates for kidney transplantation. In the phase I/II study, 25 patients were transplanted in Sweden and United States. Among them, 18 had a positive flow cytometry crossmatch (FCXM) and 2 a positive complement-dependent cytotoxicity crossmatch (CDCXM) [2]. In the phase II study (Highdes Trial), 19 patients with an incompatible living or deceased donor from the United States, Sweden, and France were included. Among them, 7, 18, 2, and 8 had respectively a positive T-cell FCXM, positive B-cell FCXM, positive T-cell CDCXM, and positive B-cell CDCCXM. The primary efficacy endpoint was the ability of Imlifidase to convert a positive XM to a negative one. Conversion of baseline positive XM to negative within 24 h after Imlifidase treatment occurred in 89.5% (n = 17) of the 19 patients [5]. In the follow-up study including all the patients transplanted after Imlifidase desensitization, the antibody-mediated rejection rate (AMR) was at 39%. Three-year death-censored graft survival was 93% in patients with AMR and 77% in the others. Three-year patient survival was 85% in patients with AMR and 94% in the others [6]. No safety signal was reported.

Based on these data, Imlifidase is now indicated as a desensitization agent of highly sensitized adult kidney transplant patients with positive crossmatch against an available ABO-compatible deceased donor. Imlifidase received a conditional marketing authorization valid throughout the European Union on 25 August 2020 (https://www.ema.europa.eu). On 23 February 2022, the French health agency authorized an early access to Imlifidase (Idefirix). On 16 August 2022, a panel of 12 transplant nephrologists and four immunologists (including two HLA experts) was convened by The French Society of Transplantation (SFT), the French-speaking Society of Nephrology, Dialysis and Transplantation (SFNDT) and the French Society of Histocompatibility and Immunogenetics (SFHI) to propose recommendations for patient selection, choice of antibodies characteristics, treatment and follow-up in order to homogenize practices. The expert panel used the Grading of Recommendations Assessment, Development and Evaluation system for a systematic weighting of the strength of the recommendation (high: A, moderate: B, low: C, very low: D) and quality of evidence (strong: 1, weak: 2) [7]. Finally, the guidelines were discussed and approved with the French agency in charge of organ regulation (Agence Nationale de la Biomedecine). The objective of these recommendations is to propose a common framework for teams using Imlifidase in order to analyze properly the benefits and tolerance of this new treatment in real-life.

AVAILABLE STRATEGIES IN HIGHLY SENSITIZED PATIENTS: THE PLACE OF IMLIFIDASE

Very recently, the ENGAGE working group (EuropeaN Guidelines for the mAnagement of Graft rEcipients) from ESOT proposed an updated definition of sensitization, stratifying the humoral risk of candidates for solid organ transplantation [8]. Among patients with day 0 donor-specific antibody (DSA), the risk of AMR is the highest in positive CDCXM patients, a situation which requires a desensitization protocol to avoid hyperacute rejection (ENGAGE category 1). Positive FCXM patients have a lower risk of AMR but these patients also require an increased immunosuppression (ENGAGE category 2). Patients with day 0 DSA but a negative crossmatch are also at increased risk of AMR but have an acceptable medium-term graft survival (ENGAGE category 3). This stratification is supported by the studies published by Orandi et al. which showed that graft survival, patient survival and risk of AMR were highly associated with the positivity of the FCXM and the CDCXM [9, 10]. Patients with a positive FCXM have a 35% risk of AMR, which increases to 50% in those with a positive CDCXM [11]. Five-year graft loss is also poor at 30% in positive FCXM recipients and 40% in positive CDCXM [10].

The use of Imlifidase should be reserved for patients unlikely to be transplanted under the available kidney allocation system including the prioritization program for highly sensitized patients (https://www.ema.europa.eu). The French kidney allocation system (KAS) has changed in 2015 and introduced a unified allocation score to be applied locally for one kidney and nationally for the other. In our KAS, highly sensitized patients have access to a national priority program. A recent paper published recently summarizes all these rules [12]. In France, the degree of sensitization (cPRA) reflects the percentage of incompatible donors with HLA antigens against which the patient has preformed anti-HLA antibodies, among all isogroup donors collected on the national territory, during the past 5 years. Highly sensitized patients are defined by a recent cPRA ≥ 70% and a peak cPRA ≥ 85%. In a recent review, Mamode et al. summarized all the available options for transplanting highly sensitized transplant candidates [13].

A living-donor transplantation must be considered for all these patients and three strategies are available: a direct transplantation with an HLA-compatible donor, an indirect transplantation thanks to a kidney exchange program, and finally a direct HLA-incompatible transplantation (**Figure 1**). Although patients transplanted with preformed DSA have globally a greater risk for AMR, this humoral risk greatly varies and can be stratified according to the results of the crossmatch assays, as proposed in the ENGAGE classification [8].

Living-donor transplantation options are often limited, and most highly sensitized patients are transplanted with a deceased donor. In the United States, 73% of transplantations are performed with a deceased donor in patients with a cPRA < 80%. This rate reaches 95%–98% in patients with a cPRA>98% [14]. If they are not transplanted with a compatible donor, transplant teams have the possibility to consider delisting unacceptable HLA antigens for which antibodies disappeared. They also have the possibility to consider delisting unacceptable HLA antigens with low level HLA antibodies (**Figure 1**). The objective of this last strategy is to perform DSA positive but

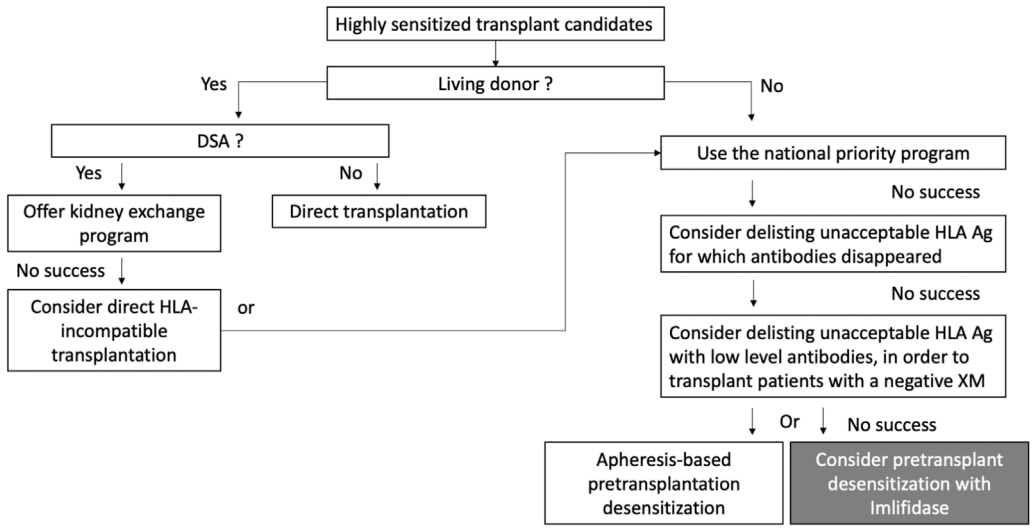

FIGURE 1 | The place of Imlifidase among the available strategies for highly sensitized patient transplantation. Adapted from Mamode et al. [13].

negative XM transplantations (i.e., ENGAGE category 3) [15–17]. But these strategies are very rarely applicable to highly sensitized candidates with persistent high-level HLA antibodies for whom positive XMs are expected (ENGAGE categories 1 and 2). For these patients, many pretransplant desensitization strategies have been tested in order to lower the titer of preformed DSA. These strategies were initially based on IVIg [18–20], then rituximab and IVIg [21], and more recently Bortezomib and apheresis [22], but their efficacy is still discussed. Sequential or single pre-transplant apheresis-based desensitization programs have also been developed by a few transplant teams [23–25]. For instance, the Vienna group proposed to 27 deceased-donor kidney transplant recipients a pre-transplant immunoadsorption for obtaining a negative CDCXM, but the rate of AMR was high (41%) [26]. Faced with the complexity of some of these strategies, complement inhibitors were also tested in a prospective trial with unconvincing results [27].

In France, Imlifidase is now indicated for replacing these strategies in the desensitization treatment of these patients who have a positive crossmatch against an HLA-incompatible deceased donor (**Figure 1**). Although additional data on long-term graft function and survival are required in patients treated by Imlifidase, the European Medicine Agency has decided that this new treatment addressed an unmet medical need (https://www.ema.europa.eu).

PATIENT SELECTION CRITERIA

Patients Eligible for This Treatment

Recipient With cPRA ≥ 98% (Calculated on the Last Serum)

Given the expected high rate of AMR, the use of Imlifidase should be reserved for patients unlikely to be transplanted. Importantly, not all highly sensitized patients have the same access to a transplant. In a French region with more than 3,000 candidates awaiting a kidney transplantation, it was observed that patients with cPRA ≥ 98% had more difficult access to a compatible donor even though they were included in the national priority program (**Figure 2**). Based on these data, we chose a threshold of cPRA ≥ 98% (calculated on the last serum) to authorize a patient to receive Imlifidase in France (IC). However, it is important to note that the French cPRA is not comparable with cPRA used in other countries. For instance, in Australia, access to transplantation is poor for those with a cPRA of 95%–98% and even worse for those with cPRA ≥ 99% [28]. In the United States, access to transplantation becomes very limited for patients with a cPRA ≥ 99% [14]. Based on that observation, the FDA considers that only patients with cPRA ≥ 99.9 should be targeted to desensitization.

Recipient Age ≤ 65 years

Orandi et al. showed that positive crossmatch patients had a significantly higher risk of death than compatible patients [10]. In recipients older than 70 years, the two main causes of death are infection and cardiovascular diseases [29]. In line with these observations, patients undergoing HLA desensitization before kidney transplantation are particularly exposed to infectious diseases and cardiovascular events [23], and Avery et al. reported that the risk of infectious disease increased with the intensity of desensitization before kidney transplantation [30]. Based on these data, we propose that recipient age should not exceed 65 years (1D).

Time on the Waiting List ≥ 3 years

The French acceptable mismatch program improved access to transplantation for highly sensitized patients with a low risk of AMR, as described in the Eurotransplant program [31]. In order

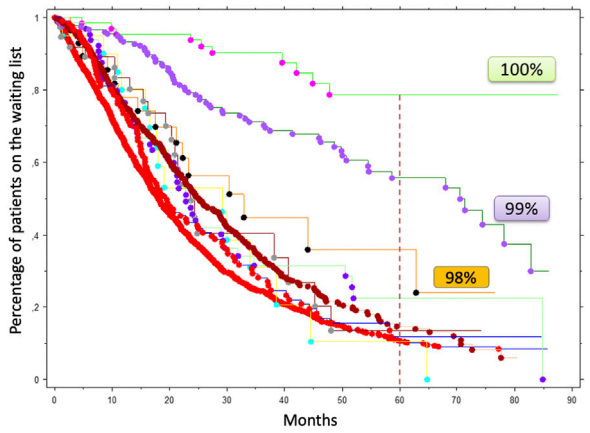

FIGURE 2 | Access to a kidney transplantation according to cPRA in a French region including more 3,096 candidates listed between July 2009 and December 2015. Patients with a cPRA > 85% were included in the national priority program. These data were kindly given by D. Bertrand and collaborators who conducted this retrospective analysis in four transplant centers (Amiens, Caen, Lille, Rouen).

to maintain some equity of access to transplantation for all candidates, we propose that the patient wait for at least 3 years on the waiting list before being offered a transplant with an Imlifidase-based desensitization (2D). It is important to note that this period of time was chosen arbitrarily based on the median time on the waiting list in France which is currently at 2 years (www.agence-biomedecine.fr).

Number of Previous Kidney Transplantations From 0 to 2 (Multidisciplinary Consensus Required If > 2 Previous Transplantations)

In order to maintain some equity of access to transplantation for all candidates, and to minimize the surgical risk, we do not recommend to perform kidney transplantation with Imlifidase in patients with a history of more than two kidney transplantations (2D).

Transplant Biopsy With a Low Risk of Complication

As the probability to develop an AMR and therefore to undergo a transplant biopsy is very high in Imlifidase-based desensitized recipients, we recommend to select patients with an anticipated low risk of biopsy-related complications (1D).

Patient Information

Patients should be informed of the implications of desensitization, how it is performed, the expected benefits and risks involved (1A).

Transplant Unit Profile

In the early post-transplantation period, AMR occurs frequently following Imlifidase desensitization. In this situation, prompt plasmapheresis sessions are highly recommended [32]. Therefore, centers must be equipped to perform round the clock apheresis treatment in the case of AMR (1A).

Donor Profile

Given the expected high rate of AMR in patients desensitized with Imlifidase, it is important to avoid a delayed graft function secondary to poor quality of the donated kidney which could interfere in the management of an early AMR. Donor characteristics associated with a high risk of delayed graft function are old age, extended criteria donor, donation after cardiac death, warm ischemia, long ischemia time, and severe acute kidney injury. According to Aubert et al. preformed DSA and cold ischemia time are the two main independent determinants of outcome of expanded criteria donor (ECD) transplantation. Recipients of ECD kidneys with circulating DSA showed a 5.6-fold increased risk of graft loss compared with all other transplant therapies ($p < 0.001$) [33]. In this context we recommend that older donors, donation after cardiac death, long ischemia time, and acute kidney injury should be avoided as much as possible (1C).

These recommendations are summarized in **Figure 3**.

DSA CHARACTERISTICS AND CROSSMATCHES

Delisting of HLA Antibodies With a Mean Fluorescence Intensity <5,000 After 1: 10 Dilution

After kidney transplantation with Imlifidase, rebound of DSA occurs frequently with an increased risk of AMR [5]. Currently, we do not have a tool able to predict this post-transplant DSA rebound. In the pooled Imlifidase 3-year follow-up analysis, the only variable associated with AMR was the pre-Imlifidase mean fluorescence intensity (MFI) level [6]. However, the Single Antigen Flow Bead (SAFB) assay displays a progressive saturation effect of the measured MFI when the antibody load increases, leading to its underestimation. Serial sera dilutions are reported to be helpful to estimate true alloantibody levels (cPRA) in highly sensitized kidney allograft candidates [34] and to evaluate DSA strength [35]. Moreover, measurement of pre-transplant serum dilutions can be used to determine unacceptable antigens, as well as the likelihood for successful HLA antibody reduction with desensitization [36]. Serum dilution and titration studies can help determining whether desensitization is likely to be successful in removing enough HLA antibody to avoid hyperacute rejection and plan the desensitization strategy. For instance, Pinelli et al. showed that transplant candidates with DSAs of titer ≥1:1,024 at baseline were unlikely to achieve sufficient DSA reduction with PP/IVIg alone [37].

Our objectives were to limit the risk of rebound and more importantly to accept DSA that could be removed efficiently by apheresis sessions in case of rebound. Therefore, we recommend to only delist, those with a SAFB MFI below 5,000 after a 1: 10 dilution (One Lambda assay) (2D). This recommendation increases significantly the cost of HLA testing and requires at the time of patient selection a delisting of all HLA antigens against which the MFI of the preformed HLA antibodies are < 5,000 after

a 1:10 dilution. We recommend to update the delisted HLA antigens every 3 months until transplant offer. All preformed DSA must be still below MFI 5000 on the last diluted serum at the time of transplant offer.

An MFI of Pre-Imlifidase Immunodominant DSA A, B, DRB1, DQB1 > 6,000 (LSAB One Lambda)

Based on the ENGAGE recommendations, our goal was to propose Imlifidase to patients with a positive pre-Imlifidase FCXM (category 2) or CDCXM (category 1). However, we chose to offer Imlifidase based on a virtual XM and not a cellular XM, in order to reduce the ischemia time. To circumvent this problem, we chose to use an MFI threshold capable of predicting the positivity of a FCXM or a CDCXM.

Vo et al. reported the rate of AMR in 226 highly sensitized patients who received transplants after desensitization, and concluded that the DSA-relative intensity scores at transplant was a strong predictor of AMR [38]. By using the assay from the One Lambda company on 432 sera also tested in T-cell XMs, Visentin et al. showed that the SAFB MFI threshold predicting a T-cell FCXM positivity was comprised between 4,400 and 6,200 for class I DSA [39]. The threshold predicting a T-cell CDCXM positivity was comprised between 8,900 and 13,600. To date, data from the other SAFB assay, from the Immucor company, are lacking in the literature. Furthermore, it has been largely demonstrated that circulating complement-activating anti-HLA DSAs had a significant deleterious impact on solid organ transplant survival and risk of rejection [40]. The C1q and C3d assay results can be efficiently predicted by the IgG SAFB MFI once complement interference is annihilated [41, 42]. For instance, Courant et al. showed that an MFI > 3,844 predicted C1q assay positivity with 87.0% sensitivity and 93.5% specificity [42].

Based on these data, we chose to offer Imlifidase only if the SAFB MFI of the immunodominant DSA (One Lambda assay) on a recent serum (less than 3 months) is above 6,000 at the time of the transplant offer (2C). We suggest that transplantations can be performed without Imlifidase if the MFI of the immunodominant DSA is less than 6,000. Other treatment options can be discussed in these situations, such as plasmapheresis and IVIg [43, 44]. A limit of this approach is the high inter-laboratory variability of MFI values.

Only DSA against A, B, DRB1, DQB1 HLA molecules were considered. It has been reported that Cw and DP DSA were associated with AMR and graft loss [45]. However, not all Cw and DP antibodies are pathogenic. For instance, 31.6% of Cw DSA are anti-denatured HLA antibodies associated with negative crossmatch and excellent graft outcome [46]. For these reasons, we did not consider DSA against Cw and DP.

A Pre-Imlifidase Virtual Positive Crossmatch on a Recent Serum Predicting a Positive Cell-Based Crossmatch

We do not recommend to perform a cell-based crossmatch before Imlifidase infusion in order to reduce the total ischemia time (1D). At the time of organ offer, the recipient must have at least one DSA A, B, DRB1, DQB1 with a MFI > 6,000 among all the preformed HLA antibodies which were delisted (because of a MFI < 5,000 after a 1:10 dilution).

A Post-Imlifidase Negative CDCXM (Performed Between 4 and 6 h After Imlifidase Infusion)

A post-Imlifidase negative CDCXM is mandatory to authorize kidney transplantation. CDCXM must be performed prospectively by integrating relevant historical sera and day-zero sera, including pre- and post-Imlifidase sera (4–6 h) (1A). If the CDCXM is positive, we recommend not infusing a second dose of Imlifidase and rejecting the transplant offer.

A Prospective or Retrospective FCXM on Recent, and Day 0 Pre- and Post-Imlifidase Serum Must Be Performed

The FCXM result has no impact on the decision of transplantation. A transplantation can be performed with a positive FCXM as long as the CDCXM is negative. A FCXM is mandatory for stratifying the humoral risk of candidates receiving Imlifidase (1C) [8].

These recommendations are summarized in **Figure 3**. Importantly, we recommend that both CDC and FCXM crossmatches are performed with an anti-Rituximab mouse monoclonal antibody (10C5 clone, ABNOVA®) if the patient has received Rituximab before transplantation (see next chapter) [47, 48].

ASSOCIATED THERAPIES

Imlifidase must be given as a single dose (0.25 mg/kg, IV in 15 min) prior to transplantation after a premedication with glucocorticoids and antihistamines. Based on 3 trials including the ongoing PAES study (NCT05369975) [2, 5], we recommend a strong associated immunosuppressive regimen including steroids, rATG, high dose IVIg, rituximab, tacrolimus and mycophenolic acid. Timing and dosing are particularly important because of the interaction between Imlifidase and immunoglobulins. Our recommendations for these associated treatments are summarized in **Figure 4**.

Steroids

Patients will receive decreasing doses of steroids starting on the day of transplantation: 500 mg at day 0, 250 mg from day 1 to day 3, 125 mg on day 4, 20 mg on day 5, then a decrease according to transplant center practice to 5 mg/day at 3 months, with no corticosteroid withdrawal (1A).

Lymphocyte-Depleting Agents

The two available desensitization studies involving Imlifidase have adopted different lymphocyte depleting strategies: horse ATG (ATGAM) or alemtuzumab. Since horse IgG are not

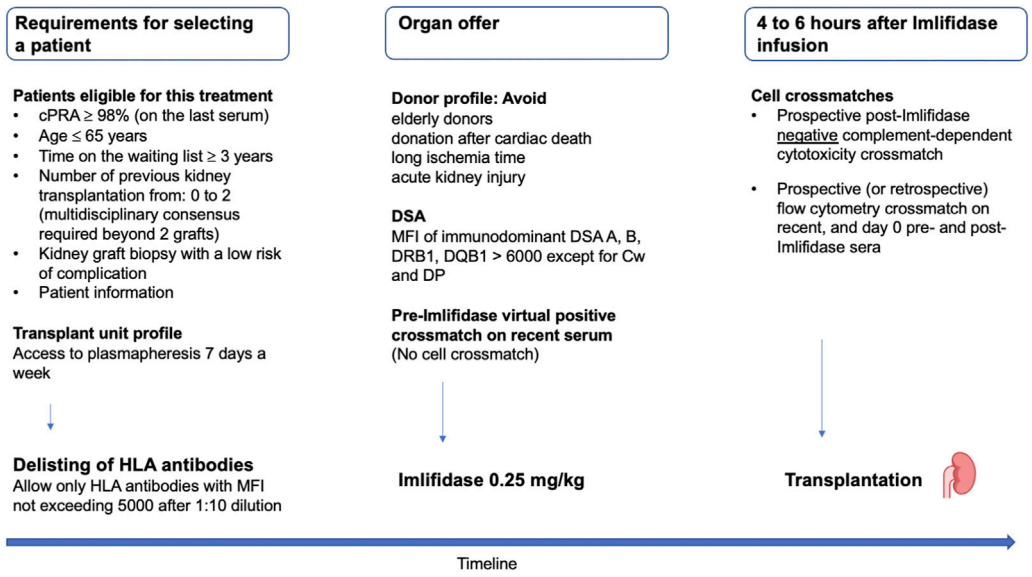

FIGURE 3 | French criteria for selecting highly sensitized patients eligible to Imlifidase, permitted DSA and the timeline of crossmatches. cPRA, calculated panel reactive antibody; DSA, donor-specific antibody; MFI, mean fluorescence intensity.

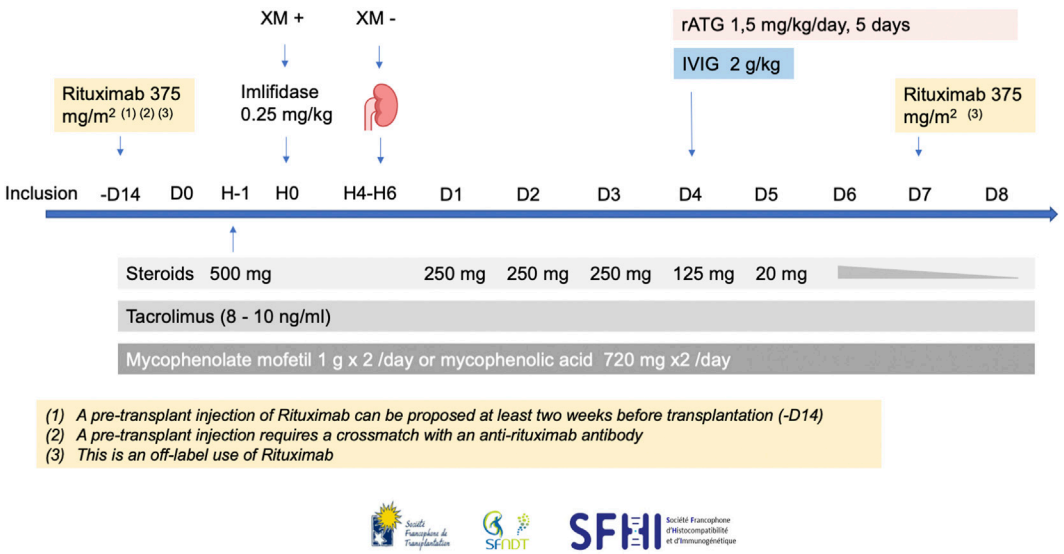

FIGURE 4 | The French consensus guidelines for therapies associated with Imlifidase in highly sensitized patients transplanted with a positive crossmatch. H, hour; rATG, rabbit antithymocyte globulin; IVIg, intravenous immunoglobulin.

cleaved by Imlifidase, ATGAM is an attractive depleting agent that can be used at day 0 with Imlifidase. However, in France, its use is not approved for kidney transplantation. Alemtuzumab, infused on day 4, is also not available in France for this indication, thus limiting its use. In these studies, it is impossible to compare efficacy between the two regimens since patients receiving ATGAM and those receiving alemtuzumab did not receive identical associated immunosuppression [2, 5].

In more recent publications, both alemtuzumab [49] and ATGAM [50] have been compared to rabbit ATG: rATG has shown repeatedly a better safety and efficacy profile than the two other induction strategies. Imlifidase, on the other hand, cleaves rabbit IgG, and so rATG cannot be infused concomitantly with Imlifidase. However, Imlifidase and rATG interaction has been studied in healthy subjects: 96 h following Imlifidase infusion, cleavage was practically inexistent [1]. We therefore recommend infusion of rATG starting on day 4 at the dose of 1.5 mg/kg/day

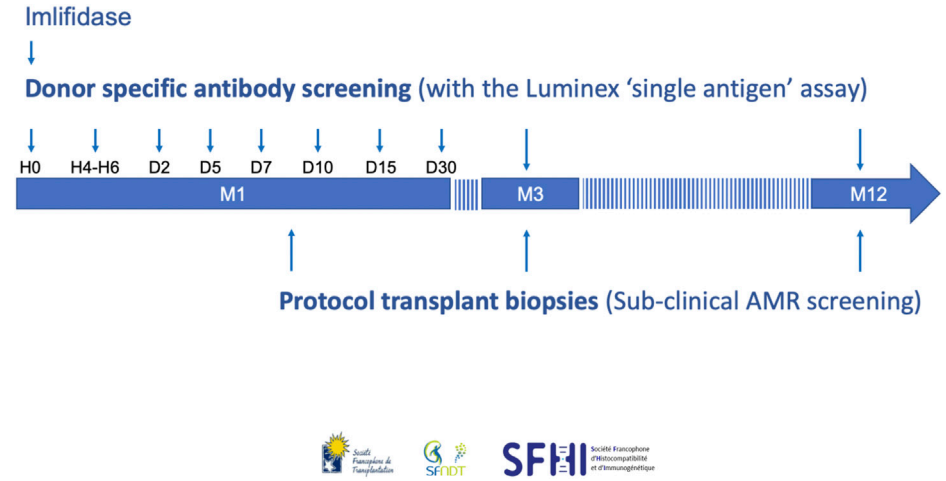

FIGURE 5 | The French consensus guidelines for monitoring and follow-up of patients transplanted with Imlifidase. AMR, antibody-mediated rejection.

for a total of 5 days (7.5 mg/kg cumulative dose) (2A). It is important to note that rATG is the lymphocyte-depleting agent used in the ongoing PAES study (NCT05369975).

IVIg

We also propose high dose IVIg infusion (2 g/kg) over day 4 and 5 in order to reduce the risk of DSA rebound (2C). This approach has been shown to reduce HLA antibodies alone [19] or in association with rituximab [21] in highly sensitized patients awaiting a kidney transplant.

Rituximab

As for Rituximab, we recommend infusing patients on day 7 post transplantation (375 mg/m^2 per dose), since it has been shown that it could attenuate the post-transplant DSA rebound [2, 51]. We also propose to infuse rituximab at least 2 weeks before transplantation (2C). However, even a small amount of rituximab in the recipient sera, can render positive a negative crossmatch [52]. In this case it is imperative that the pre-transplant infusion is performed only in centers where the HLA laboratory has the necessary know-how to counter rituximab in crossmatch testing [47, 48]. If this technique is not available, it is recommended not to infuse rituximab before transplantation. However, it is worth noting that the use of rituximab in addition to rATG could increase the risk of leukopenia and infections.

Standard Maintenance Therapy

Standard maintenance therapy associating tacrolimus and mycophenolate mofetil should be started on day of transplantation with recommended tacrolimus trough levels between 8 and 10 ng/mL and high MPA exposure if tolerated (1A).

Anti-Infectious Prophylaxis

Because of the hefty immunosuppressive regimen, we strongly encourage patient vaccination for *Pneumococcus pneumoniae* (1A), *Neisseria meningitidis* (serotypes ACWY and B) (1A), Influenza virus (1A) and *SARS COV-2* (1A) prior to inclusion in the program. Once transplanted, patients should undergo CMV and pneumocystis prophylaxis. Further bacterial prophylaxis may also be administered (penicillin). The management of CMV or BK virus infections should be performed according to the most recently published recommendations.

MONITORING AND FOLLOW-UP

Given the DSA rebound and the high rate of AMR, we propose guidelines for the post-transplant management of Imlifidase-treated patients. Our recommendations for serologic and histological monitoring are summarized in **Figure 5**.

Donor Specific Antibody

We recommend to test for DSA with the Luminex SAFB technique at the following timepoints:

- Before Imlifidase injection (H0), and 4–6 h (H4-H6) post-injection in order to help interpretation of the pre- and post-Imlifidase XMs (1A).
- At days 2, 5, 7, 10, 15, and month 1, because rebound occurred between day 3 and 14 in the phase II study (1A) [5]. Moreover, a rise in DSA level at week 1 and day 10 was previously associated with AMR [53, 54].
- At months 3 and 12 (1C), because DSA persistence was associated with AMR and a higher risk of chronic AMR in patients transplanted with preformed DSA [55, 56]. At 3 months, persistent DSA was also associated with impaired graft outcome [56, 57].

Moreover, we also recommend that sera be harvested daily during the first week post-transplant and stored, in order to

retrospectively and accurately date the onset of a possible rebound or for possible academic purposes (2D).

Protocol Kidney Biopsies

Systematic surveillance biopsies of the kidney graft are also recommended in all the patients at the following timepoints to detect subclinical rejection: between day 7 and day 10 to capture potential kidney injury at the time of the DSA rebound, and then months 3 and 12 (2C). The incidence of subclinical AMR during the first-year post-transplant in HLA-incompatible kidney transplant recipients has been reported at 80% and more by several teams [58–60]. This incidence is unknown in HLA-incompatible patients treated with Imlifidase. It is then important to clarify this point since subclinical AMR detected at the 1-year screening biopsy leads to a reduced graft survival at 8 years post-transplant (56%) independently of eGFR and proteinuria [61]. Moreover, as subclinical AMR is associated with graft loss, early treatment could be initiated to improve graft outcome [62].

CONCLUSION

Imlifidase could be a major breakthrough in kidney transplantation, because this is the first treatment authorized in our field since belatacept more than 10 years ago and could allow transplanting patients so far considered as untransplantable. We urgently need more clinical data coming from clinical trials as well as by unifying efforts across centers and countries, that may enable enhancing the evidence on how to refine the use and implementation of Imlifidase. These French guidelines are partly subjective but are part of this international effort. The experience acquired in the few coming years will help revising and refining them.

AUTHOR CONTRIBUTIONS

LC was Chairman of the Working Group and drafted the first version of the manuscript. PM and DB wrote specified sections of the manuscript. LA, DA, GB, SC, MF, GG, NK, CL, CM, AK, JN, OT, AT, and J-LT all participated in the discussions that contributed to the development of these recommendations, reviewed and provided input into the entire manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

LC has received lecture fees from Astellas, Chiesi, Novartis, Sandoz, Ostuka, GSK, Biotest, and participated on advisory boards for Biotest, Hansa, and Novartis. LA, DA, GG, CL, AK, J-LT, JN, and DB participated on advisory boards for Hansa. NK has received speakers fees from, and participated on advisory boards for Astellas, AstraZeneca, Biotest, CSL Behring, Chiesi, ExeViR, Hansa, Merck Sharp and Dohme, Glasgow Smith Kline, Novartis Pharma, Sanofi, Sandoz, and Takeda.

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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Helical TomoTherapy Total Lymphoid Irradiation and Hematopoietic Cell Transplantation for Kidney Transplant Tolerance in Rhesus Macaques

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Development of a post-transplant kidney transplant tolerance induction protocol involving a novel total lymphoid irradiation (TLI) conditioning method in a rhesus macaque model is described. We examined the feasibility of acheiving tolerance to MHC 1-haplotype matched kidney transplants by establishing a mixed chimeric state with infusion of donor hematopoietic cells (HC) using TomoTherapy TLI. The chimeric state was hypothesized to permit the elimination of all immunosuppressive (IS) medications while preserving allograft function long-term without development of graft-versus-host-disease (GVHD) or rejection. An experimental group of 11 renal transplant recipients received the tolerance induction protocol and outcomes were compared to a control group (n = 7) that received the same conditioning but without donor HC infusion. Development of mixed chimerism and operational tolerance was accomplished in two recipients in the experimental group. Both recipients were withdrawn from all IS and continued to maintain normal renal allograft function for 4 years without rejection or GVHD. None of the animals in the control group achieved tolerance when IS was eliminated. This novel experimental model demonstrated the feasibility for inducing of long-term operational tolerance when mixed chimerism is achieved using a TLI post-transplant conditioning protocol in 1-haplotype matched non-human primate recipients of combined kidney and HC transplantation.

Keywords: kidney transplantation, tolerance induction, chimerism, hematopoietic cells, TomoTherapy

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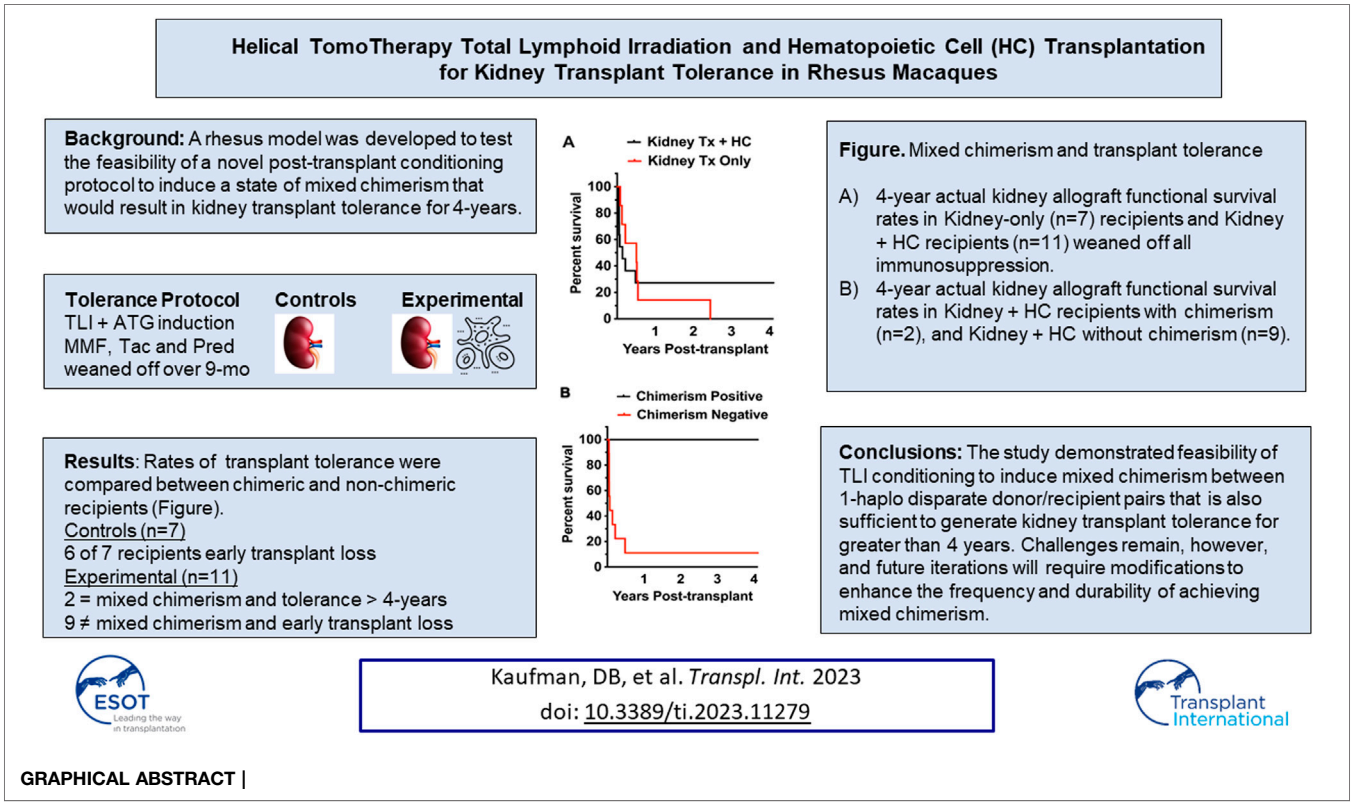
Abbreviations: ATG, Anti-thymocyte globulin; DC, Dendritic cells; DSA, Donor specific antibody; GVHD, Graft-versus-host disease; TomoTherapy TLI, Helical TomoTherapy-based total lymphoid irradiation; HC, Hematopoietic cell; IS, Immunosuppression; MLR, Mixed lymphocyte reaction; MMF, Mycophenolate Mofetil; MDSC, Myeloid derived suppressor cells; NIAID, National Institute of Allergy and Infectious Disease; NIH, National Institute of Health; POD, Post-operative day; RhCMV, Rhesus Cytomegalovirus; STR, Short tandem repeats; TLI, Total lymphoid irradiation; WNPRC, Wisconsin National Primate Research Center.



GRAPHICAL ABSTRACT |

INTRODUCTION

Complete elimination of immunosuppressive (IS) medications in solid organ transplant recipients results in allograft rejection and graft loss unless host immune tolerance to the donor organ is induced. A promising development in allograft tolerance induction is the creation of a chimeric immune state within the transplant recipient comprised of both host and donor immune cellular elements [1–15]. This can be accomplished by application of a conditioning protocol to the recipient followed by hematopoietic cell (HC) transplantation from the organ donor into recipient. Establishing a chimeric state allows IS elimination without organ allograft loss from rejection [1–16].

Of particular relevance to this report is the success in human studies applying a post-transplant, non-myeloablative, total lymphoid irradiation (TLI) and anti-thymocyte globulin (ATG) conditioning protocol to achieve engraftment of donor HCs that produce a stable mixed chimeric state without graft versus host disease (GVHD) [2–8, 17–19]. The development of a chimeric state permitted elimination of all IS without inducing cellular- or antibody-mediated immune injury among HLA-identical living related pairs [5–7].

The non-human primate (NHP) model described in this study replicated the human protocol. The purpose of this translational study was to inform future clinical development of the TLI tolerance induction protocol as applied to donor/recipient pairs with greater MHC disparity than the current clinical studies between HLA-identical transplants. Human studies involving TLI-induced tolerance had not previously been conducted in recipients of 1-haplo matched living donor kidneys.

This pre-clinical primate study examined the feasibility of a novel post-transplant, non-myeloablative conditioning protocol comprised of helical tomotherapy-based TLI (TomoTherapy TLI) and ATG in conjunction with donor peripheral blood mobilized HC infusion to induce a state of mixed chimerism in a rhesus macaque 1-haplotype MHC matched living related kidney transplant model [20]. We hypothesized that the chimeric state would result in operational tolerance for 4-years and without GVHD. We evaluated the extent to which this tolerance induction protocol induced recipient immunomodulation, the frequency and durability of achieving mixed chimerism, rates of GVHD and rejection, generation of Class I and II donor-specific antibody (DSA), CMV reactivation, and the rate of long-term (4-year) kidney transplant operational tolerance.

MATERIALS AND METHODS

Animals and Determination of Donor-Recipient Pairs

Male and female rhesus macaques were obtained from the NIAID colony maintained by Alpha Genesis Inc. (Yemassee, SC) (**Table 1**). All animals were treated in accordance with the 8th edition of the Guide for the Care and Use of Laboratory Animals published by National Research Council and the procedures and

TABLE 1 | Hematopoeitic cell therapy and transplant outcome by experimental group.

ID	Weight (kg)	Sex	Relationship	MHC type	TNC (x 106 kg)	$CD3^+$ cells (x 106 kg)	$CD34^+$ cells (x 106 kg)	Kidney survival (days)	Outcome
Kidney Transplant Only									
C1	5.7	F	Mother/Child	A016, A003, B024a, B017a, DR35, DR04a	-	-	-	45	AMR
C1-D	11.4	F		A016, A001, B024a, B043b, DR35, DR03f					
C2	11.0	M	Mother/Child	A004, A008, B012b, B047a, DR04a, DR09a	-	-	-	188	Rejection/ volvulus
C2-D	5.3	F		A004, A019, B012b, B048, DR04a, DR03f					
C3	6.4	F	Mother/Child	A004, A023, B017a, B012b, DR11c, DR15ab	-	-	-	1089	CR/AMR
C3-D	6.6	F		A004, A023, B017a, B055, DR11c, DR04a					
C4	4.9	F	Siblings	A001, A008, B024a, B012a, DR15a/b, DR03f	-	-	-	199	PTLD
C4-D	5.5	M		A001, A008, B024a, B069b, DR15a/b, DR04a					
C5	6.1	M	Siblings	A008, A004, B-unk, B001a, DR05a, DR04a	-	-	-	29	Failure to Thrive, AKI
C5-D	5.1	F		A008, A006, B-unk, B043a, DR05a, DR03f					
C6	5.7	M	Mother/Child	A004, A002a, B002, B012a, DR06, DR03f	-	-	-	79	AMR
C6-D	7.9	F		A004, A004, B002, B028, DR06, DR14a					
C7	5.2	M	Mother/Child	A018a, A002a, B002, B012a, DR27b, DR03f	-	-	-	185	Peritubular capillaritis
C7-D	9.6	F		A018a, A004, B002, B048, DR27b, DR15c					
Kidney + Hematopoietic Cell Transplant									
E1	5.7	M	Mother/Child	A025, A023, B017a, B012b, DR16, DR15c	440	57	2.7	>3416	Survival
E1-D	6.7	F		A025, A004, B017a, B048, DR16, DR15c					
E2	9.0	M	Mother/Child	A008, A224a, B055, B001a, DR06, DR03a	1250	163	15.4	176	AMR
E2-D	10.0	F		A008, A004, B055, B012b, DR06, DR03f					
E3	10.0	M	Mother/Child	A002a, A065, B017a, B003a, DR03f, DR04c	1111	479	4.1	19	Engraftment syndrome
E3-D	7.2	F		A002a, A006, B017a, B047a					
E4	5.4	F	Mother/Child	A001, A004, B012a, B012b, DR04a, DR05a	1464	173	6.6	18	Engraftment syndrome
E4-D	7.2	F		A001, A001, B012a, B043b, DR04a, DR03f					
E5	6.2	F	Siblings	A006, A004, B001a, B001a, DR03a, DR04a	400	176	1.5	1652	CR/AMR
E5-D	9.2	M		A006, A006, B001a, B043a, DR03a, DR03f					
E6	6.1	M	Mother/Child	A004, A004, B015a, B055, DR03a, DR03e	1900	617	4.5	79	Infection (Parvovirus)
E6-D	7.0	F		A004, A224a, B015a, B045a, DR03a, DR16					
E7	10.9	M	Siblings	A016, A002a, B001a, B015a, DR13a, DR15a	320	157	4.1	24	Infection (CMV)
E7-D	5.5	M		A016, A001, B001a, B001a, DR13a, DR01c					
E8	5.2	F	Siblings	A002a, A002a, B012a, B012a, DR03f, DR03f	680	97	0.7	16	Engraftment syndrome
E8-D	9.3	M		A002a, A002a, B012a, B001a, DR03f, DR02					
E9	10.6	M	Mother/Child	A026, A004, B001a, B001a, DR16, DR04a	106	12.5	1	50	AMR
E9-D	9.8	F		A026, A004, B001a, B012b, DR16, DR04a					
E10	6.1	F	Mother/Child	A004, A028, B012a, B012a, DR03f, DR04a	1144	356	4	18	Engraftment syndrome
E10-D	8.9	F		A004, A028, B012a, B048, DR03f, DR01a					
E11	5.7	M	Siblings	A001, A004, B001a, B002, DR01c, DR06	1373	183	8.4	1814	CR/AMR
E11-D	8.9	M		A001, A016, B001a, B001a, DR01c, DR13a					

protocol were approved by the University of Wisconsin-Madison institutional animal care and use committee.

Animals were 4.9–10.0 kg and 3.3–12.1-years-old at the time of the procedures. Both males and females were used as donors and recipients. MHC Class I and Class II typing of recipient and donor animals were performed by the WNPRC Genetics Services Unit as previously described [21, 22]. These MHC haplotyping results, along with pedigree analysis, were used to determine appropriate 1-haplotype matched donor/recipient pairs for each transplant. The kidney allografts came from blood group compatible, MHC 1-haplotype matched donors (either full siblings or maternal donors). Anti-donor T and B cell flow cytometric crossmatches were negative in all recipients pre-transplant.

Investigative Protocol Design

Rates of kidney transplant tolerance were compared between the controls ($n = 7$, no HC infusion) and the experimental ($n = 11$, HC infusion) groups. All recipients received the same post-transplant conditioning protocol that included TLI and ATG. All animals had the IS eliminated according to the same tapering schedule (**Figure 1**). Seven animals in the control group received kidney transplants alone, and 11 animals in the experimental group received a combined kidney transplant plus the mobilized peripheral HC product. We monitored for donor immune cell engraftment in the experimental (kidney + HC) group by determining the frequency and durability of the chimeric state within multiple immunologic lineages.

Conditioning and Immunosuppression Protocol

A novel method of TLI delivery using helical TomoTherapy (TomoTherapy TLI) was implemented as previously reported [20]. Briefly, TomoTherapy TLI was administered to the recipients on post-operative day 1 following kidney transplantation. The animals received 120cGy radiation divided over 10 doses similar to the dosing protocol in human trials (**Figure 1**). Donor peripheral blood mobilized HCs were collected as previously described for infusions occurring on post-transplant day 11 [23]. The native kidneys were removed immediately following renal allo-transplantation and submitted to pathology for documentation of their removal.

Induction and Maintenance Immunosuppression

The immunosuppression protocol is illustrated in **Figure 1**. All rhesus transplant recipients received induction therapy consisting of five consecutive daily doses of 4 mg/kg anti-thymocyte globulin [ATG - either Thymoglobulin®, or the rhesus specific ATG generated by the NIH Nonhuman Primate Reagent Resource (supported by HHSN272200900037C and OD010976)] beginning at the time of kidney transplantation (day 0). Animals also received methylprednisolone (2 mg/kg), acetaminophen (5 mg/kg), and diphenhydramine (1 mg/kg) intravenously immediately prior to ATG infusion to minimize adverse reactions to the medication. Post-transplant maintenance IS consisted of corticosteroids, mycophenolate mofetil (MMF) administered orally at 15 mg/kg BID, and tacrolimus administered intramuscularly at 0.03 mg/kg BID with levels monitored 1–2 times per week to maintain a 12-hour trough level of 8–10 ng/mL.

Immunosuppression Elimination Protocol

All recipients underwent IS withdrawal as planned (**Figure 1**). The corticosteroids were weaned and eliminated over post-transplant week 1. Beginning on day 90 post-transplant MMF was tapered 25% every 2 weeks. Subsequently, 2 weeks after completing the MMF withdrawal (month 4.5), the tacrolimus taper began by reducing the dosage approximately 25% each month to achieve decreasing trough levels of 6–8 ng/mL (month 5), 4–6 ng/mL (month 6), and 2–4 ng/mL (month 7) before being eliminated by month 8. Thereafter no IS therapy was administered to the recipient, nor was acute rejection treated with adjuvant IS at any point during the protocol.

Helical TomoTherapy for Total Lymphoid Irradiation

Details of the Helical TomoTherapy TLI protocol has been previously reported [20]. TLI was planned and delivered by imaged-guided, intensity modulated helical TomoTherapy (TomoTherapy Hi-Art II, Accuray Inc, Sunnyvale CA). The total lymphoid target included the inguinal, iliac, sublumbar,

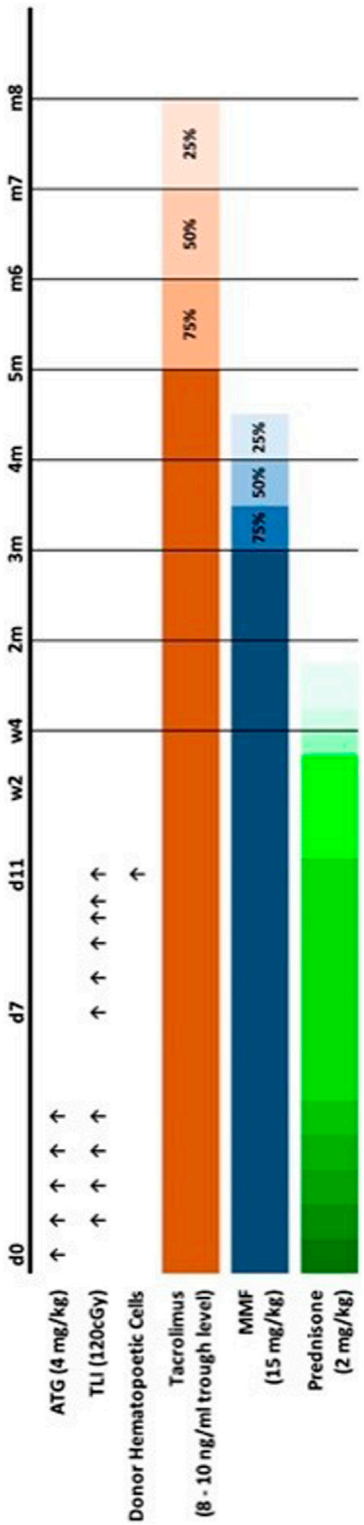


FIGURE 1 | TomoTherapy TLI/ATG tolerance induction protocol and maintenance immunosuppression weaning.

para-aortic, axillary and mandibular lymph nodes, as well as the spleen and anterior mediastinal/thymic tissues.

Donor Peripheral Blood Mobilized CD34$^+$ Hematopoietic Cell and CD3$^+$ T-cell Collection

Details of the apheresis procedure has been previously reported [23]. Briefly, donor animals received G-CSF (50 mcg/kg/d) for four consecutive days prior to, and on the day of, apheresis. In addition, one dose of plerixafor (Mozobil) (1 mg/kg) ~3 h prior to apheresis of peripheral blood hematopoietic cells was administered. Flow cytometric analyses were performed on the apheresis product before freezing and prior to infusion for determination of frequency and total numbers of donor peripheral blood CD34$^+$ and CD3$^+$ cells.

Chimerism Assessment

The chimeric state was assessed in the recipients by measuring the proportion of donor cells and the immunologic subset of each subtype. Chimerism was measured in the peripheral blood and bone marrow compartments of the recipient using a PCR-based assay. DNA was purified from peripheral whole blood cells (Qiagen Blood kit; with minor modifications) to be used as template for PCR with fluorescent-labeled primers specific to microsatellite loci (Dr. Cecilia Penedo, Veterinary Genetics Laboratory, UC Davis, CA) [24, 25]. To assess chimerism in specific cellular compartments, lymphocytes were fractionated from granulocytes using Lymphocyte Separation Media (Mediatech, Manassas, VA). DNA was then purified from the granulocyte fraction and MACS (Miltenyi Biotec, Auburn, CA) separated subsets of CD3$^+$ T cells, CD20$^+$ B cells, and non-CD3/CD20 cells (commercial human separation kits with known NHP cross-reactivity were utilized). The limit of detection was approximately 2%–4% by STR analysis.

Donor Specific Antibody Monitoring

A standard flow cytometry-based assay was utilized to measure pre- and post-transplant donor-specific antibodies (DSA) in recipients. Plasma isolated from peripheral blood before and serially after transplantation was diluted 1:25 and incubated separately with donor (experimental) and recipient (control) PBMC. Cells were washed and stained for T cells (CD3) and B cells (CD20), as well as with the anti-rhesus IgG1/IgG3 antibody, 1B3-FITC (NHP-Reagent Resource, Boston, MA). After incubation and washing, cells were fixed and flow was acquired using an Accuri C6 flow cytometer (BD biosciences, San Jose, CA). Data was analyzed using FlowJo software (FlowJo LLC, Ashland, OR). Post-transplant FITC MFI shift on donor T (expressing only MHC class I) or B cells (expressing both MHC class I and II) of more than 2-fold compared to pre-transplant plasma or self-cell controls was considered positive for antibody.

Rhesus Cytomegalovirus (rhCMV) Monitoring

Plasma samples were analyzed for evidence of rhCMV reactivation by detection of rhCMV DNA. The plasma samples were purified on a QIASymphony DNA Extraction System according to manufacturer protocols (QIAGEN) [26, 27]. DNA from a volume of 350 µL of plasma were extracted and eluted in an equivalent volume so that there was no dilution or concentration of any viral DNA. RhCMV genome copy numbers were quantified by qPCR assay methods based on a gB primer/probe set with a broad linear range of sensitivity [27]. Samples (5 µL of purified DNA) were analyzed in triplicate with a QuantStudio Flex 6 sequence detection system (Applied Biosystems). Each qPCR plate contained a 10-fold serial dilution of a CsCl-purified plasmid standard (10^6–10^0 plasmid molecules per 5 mL) containing the amplicon in order to provide a standard curve for each plate. Plate results were considered valid only if efficiencies were 90%–110%. qPCR results were normalized to RhCMV genomes/mL of plasma.

Immune Suppressive Function of Recipient Myeloid-Derived Suppressor Cells

Ficoll gradient enriched PBMCs were labelled with CFSE according to manufacturer protocols (Invitrogen/Thermo Fisher Scientific). T cells were enriched from CFSE labeled cells using a negative selection kit that eliminates all non-T cells (Stem Cell Technologies) according to manufacturer's protocol. Recipient Lin$^-$CD11b^{hi}DR^{lo} MDSCs, Lin$^-$CD11b^{hi}DR^{hi}CD14^{hi} monocytes, and Lin$^-$CD11b^{lo}DR^{hi}CD11c$^+$CD14$^-$ DCs were sorted on a FACSAria sorter (BD Biosciences) to >90% purity to be added to cultures to be assayed for suppressor activity (**Supplementary Figure S1**). Following enrichment and labeling with CFSE, T cells were resuspended to 1×10^6 cells/mL in complete IMDM medium, and placed in 96 well U bottom tissue culture plates (1×10^5/well) for 5 days at 37°C in 5% CO_2/air either alone or in the presence of anti-CD2/CD3/CD28 NHP T cell activation microbeads (Miltenyi Biotec). These anti-CD2/CD3/CD28 T cell beads were added at a ratio of one bead per eight T cells. MDSCs, monocytes, or DCs (5×10^4 cells/well, 1 suppressor cell per 2 T cells) were added to parallel wells containing T cells plus beads for co-culture as described above. Cultured cells were stained with fluorochrome labeled antibodies specific to CD3, CD4, and CD8 as well as T cell activation/differentiation markers. CFSE was measured to determine T cell proliferation (CFSE low indicating replicative cycles). Suppression of proliferation was calculated using the following formula: 1-[(%CFSEdim T cells from T+beads+suppressor cell co-cultures)/(%CFSEdim T cells from T+beads co-cultures)] x100.

RESULTS

Outcomes and Association of Mixed Chimerism With Long-Term Graft Acceptance

In the control group of kidney transplant-only transplants, 6 of 7 recipients lost renal allograft function early during the period of IS elimination (**Table 1**; **Figure 2A**). Graft survival in those

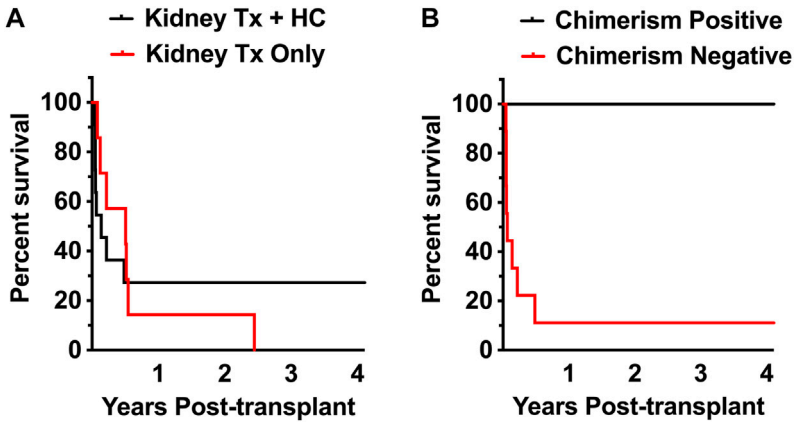


FIGURE 2 | Peripheral donor cell chimerism is associated with kidney allograft tolerance. **(A)** 4-year actual kidney allograft functional survival rates in Kidney-only (n = 7) recipients and Kidney Tx + HC recipients (n = 11) weaned off all immunosuppression. **(B)** 4-year actual kidney allograft functional survival rates in Kidney Tx + HC recipients without chimerism (n = 9), and Kidney Tx + HC with chimerism recipients (n = 2).

animals ranged from 29–199 days. One animal demonstrated prolonged graft survival 1,069 days, though it eventually failed to chronic antibody-mediated rejection.

Animals in the experimental group (n = 11) received between 1–19 × 10^8 total nucleated cells/kg comprised of 0.7–16 × 10^6 $CD34^+$ cells/kg and 12–617 × 10^6 T cells/kg and (**Table 1**). Renal allograft functional survival rates are shown in **Figure 2**. Graft survival ranged from 16 days to >4 years in the experimental Kidney Tx + HC cohort. Three of 11 Kidney Tx + HC animals had graft survival greater than 4-years (**Figure 2A**). The two recipients that achieved mixed chimerism each acheived kidney transplant tolerance greater than 4-years and without GVHD (**Figure 2B**).

Donor cell chimerism in the experimental group could be ascertained in whole blood using STR analysis in 6 Kidney Tx + HC animals that exhibited greater than 30 days of survival (**Figure 3A**). Two of the 6 Kidney Tx + HC animals demonstrated transient mixed chimerism. The highest level of engraftment of donor-derived lymphocytes peaked at 20% by day 35, and was lost by 4 months post-induction. Bone marrow analysis revealed that animals with peripheral chimerism also had detectable chimerism within the lymphohematopoietic compartment (**Figure 3B**). None of the animals in the experimental group developed GVHD.

TomoTherapy TLI and ATG Conditioning on Leukocyte Depletion

The efficacy of the tolerance induction protocol on recipient lymphopenia was assessed. TLI/ATG conditioning resulted in

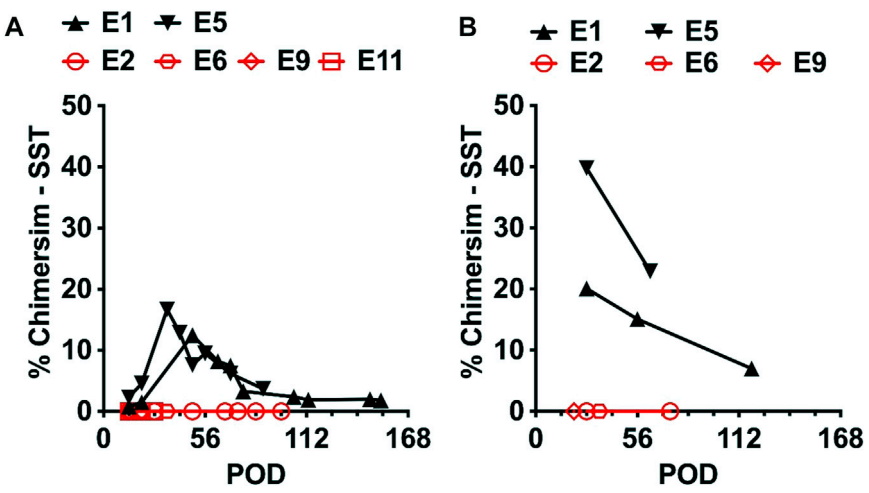


FIGURE 3 | Kinetics of mixed chimerism in the Kidney Tx + HC experimental group (N = 6) as measured by short tandem repeats (STR) in the: **(A)** peripheral blood, and **(B)** bone marrow. Animals E1 and E5 achieved transient mixed chimerism in the peripheral blood and bone marrow. Animals E2, E6, E9 and E11 did not achieve chimerism. Bone marrow was not examined for chimerism in animal E11.

prolonged leukocyte depletion (**Figures 4A–C**) in the peripheral blood. Lymphocyte numbers dropped precipitously by day 2 following initiation of TLI/ATG, reaching the average nadir of <200/μL between days 4 and 7. Recovery, defined as a statistically significant increase in lymphocyte numbers compared to days 4 and 7, was delayed until day 25. Nadir for neutrophils (days 16–18) and monocytes (days 11–14) occurred later compared to lymphocytes, with recovery beginning at approximately the same time as lymphocytes. Upon observation of lymphocyte recovery by day 25, CD8 T cells were first to emerge, followed by NK and B cells, respectively (data not shown). CD4 T cell recovery occured at a similar pace, but only to 30% of pre-transplant values (data not shown).

TomoTherapy TLI and ATG Conditioning on Immune Modulation

We examined a unique mechanism of host immune modulation in the rhesus model related to TLI/ATG conditioning that is known to promote chimerism and prevent GVHD in humans and small animals [28, 29]. TLI/

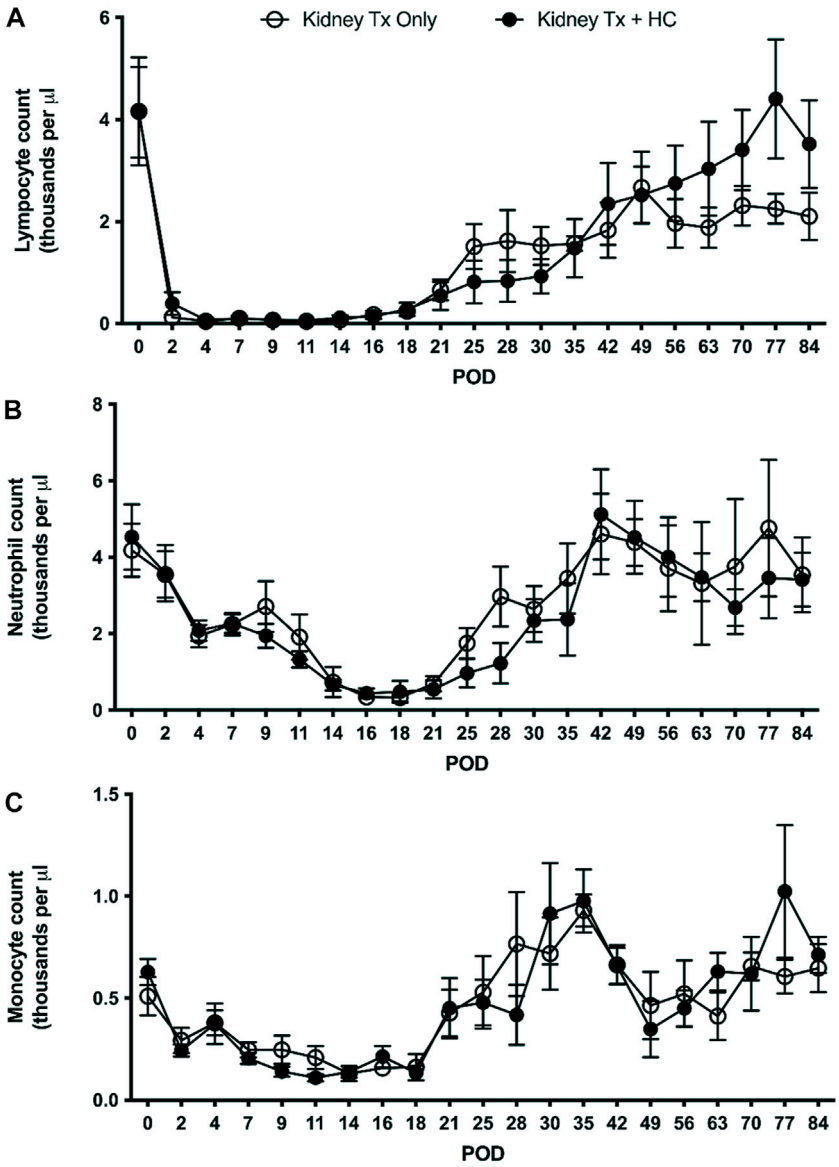


FIGURE 4 | TomoTherapy TLI/ATG effect on blood leukocyte depletion in recipient cell subsets. The number of **(A)** lymphocytes, **(B)** neutrophils and **(C)** monocytes per microliter were determined by complete blood cell count every 2–7 days for Kidney Tx only and Kidney Tx + HC in recipients exhibiting at least 30 days of follow-up.

ATG-induced suppressive activity of host peripheral blood myeloid derived suppressor cells (MDSCs; $Lin^{-}CD11b^{hi}HLA\text{-}DR^{lo/-}$), monocytes ($Lin^{-}CD11b^{hi}HLA\text{-}DR^{hi}CD14^{hi}$) and dendritic cells (DCs; $Lin^{-}CD11b^{lo}HLA\text{-}DR^{hi}CD11c^{+}CD14^{-}$) on host $CD4^{+}$ and $CD8^{+}$ T cells were tested. In these studies the percent change of CD8 and CD4 T cell subset stimulated proliferation induced by MDSCs, monocytes, DCs, before and at serial timepoints (1.5–3 months, 6–12 months, 12–24 months) after transplantation were measured *in vitro* in several Kidney Tx + HC recipients that received the TomoTherapyTLI/ATG tolerance induction protocol. During the first 4 months post-therapy, both MDSCs and monocytes were able to strongly suppress proliferation of bead-activated autologous CD4 and CD8 T cells (**Figure 5**). DCs, in contrast, demonstrated less suppressive activity, which was only observed among CD4 T cells populations. Mean percentages of each cell type were compared using the two-tailed Student's t-test to determine statistical significance. The suppression effects abated in samples drawn from transplant recipients 6–12 months post-transplant as expected and that has been observed in human studies [29].

Effect of DSA and CMV on Chimerism

The effect of post-transplant DSA and reactivation of CMV on rates of chimerism were assessed in the six animals in the experimental group that had survival of at least 50 days. Achieving mixed chimerism was associated with an absence of anti-donor specific antibody as measured by B-cell and T-cell flow crossmatch (**Figures 6A, B**). Conversely, animals that failed to achieve mixed chimerism developed HLA Class II DSA. With respect to CMV reactivation, all animals had detectable increases in peripheral blood CMV titer. No level of CMV titer correlated with either achieving or failing to achieve chimerism (**Figure 6C**).

Post-HC Infusion Engraftment Syndrome

Four animals in the Kidney Tx + HC experimental group developed immediate, aggressive and irreversible engraftment syndrome. All 4 animals exhibited rapid acute renal allograft functional decline secondary to non-immune mediated injury (**Figure 7A**). Histological analysis of the kidneys revealed hyaline casts, interstitial hemorrhage and tubular necrosis (**Figure 7B**, representative sample).

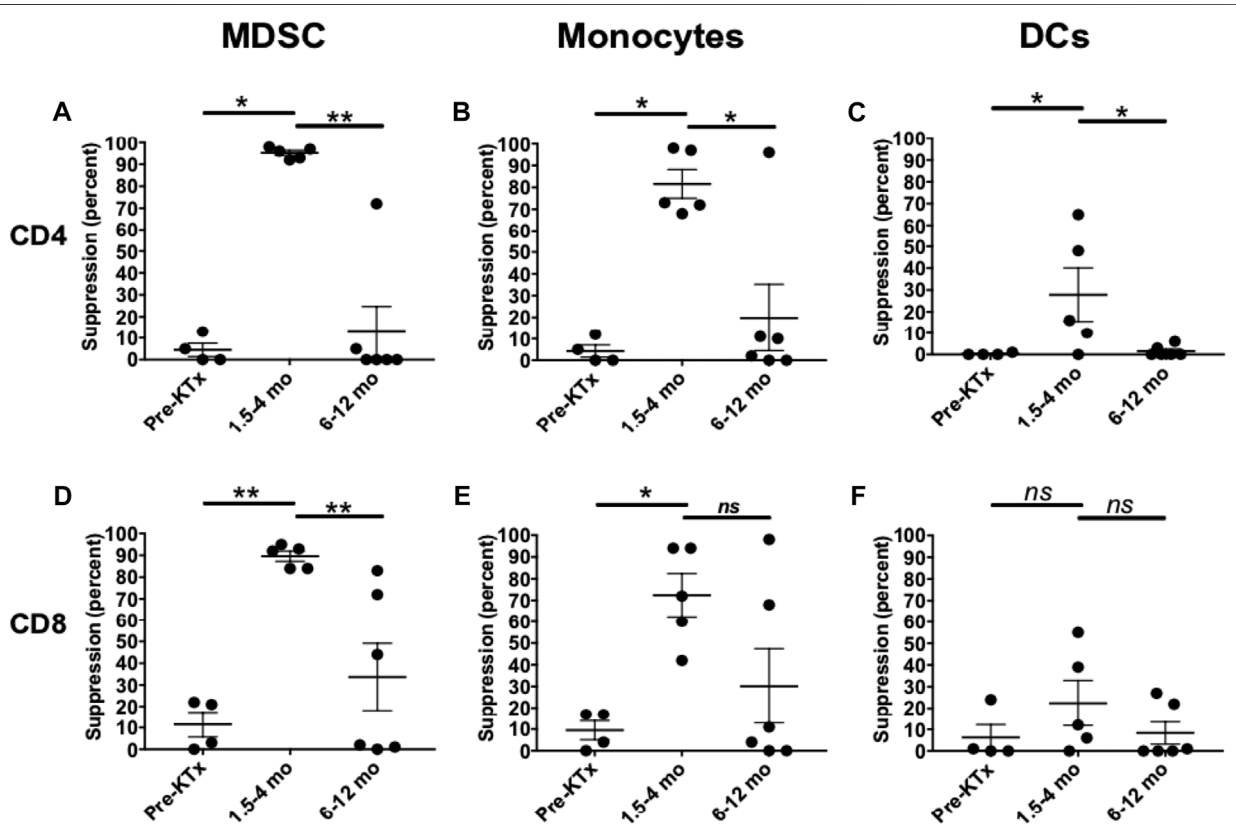


FIGURE 5 | Recipient peripheral blood MDSC and monocytes exhibited immunosuppressive activity following TomoTherapy TLI/ATG tolerance induction. MDSC **(A, D)**, monocytes **(B, E)** and DCs **(C, F)** were isolated at various timepoints and co-cultured with CFSE labelled autologous T cells in the presence of T cell stimulation beads for 5 days. The % suppression of the proliferative response of CD4 T cells **(A–C)** or CD8 T cells **(D–F)** cultured without myeloid cell subsets was determined. Statistical comparisons were made using Student's t-test, (* = $p < 0.05$; ** = $p < 0.01$).

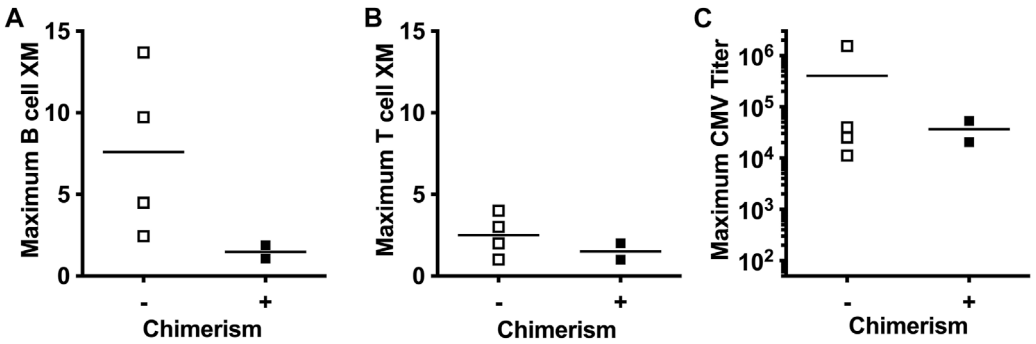


FIGURE 6 | Achieving peripheral donor cell chimerism is associated with absence of generation of antidonor antibody and not CMV reactivation titers. Peak post-transplant anti-donor antibody levels in Kidney Tx + HC recipients with (+) and without (–) Chimerism, as measured by B-cell **(A)** and T-cell **(B)** flow crossmatch. **(C)** Peak post-transplant CMV titers in Kidney Tx + HC recipients with (+) and without (–) Chimerism.

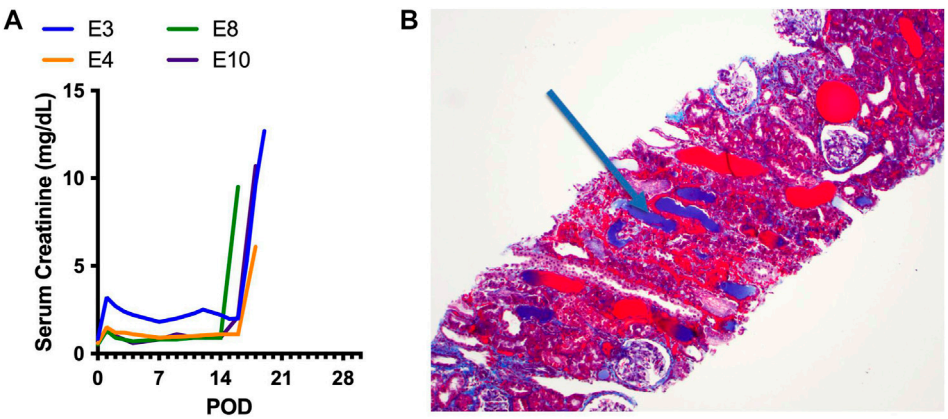


FIGURE 7 | Clinical course of engraftment syndrome. **(A)** Four animals that received a Kidney Tx + HC infusion experienced a rapid increase in creatinine 5–7 days after HC infusion (post-transplant days 16–18). **(B)** Histological analysis of a representative early kidney biopsy demonstrating hyaline casts, interstitial hemorrhage, and tubular necrosis.

DISCUSSION

To achieve kidney transplant tolerance in this rhesus kidney transplant model we used a TLI tolerance induction protocol to create mixed chimerism based on the Stanford clinical protocol [3, 4, 7, 8, 17–19, 30]. TLI tolerance induction protocols in human studies have demonstrated that stable mixed chimerism permits complete elimination of all IS and operational tolerance in well-matched HLA-identical donor/recipient pairs [2–9]. This rhesus model was developed to test the feasibility of TLI condtioning to create mixed chimerism in more disparate 1-haplotype mismatched recipients of combined kidney and HC transplants. To optimize the translational impact of this study we applied a novel TomoTherapy TLI delivery system first developed and reported in primates by this group that also utilized conditioning and maintenance agents approved for human use by the FDA [20]. We then tested the hypothesis that animals achieving chimerism would become operationally tolerant to the kidney allograft for up to 4-years.

The biological underpinings of TLI conditioning to generate chimerism relates to its immunomodulatory effects that have been studied extensively in small animal models and in human pilot studies [13, 29, 31]. In murine studies TLI conditioning was found to activate host MDSCs as characterized by the increased expression of arginase-1, IL-4Rα and programmed death ligand 1 [28]. It was determined that host MDSCs were required for chimerism and tolerance induction in the combined organ and BM transplant model after TLI/ATG conditioning [28]. Depleting this population by monoclonal antibody therapy abrogated chimerism and tolerance while adding back these cells led to restoration of both phenomena. In addition, *in vitro* immunomodulatory properties of the MDSCs demonstrated development of immune suppressive capacity that inhibited the proliferation of host CD8 and CD4 T cell subtypes in response to donor-specific stimulation in an allo-MLR [28]. Importantly, these findings were recapitulated in human subjects involved in TLI/ATG-based chimerism induction studies [29].

In this rhesus model, the three types of myeloid cells (MDSCs, monocytes, and DCs) were detectable in the peripheral blood of the transplant recipients. Importantly, the host MDSCs, monocytes and DCs *in vitro* demonstrated development of immunomodulatory function through suppression of the T cell proliferative response to the presence of potent anti-CD3/CD28 T cell activation beads in culture similar to that previously reported in the human studies [29]. These findings demonstrated for the first time that the TomoTherapy TLI/ATG conditioning methodology applied to the rhesus model was consistent with the immunomodulatory effects achieved with conventional TLI/ATG methodology applied in the human pilot trials [29].

The efficacy of the TomoTherapy TLI/ATG conditioning protocol to generate chimerism had been previously established in rhesus macaques receiving HCs but without the renal transplant [20]. Building on those earlier studies, this study demonstrated that TLI conditioning followed by HC infusion would also generate mixed chimerism in the experimental kidney transplant cohort and proved the hypothesis that chimeric animals would exhibit operational tolerance for over 4-years after IS was withdrawn.

Though our study demonstrated the feasibility of the TLI conditioning protocol to acheive chimerism among the more widely disparate donor/recipients pairs, it proved to be more challenging than what has been observed in well-matched HLA-ID pairs in the human studies, and akin to the challenges in ongoing human 1-haplo matched studies (Stephan Busque, personal communications). However, the observation that operational tolerance was achieved with transient mixed chimerism in this model is consistent with others' observations that sustained chimerism is not a requirment to successful withdraw maintenance IS without inducing rejection [12, 32].

The chimerism rates in 1-haplo matched rhesus donor/recient pairs was observed much less frequently than that reported in well-matched HLA-identical human recipients. Six experimental animals survived >30 days post-transplant and were evaluable for chimerism, 2 of which achieved transient mixed chimerism in the peripheral blood for up to 112 days. The chimeric monocyte and T-cell subpopulations demonstrated levels similar to those observed in human studies (10%–20%), though at absolute levels less than subjects that achieved stable mixed chimerism. Both also demonstrated transient mixed chimerism in the bone marrow. Interestingly, transient mixed chimerism in the rhesus model was associated with IS-free graft survival indicating that persistent chimerism was not necessarily required for the induction of operational tolerance in this model.

Two potential impediments to achieving mixed chimerism were investigated. These included the development of *de novo* DSA and the occurrence of CMV reactivation. Importantly, the avoidance of DSA correlated with the induction of chimerism. Peak antibody generation against MHC Class II was higher than Class I, indicating that Class II-targeted DSA may be an important barrier to HC engraftment. In this rhesus model all animals experienced CMV reactivation despite prophylactic treatment with anti-virals. However, CMV reactivation was generally not clinically significant and resolved on standard anti-viral therapy without complication. The high frequency of CMV reactivation is an important characteristic of this and other tolerance induction strategies in the rhesus model [33]. Interestingly, the intensity and duration of reactivation, as determined by peripheral blood viral load measurements, were similar in chimeric and non-chimeric animals. This contrasted to results in the Cynologous model indicating that CMV viremia could have a detrimental effect on durable engraftment [34]. This will require further study across the various non-human primate study groups, as this effect could be animal specific and protocol dependent.

The study also demonstrated that four animals in the experimental group experienced rapid and irreversible renal failure with histological findings of interstitial hemorrhage and tubular necrosis consistent with engraftment syndrome. Differences in the donor HC product, as indicated by total number of nucleated cells, T cells, or $CD34^+$ cells infused, did not correlate with the development of engraftment syndrome. Furthermore, comparison of the level and duration of leukocyte depletion among all animals within this cohort indicated that the induction agents were consistently effective regardless of the development of engraftment syndrome and did not likely account for the resulting rapid graft loss in such cases. Based on these observations, changes to the maintenance IS regimen, such as intensifying the corticosteroids, represents an opportunity to improve outcomes in future iterations to mitigate occurrences.

Future iterations of the TomoTLI/ATG, non-myeloablative, post-transplant tolerance induction protocol in the rhesus model will require several modifications to enhance the frequency and durability of achieving mixed chimerism. Specifically, modifications to the early maintenance IS regimen will be needed to reduce the risk of engraftment syndrome. In, addition, including a short course of co-stimulatory blockade, such as belatacept, may prevent *de novo* DSA development, as has been observed after adding it to the TomoTherapy TLI protocol in HC-transplant recipients [20].

Another interesting observation was the prolonged survival after IS elimination in one control animal and in an animal in the experimental group that did not achieve chimerism. Eventual kidney failure occurred in both and revealed histologic findings of acute and chronic antibody-mediated rejection in addition to moderate interstitial fibrosis and tubular atrophy. All the recipients received TLI/ATG. It is known from human studies that TLI/ATG added to a conventional immunosuppression protocol in high-risk renal re-transplant recipients has been associated with prolonged survival and development of donor-specific hyporesponsiveness [35–37]. Though the numbers are small, by trend, it appears that transient mixed chimerism is sufficient for prolonged tolerance, but not an absolute necessity. An occasional recipient receiving ATG/TLI induction does achieve prolonged survival after immunosuppression has been weaned off in this model.

In summary, there were several important insights gained from this model. Promising results were demonstrated in this

tolerance induction protocol applied to 1-haplotype matched donor/recipient pairs that permited the elimination of all IS without rejection or GVHD while maintaining 4-years of operational tolerance of the renal allograft. Opportunities to enhance the results were also presented. Eliminating engraftment syndrome and improving the rate of mixed chimerism will be crucial to the extension of this protocol to more disparate MHC barriers. Additionally, mechanistic studies will need to be continued and expanded in order to elucidate the immunologic mechanisms underlying mixed-chimerism based tolerance, so as to leverage potential targets and manipulations for future induction strategies. Knowledge gained through this rhesus tolerance induction model could possibly have direct relevance to a wide variety of donor transplants including deceased donation cases, which would dramatically expand the implementation of tolerance induction protocols beyond the limited pool of living related pairs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and use Committee, University of Wisconsin-Madison.

AUTHOR CONTRIBUTIONS

DK: Designed and conducted transplant experiments, Manuscript composition and editing. LF: Designed and conducted the TomoTherapy TLI treatments, manuscript editing. JF: Conducted immunological studies, prepared the figures and table. JP: Conducted transplant studies, primate transplant care and TLI treatments. JC: Conducted primate transplant care. LH: Conducted apheresis and HSC collection and infusions, and study manager. WH: Conducted chimerism studies and measurements. NC: Conducted the TomoTherapy TLI treatments. WZ: Conducted pathology analysis of kidney transplants. CL: Manuscript editing. AD'A: Conducted transplant studies. LF: Conducted transplant studies. KB: Conducted apheresis procedures and primate care KJ: Conducted immunological studies. WB: Conducted immunological studies. SS: Designed TLI, analyzed data. All authors contributed to the article and approved the submitted version.

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AUTHOR DISCLAIMER

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Rhesus anti-thymocyte globulin reagents used for these studies was provided by the NIH Nonhuman Primate Reagent Resource (P40 OD028116, U24 AI126683).

CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11279/full#supplementary-material

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ORIGINAL RESEARCH
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Factors Associated With Genotypic Resistance and Outcome Among Solid Organ Transplant Recipients With Refractory Cytomegalovirus Infection

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Genotypically resistant cytomegalovirus (CMV) infection is associated with increased morbi-mortality. We herein aimed at understanding the factors that predict CMV genotypic resistance in refractory infections and disease in the SOTR (Solid Organ Transplant Recipients) population, and the factors associated with outcomes. We included all SOTRs who were tested for CMV genotypic resistance for CMV refractory infection/disease over ten years in two centers. Eighty-one refractory patients were included, 26 with genotypically resistant infections (32%). Twenty-four of these genotypic profiles conferred resistance to ganciclovir (GCV) and 2 to GCV and cidofovir. Twenty-three patients presented a high level of GCV resistance. We found no resistance mutation to letermovir. Age (OR = 0.94 per year, IC95 [0.089–0.99]), a history of valganciclovir (VGCV) underdosing or of low plasma concentration (OR= 5.6, IC95 [1.69–20.7]), being on VGCV at infection onset (OR = 3.11, IC95 [1.18–5.32]) and the recipients' CMV negative serostatus (OR = 3.40, IC95 [0.97–12.8]) were independently associated with CMV genotypic resistance. One year mortality was higher in the resistant CMV group (19.2 % *versus* 3.6 %, p = 0.02). Antiviral drugs severe adverse effects were also independently associated with CMV genotypic resistance. CMV genotypic resistance to antivirals was independently associated with a younger age, exposure to low levels of GCV, the recipients' negative serostatus, and presenting the infection on VGCV prophylaxis. This data is of importance, given that we also found a poorer outcome in the patients of the resistant group.

Keywords: risk factors, cytomegalovirus, solid organ transplantation, opportunistic infections, antiviral resistance

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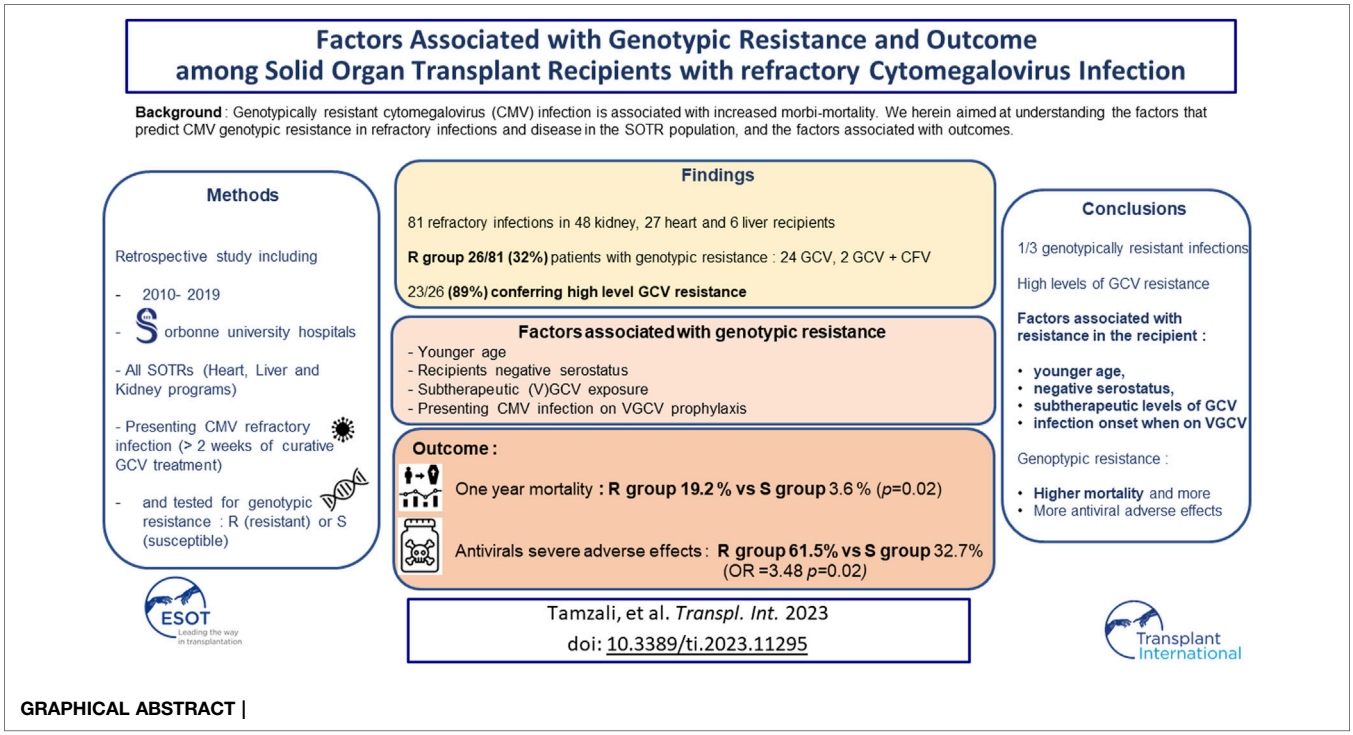

GRAPHICAL ABSTRACT |

INTRODUCTION

Cytomegalovirus (CMV) infection is the most common opportunistic infection in SOTRs (Solid Organ Transplant Recipients), occurring in 10%–40% of the patients, depending on the anti-CMV prophylaxis strategy [1–4]. The morbidity and mortality of CMV infection is now well-established, and its mechanisms more and more understood [1, 5–7].

The topic of ganciclovir (GCV)-resistant CMV infection has emerged in the last decades in the SOTR population [7], with a growing number of cases and an even increased morbi-mortality [5, 7–9]. Among some patients treated with GCV harboring a high viral load, CMV UL97 phosphotransferase mutated strains may emerge, conferring low to high level of resistance to the antiviral. When a low dose of GCV is persistently prescribed because of a low-level resistance, additional mutations in the UL54 DNA polymerase can be selected which confers high-level resistance to GCV and sometimes a cross-resistance to cidofovir (CDV) and foscarnet (FOS) [10]. High dose of GCV or second line antivirals (FOS, CDV) frequently lead to adverse effects, including cytopenia and kidney failure, both potentially affecting treatment success (due to early discontinuation or doses reduction) and impairing transplant and patients' outcomes [5]. Nowadays, CMV resistance to antivirals is generally assessed with genotypic assays (UL97 and UL54 genes sequencing). This implies important technical work, can necessitate up to 2 weeks, sometimes resulting in an empiric dose increase or in a second line antiviral therapy switch. Therefore, understanding the determinants of CMV resistance and its consequences on outcome is critical, especially in SOTRs who already present comorbidities and possibly multiple medications.

We aimed at assessing risk factors for genotypically resistant CMV in the population of clinically refractory SOTRs and the factors associated with outcome (i.e., 1-year mortality and treatments adverse effects).

MATERIALS AND METHODS

Study Design, Setting, and Participants

We performed a retrospective cohort study, by including all SOTRs (kidney, heart and liver transplant programs only in this University Hospital) with a CMV genotypic resistance testing for CMV refractory infection or disease (inclusion only if refractory, see further) between 1st January 2010 and 31st December 2019 in two centers (Pitié-Salpêtrière University Hospital and Tenon University Hospital, Assistance Publique-Hôpitaux de Paris, France).

The patients were divided in two groups according to the results of the resistance testing: susceptible (S) in case of the absence of resistance mutations and resistant (R) in case of the presence of at least one resistance mutation towards GCV, FOS or CDV.

Clinical biological, therapeutic data and outcome were collected from medical charts and the department of virology database. Data about follow-up was collected until 1-year after CMV infection onset.

Clinical Data and CMV Definitions

CMV infection, disease and refractory infection were defined according to the current international recommendations on CMV management and definitions for use in clinical trials [11, 12].

Because of working in the context of refractoriness, CMV infection was defined as the positivity of one quantitative Polymerase Chain Reaction (PCR) on whole blood with a CMV load ≥3.0 log(IU/mL) using the artus® CMV RGQ MDX kit (Qiagen) without any symptoms of the disease.

CMV disease was defined as CMV infection with any involvement of the following organs: eye, liver, digestive tract, bone marrow, lung, kidney, or central nervous system (CNS). Patients presenting with anomalies compatible with CMV syndrome or tissue invasive CMV disease were classified as CMV disease [13].

Refractory CMV infection was qualified in case of meeting the definition of a probable or certain CMV refractory infection as defined by Chemaly et al. [12] i.e., a persistent (<1.0 log decrease, above 1,000 IU/mL) or increased viral load after 14 days of appropriate antiviral treatment. Refractory CMV disease was qualified in case of meeting the definition of Chemaly et al [12]. of a probable or certain CMV disease, i.e., of a lack of improvement of the symptoms after 14 days of appropriate antiviral treatment.

Immunosuppression alleviation was defined as the discontinuation or reduction of 50% or more of the dose of one the following drugs: calcineurin inhibitors, antimetabolites, mammalian Target Of Rapamycin inhibitors and belatacept.

Valganciclovir (VGCV) underdosing was defined either as a daily dose ≤50% of the appropriate dose for more than a week, taking into account the estimated Glomerular Filtration Rate according to the GPR tool [14], or as a documented low GCV plasma concentration defined as a trough level <1 µg/mL when investigating refractoriness.

Serious adverse events were defined as per the food and drugs administration definition (www.fda.org). The cases were adjudicated by two independent clinicians based on chart review.

CMV Prophylaxis, Infection Monitoring and Genotypic Antiviral Resistance Testing

All CMV seronegative transplant recipients receiving a graft from a CMV seropositive donor received oral VGCV prophylaxis for 6 months regardless the type of organ transplanted (no systematic screening until VGCV discontinuation). All CMV seropositive liver transplant recipients received systematic prophylaxis for 3 months (no systematic screening until VGCV discontinuation). CMV seropositive kidney transplant recipients could received a 3-month prophylaxis (no systematic screening until VGCV discontinuation) or be screened weekly for CMV replication for 3 months, then monthly until 1 year and every 2 months until 2 years after transplantation (according to the center). CMV seropositive heart transplant recipients were screened weekly for CMV replication for 3 months, then monthly until 1 year and every 2 months until 2 years after transplantation. All seronegative CMV recipients receiving grafts from a seronegative donor were routinely screened weekly for CMV replication for 3 months, then monthly until 1 year and every 2 months until 2 years after transplantation (no prophylaxis regardless the type of organ transplanted).

After transplantation, CMV replication was monitored at every visit with systematic CMV PCRs in whole blood for all SOTRs without ongoing VGCV prophylaxis for at least 1 year. In case of symptoms or biological anomalies suggesting potential CMV disease, patients were also tested for CMV replication in blood samples and possibly organ biopsies.

CMV DNA was quantified in clinical samples using the artus® CMV RGQ MDX kit (Qiagen).

In case of refractory infection, GCV plasma trough concentration measuring and CMV genotypic resistance testing towards GCV, FOS, and CDV were performed by UL97 and UL54 gene sequencing, as previously described [6, 15].

We also performed retrospectively UL56 and UL89 gene sequencing for the screening of CMV resistance to letermovir (LMV), as previously described [16].

Statistical Analysis

Quantitative variables are presented as mean ± standard deviation or median [IQR] according to their normal or skewed distribution. Comparisons were made using the Student's t-test or the Mann Whitney Wilcoxon test according to their normal or skewed distribution. Qualitative variables are presented as numbers (percentages). The data were compared using the Fisher or Chi^2 test.

We studied the association between genotypic resistance, 12-month mortality, antiviral toxicity and other collected variables. Risk factors for CMV resistance were searched using univariate logistic regression, including all characteristics differently distributed between the cases (CMV resistance to antivirals) and controls (CMV susceptibility to antivirals) (respectively the R and the S groups) with a p-value <0.1. A two-sided p-value <0.05 was considered statistically significant in the univariate analysis. The choice of the adjustment variables for multivariate analysis was made according to the existing literature, the results of the univariate analysis and the models with the lowest Akaike Information Criterion. The factors associated with 1-year mortality and treatment adverse effects were searched using the same method.

Survival analyses were performed using the Kaplan-Meier method. A Log-rank test was performed for the comparison between the two groups, with a p-value threshold at <0.05.

The statistical analysis was made using GraphPad PRISM® (GraphPad Software, San Diego, CA, United States) and RStudio® (R Software Boston, MA 02210).

Ethics

All the patients undergoing healthcare at Assistance Publique-Hôpitaux de Paris agree to the retrospective use of their data by Assistance Publique-Hôpitaux de Paris healthcare providers for research purposes except if mentioning otherwise (http://eds.aphp.fr).

RESULTS

Patients Population

Between 1st January 2010 and 31st December 2019, 3,711 patients received a solid organ transplant in Sorbonne University Hospitals, resulting in a cohort of 32,394 patients-years (patients with a functioning graft). CMV genotypic resistance testing was performed for 81 different SOTRs for refractory CMV infection or CMV disease in the two centers, including 48 kidney transplant recipients (59%), 27 heart transplant recipients (34%) and 6 liver transplant recipients (7%) (**Table 1**).

CMV resistance to at least one antiviral (GCV, FOS, CDV) was detected in about one-third of transplant recipients (26/81, 32%), **Tables 1**, **2**.

Patients Characteristics and CMV Infection Presentation

The patients in the R group were younger (48.7 *versus* 58.8%, $p = 0.022$), more frequently CMV seronegative at the time of transplantation (53.8 *versus* 20.0%, $p = 0.005$), and presented more CMV primary infections than patients in the S group (53.8 *versus* 20.0%, $p = 0.001$). Three patients acquired primary infection when having CMV seronegative donors (one in the R and two in the S group). As expected, the peak viral load was significantly higher in the R group (25,088 [9,422–153,922] *versus* 13,186 [3,132–32,147] IU/mL, $p = 0.02$). Sixty-one percent of the patients in the R group presented a history of VGCV underdosing or a low plasma concentration *versus* 23.6% in the S group ($p = 0.02$) (**Table 2**).

In the multivariate analysis, a younger age (OR = 0.94 per year, IC95 [0.089–0.99]), a history of VGCV underdosing or low plasma concentration (OR = 5.6, IC95 [1.69–20.7]), being on VGCV at infection onset (OR = 3.11, IC95 [1.18–5.32]) and the recipients' CMV negative serostatus (OR = 3.40, IC95 [0.97–12.80]) were identified as risk factors for CMV genotypic resistance (**Table 3**).

CMV Resistance to Antivirals

Resistance mutations in CMV UL97 phosphotransferase and UL54 DNA polymerase found in the 26 patients of the R group are summarized in **Table 2**. Thirteen mutations were found in 26 patients. Two patients presented two mutations (A594V in UL97 and K545S in UL54 for one and M460I in UL97 and F412L in UL54 for the other). Twenty-four of these genotypic profiles conferred resistance to GCV and two conferred resistance to GCV and CDV. All patients but three presented a high level of GCV resistance, one presented a low level of GCV resistance (A594P in UL97) [10, 17, 18] and two presented mutations with undetermined level of resistance (Del GKLTH 598-602 and Del 598-603 GKLTHC in UL97) [11].

We also retrospectively sequenced the CMV UL56 and UL89 terminase complex genes and found no resistance mutation to LMV among the 45 patients with available samples. All patients were naive of LMV and maribavir (MBV).

CMV Management

All patients with refractory non-resistant CMV infection (group S) were treated with oral VGCV or intravenous GCV except for two who were treated with FOS (given clinical refractoriness). The treatments in the R group were distributed as follows: fifteen patients received FOS only, five (V)GCV (three with low levels of GCV resistance and one with severe acute renal failure treated with VGCV and immunomodulation only with favorable outcome), two FOS + MBV, one MBV alone, one specific anti-CMV immunoglobulins, and one no antiviral treatment (immunosuppression [IS] reduction only).

In the S group, 38/53 (71.6%) patients with available information underwent IS alleviation *versus* 23/24 (95.8%) in the R group ($p = 0.03$). The detail about IS alleviation is displayed in **Table 4**.

Outcome

Time to viral clearance was longer in the R group: 105 [67.00, 240.00] *versus* 50 [30.00, 97.50] days, ($p < 0.001$). Three patients in the R group (12%) presented persisting CMV DNAemia by the end of the 1-year follow-up *versus* none in the S group ($p = 0.03$).

Thirty-four patients developed serious anti-CMV treatment toxicity, 16 (61.5%) in the R group and 18 (32.7%) in the S group ($p = 0.03$). Toxicities were mainly represented by FOS-related acute kidney injury in the R group (11/16, 69%) and GCV-related cytopenia in the S group (16/18, 89%). CMV genotypic resistance was independently associated with antiviral drug toxicity (OR 3.48 IC95 [1.21–10.07], **Table 5**).

The overall 1-year mortality was 8.6% (7/81 patients). The mortality rate was significantly higher in the R group (19.2% *versus* 3.6%, $p = 0.015$). The Hazard Ratio for mortality in the R group was HR = 7.4, IC95 [1.5–37.5]).

DISCUSSION

This study focuses on clinically refractory CMV infection/disease in SOTRs, the factors associated with CMV genotypic resistance, and the outcomes, including antiviral drug toxicity and mortality. This cohort included twenty-five cases of genotypically proven resistant CMV infection in transplant recipients, and twice as many clinically refractory infections without resistance. This is one of the largest descriptive cohorts of this type [5–9, 19–21].

In this study, only one-third of the clinically refractory CMV infections were explained by genotypic resistance. All patients with resistant CMV but three presented high level of GCV resistance. Most of the patients presented UL97 isolated mutations, and some UL97 and UL54 mutations, in accordance with previous studies [5, 8]. In cases of critical CMV disease with suspicion of resistance, FOS treatment should be considered. MBV is an alternative recently approved by the Food and Drug Administration for refractory and resistant CMV infection [22] with a better tolerance profile [23].

All patients presented genotypically susceptible CMV to LMV. However, LMV treatment failures–explained by the emergence of LMV resistance in GCV-resistant CMV infections treated with

TABLE 1 | Comparison of patient characteristics, CMV infection and outcome in the R (Resistant) and S (Suceptible) CMV groups.

	S group *N* = 55	R group *N* = 26	*p*
Clinical characteristics			
Age (years, mean ± SD)	54.8 ± 10.0	48.7 ± 12.2	0.02
Sex (male, *n*, %)	42 (76.4)	15 (57.7)	0.1
Transplanted organ (*n*, %)			0.9
Kidney	32 (58.2)	16 (61.5)	
Heart	19 (34.5)	8 (30.8)	
Liver	4 (7.3)	2 (7.7)	
Rank of transplantation (*n*, %)			
1	51 (92.7)	24 (92.3)	0.9
≥2	4 (7.3)	2 (7.7)	
CKD stage ≥ IV[a] (*n*, %)	15 (27.3)	7 (26.9)	1
Induction with antithymocyte therapy (*n*, %)	47 (85.5)	23 (88.5)	1
Immunosuppressive regimen (*n*, %)			
Calcineurin Inhibitors	55 (100.0)	26 (100.0)	1
MMF	55 (100.0)	26 (100.0)	1
Corticosteroids	51 (92.7)	24 (92.3)	1
CMV serostatus and prevention			
CMV serostatus			
Donor negative, recipient negative (D–/R–), *n* (%)	2 (3.6)	1 (3.8)	
Donor positive, recipient negative (D+/R–), *n* (%)	9 (16.4)	13 (50.0)	0.002
Donor positive, recipient positive (D+/R+), *n* (%)	44 (80)	12 (46.2)	
Post-transplant CMV prevention strategy (*n*, %)			
Preemptive treatment	33 (60.0)	8 (30.8)	0.03
Prophylaxis	22 (40.0)	18 (69.2)	
VGCV prophylaxis underdosing or low plasma concentration[b] (*n*, %)	13 (23.6)	16 (61.5)	0.002
CMV infection characteristics			
Post-transplant delay before CMV infection onset (months, median [IQR])	1 [0–5]	3 [1–5]	0.2
Patient on VGCV prophylaxis at CMV infection onset (*n*, %)	7 (12.7)	9 (34)	0.03
Peak viral load (UI/mL, median [IQR])	13,186 [3,132 32,147]	25,088 [9,422–153922]	0.02
CMV infection (*n*, %)	17 (31.0)	6 (23.0)	0.8
CMV disease (*n*, %)	38 (69.0)	20 (77.0)	
Organs involved in CMV disease (*n*, %)			
Bone marrow	25/38 (65.8)	20/20 (100.0)	0.002
Digestive tract (elevated LFTs, colitis)	22/38 (57.9)	15/20 (75.0)	0.25
Lungs	0/38 (0)	3/20 (15.0)	0.036
CNS	0/38 (0)	1/20 (5.0)	0.3
Other	0/38 (0)	2/20 (1kidney, 1 eye) (10.0)	0.1
Outcome			
Time for viral clearance (days, median [IQR])	50.0 [30.0, 97.5]	105.00 [67.0, 240.0]	<0.001
Antivirals serious adverse events[c], (*n*, %)	18 (32.7)	16 (61.5)	0.03
One year mortality (*n*, %)	2 (3.6)	5 (19.2)	0.02[d]

CKD, chronic kidney disease; CNS, central nervous system; eGFR, estimated Glomerular Filtration Rate; LFTs, liver function tests; MMF, mycophenolate mofetil; VGCV, valganciclovir.

[a]*Defined by an estimated CKD EPI eGFR <30 mL/min/1.73 m^2.*

[b]*Underdosing was defined as a daily dose <50% of the recommended dose for eGFR according to http://sitegpr.com and low plasma concentration was defined as a Valganciclovir trough level < 1 µg/mL*

[c]*Defined as the presence of at least one of the following events attributed to CMV, treatment among: cytopenia, acute kidney injury, elevated liver enzymes, neuropathy, mental status alterations, seizures.*

[d]*Result by the Kaplan-Meier method/log rank test.*

LMV [24–26] - tend to suggest that this molecule should rather be used as a secondary prevention of GCV-resistant CMV infection in the SOTR population or in case of contraindications in certain patients, like severe neutropenia for instance.

Clinical refractoriness in the S group can be explained by i) inobservance of VGCV treatment, ii) subtherapeutic levels of (V) GCV due to malabsorption, under-prescription or renal function improvement or misevaluation iii) the inclusion criterion requiring 2 weeks of treatment which might be too short to define clinically refractory CMV infection. Subtherapeutic levels of (v)GCV can also lead to genotypic resistance development [11, 27, 28]. Some authors suggest that 3 weeks of treatment are necessary before CMV viremia shows significant decrease and recommend this delay before testing for CMV genotypic resistance [5, 19]. Refractoriness without genotypic mutation is partially understood. The inability of the immune system to clear viremia despite VGCV treatment is the main hypothetical mechanism [5, 7].

TABLE 2 | Mutations found in the genes of interest for CMV antiviral resistance.

UL97 phosphotransferase mutations (24)	UL54 DNA polymerase mutations (4)	UL56/UL89 terminase complex mutations (0)
L595S (10)		
A594V (4)		
C603W (3)		
L595F (2)	F412L (1)	
M460V (1)	K545S (1)	—
A594P (1)	K513N (1)	
Del 598-603 GKLTHC (1)	K545S (1)	
M460I and C603W (1)		
Del GKLTH 598-602 (1)		

The number of patients presenting the mutation is indicated within parentheses.

TABLE 3 | Factors associated with CMV genotypic resistance in multivariate analysis.

Variable	OR	IC 95%	*p*
Age	0.94	0.089–0.99	0.02
Systematic VGCV prophylaxis	1.35	0.33–5.36	0.67
On VGCV prophylaxis at infection onset	3.11	1.18–5.32	0.03
Recipient CMV negative serostatus	3.40	0.97–12.8	0.06
History of VGCV underdosing or low plasma concentration	5.61	1.69–20.7	0.006

CMV, cytomegalovirus; eGFR, estimated Glomerular Filtration Rate; VGCV, valganciclovir.

TABLE 4 | Immunosuppression alleviation to deal with CMV refractory infection.

Strategy	S group (*N* = 55)	R group (*N* = 26)	*p*
No change, (*n*, %)	17 (30.9)	2 (7.6)	0.01
MMF 50% dose reduction, (*n*, %)	26 (47.3)	8 (30.8)	
MMF discontinuation, (*n*, %)	10 (18.2)	14 (53.8)	
Switch MMF to mTORi, (*n*, %)	1 (1.8)	2 (7.6)	
Switch Tacrolimus to CsA and MMF 50% dose reduction, (*n*, %)	1 (1.8)	2 (7.6)	

CsA, ciclosporin A; MMF, mycophenolate mofetil; mTORi, mammalian target of rapamycin inhibitors.

TABLE 5 | Factors associated with antiviral treatment toxicity in multivariate analysis.

Variable	OR	IC 95%	*p*
Age	0.99	0.095–1.03	0.6
Systematic (V)GCV prophylaxis	0.74	0.26–1.97	0.6
eGFR<30 mL/min	1.70	0.60–4.89	0.3
CMV genotypic resistance	3.48	1.21–10.07	0.02

eGFR, estimated glomerular filtration rate; (V)GCV, valganciclovir or ganciclovir.

We found that both being on VGCV prophylaxis at infection onset and the exposure to low levels of VGCV were associated with CMV resistance: patients presenting refractory infection in this particular setting should be considered as more at risk of genotypically resistant infection. We also found significantly more patients with a history of VGCV prophylaxis in the R group: pre-emptive strategy could reduce resistance development through reduced GCV exposure [5, 6, 11], but CMV prophylaxis strategy did not result significative in multivariate analysis accordingly with the recent findings of Acquier et al. [29] in a cohort of kidney transplant recipients. It has been known for a few years that VGCV exposure *per se* is not a risk factor for infection but that a longer exposure is associated with resistance development [29, 30]. The threshold of exposure is usually recognized as 6 weeks [1] but a recent study found a threshold of 8 weeks of treatment with active replication as a risk factor [29] rather than prophylaxis itself or the cumulated time on prophylaxis and curative treatment Finally, the potential effect of VGCV exposure on resistance development should be balanced with the effects of more episodes of CMV replication in patients with pre-emptive strategies (immune exhaustion and opportunistic infections for instance).

Consistently with Fisher's study [8] more than 50% of the patients with resistant CMV were seronegative before transplantation: this correlates both with longer VGCV exposure (a longer treatment is recommended [7, 11, 19]) and higher viral loads in the context of primary infection, two reported risk factors for resistance [5–7]. Resistance was also found associated with a younger age, consistently with previous data [31]: seronegative patients tend to be younger, as the risk of CMV seropositivity increases with age, and this association may result of a confusion bias. Younger or seronegative patients presenting refractory infection should be considered more at risk of genotypically resistant CMV infection.

CMV resistance was associated with a poorer outcome, including a fivefold higher 1-year mortality in this group, accordingly with previous studies [8]. CMV replication persisted three times as long in the R group than in the S group which may have resulted in either CMV-specific complications or in increased immune exhaustion [5, 32]. Both 3-month (early) and 1-year mortality were previously reported increased in resistant CMV infections, pleading for a direct attributable effect of the persistence of viral replication on mortality. Second-line antiviral treatment toxicities may also have played a role. Treatment serious adverse effects were found associated with resistance but are obviously not related to resistance itself but more probably to FOS toxicity mainly (65.6% vin the R group *versus* 4.6% in the S group): acute kidney injury being the main adverse effect in the resistant group and being a major factor of morbi-mortality in various infectious diseases, as it leads to therapeutic difficulties and specific complications [33, 34]. GCV higher dosing may also have played a role in these toxicities.

Another potential factor to explain poorer outcome in the R group may be immunosuppression alleviation, more frequent in this group, likely due to in the difficulty to achieve viral clearance in this group. It may have resulted in more organ rejection but we did not collect rejection events in our study.

This study shows some limitations: it is a small scaled retrospective study, comparing two very heterogenous groups of patients. We could not build solid multivariate models due to the small number of patients and events, which limits the applicability of these results. Also, because

genotypic testing was only performed upon clinicians' demand in case of the suspicion of a refractory infection rather than systematically, it is therefore plausible that resistance may have been underdiagnosed in this population. We believe it allows to show some factors associated with genotypic resistance to help the clinician identify patients at risk during the turnaround time of genotypic resistance testing. It also shows coherence with the previously published results on the topic.

In conclusion, this study mainly shows among SOTRs with CMV refractory infection an association of CMV genotypic resistance with the recipients negative serostatus, the exposure to low levels of VGCV and a younger age. On the other hand, GCV-resistant CMV infection is (in this cohort and in the literature) associated with increased (probably attributable) morbimortality. The recent evolutions in CMV antiviral strategies could make a difference in the prognosis of this infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CNIL France. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YT, designed the study, collected and analyzed the data and wrote the article. DB designed the study, performed all the virology work up and reviewed the article. VP designed the study and reviewed the article. LA analyzed the data. JT, GC, FC, BB, NO, and FG reviewed the article. 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.2023.11295/full#supplementary-material

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A Multi-Modal Approach to Islet and Pancreas Transplantation With Calcineurin-Sparing Immunosuppression Maintains Long-Term Insulin Independence in Patients With Type I Diabetes

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Long-term success in beta-cell replacement remains limited by the toxic effects of calcineurin inhibitors (CNI) on beta-cells and renal function. We report a multi-modal approach including islet and pancreas-after-islet (PAI) transplant utilizing calcineurin-sparing immunosuppression. Ten consecutive non-uremic patients with Type 1 diabetes underwent islet transplant with immunosuppression based on belatacept (BELA; $n = 5$) or efalizumab (EFA; $n = 5$). Following islet failure, patients were considered for repeat islet infusion and/or PAI transplant. 70% of patients (four EFA, three BELA) maintained insulin independence at 10 years post-islet transplant, including four patients receiving a single islet infusion and three patients undergoing PAI transplant. 60% remain insulin independent at mean follow-up of 13.3 ± 1.1 years, including one patient 9 years after discontinuing all immunosuppression for adverse events, suggesting operational tolerance. All patients who underwent repeat islet transplant experienced graft failure. Overall, patients demonstrated preserved renal function, with a mild decrease in GFR from 76.5 ± 23.1 mL/min to 50.2 ± 27.1 mL/min ($p = 0.192$). Patients undergoing PAI showed the greatest degree of renal impairment following initiation of CNI (56% ± 18.7% decrease in GFR). In our series, repeat islet transplant is ineffective at maintaining long-term insulin independence. PAI results in durable insulin independence but is associated with impaired renal function secondary to CNI dependence.

Keywords: immunosuppression, immune tolerance, insulin independence, islet transplant, pancreas transplant

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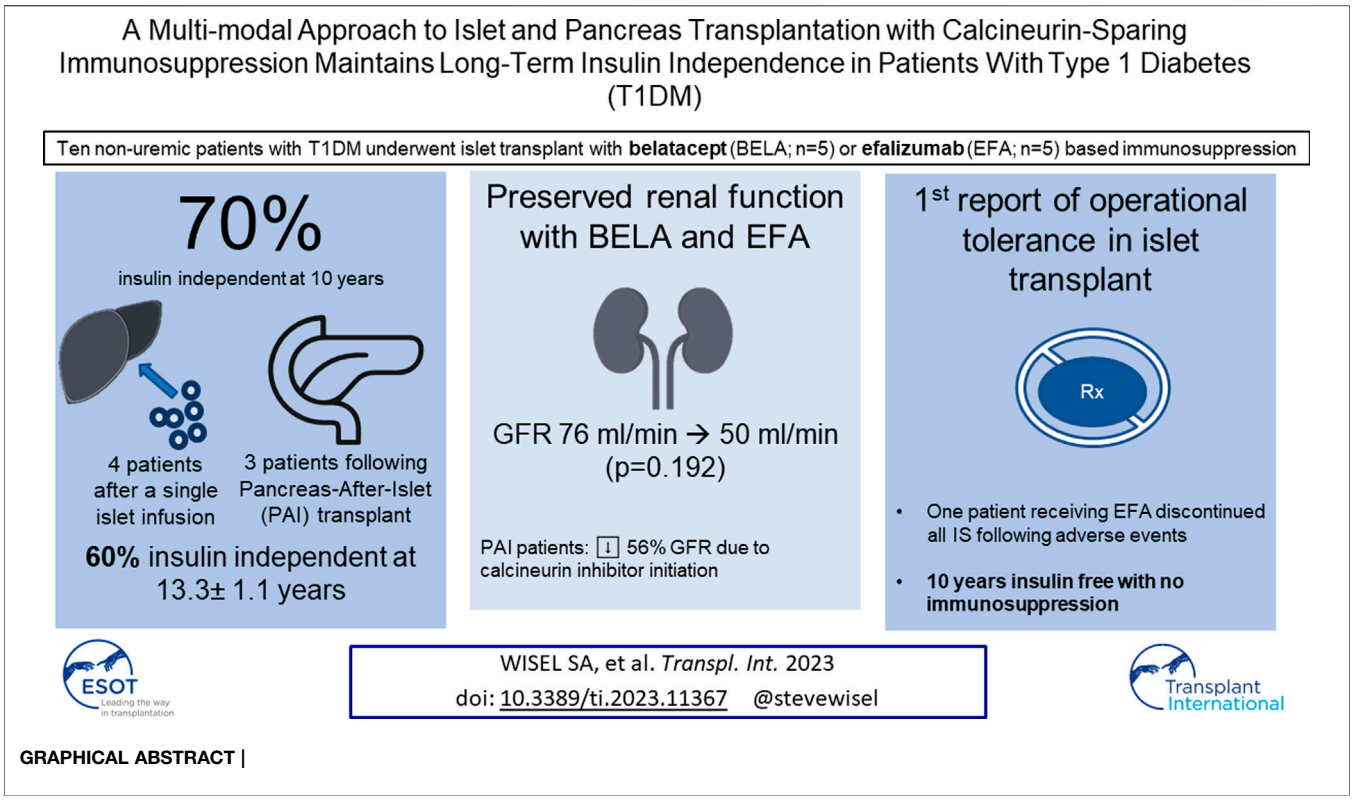
Abbreviations: BELA, belatacept; CKD, chronic kidney disease; CNI, calcineurin inhibitors; CTLA-4, cytotoxic T-lymphocyte-associated protein-4; EFA, Efalizumab; GFR, glomerular filtration rate; HbA1c, hemoglobin A1c; IAP, Islet-after-pancreas transplantation; ICAM-1, intercellular adhesion molecule-1; IEQ, islet equivalents; LFA-1, leukocyte function antigen-1; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; PAI, Pancreas-after-islet transplantation; PBMC, peripheral blood mononuclear cells; PML, progressive multifocal leukoencephalopathy; PRA, panel reactive antibody; Treg, regulatory T cells.



GRAPHICAL ABSTRACT |

INTRODUCTION

Over five million Americans are expected to be living with Type 1 diabetes by 2050 [1]. For most patients, insulin therapy remains the mainstay of management to control blood glucose and minimize microvascular complications [2, 3]. Continuous glucose monitors, wearable insulin pumps, and mobile applications have improved patient compliance and outcomes in managing Type 1 diabetes with exogenous insulin [3, 4]. However, beta cell replacement—either by solid organ pancreas transplant or islet transplantation—remains the sole curative intervention for Type 1 diabetes and avoids the life-threatening complication of hypoglycemic episodes associated with intensive insulin therapy [5–9]. Long-term islet function beyond 5 years remains a challenge, and patients may require additional islet infusions to restore insulin independence. Previous studies from our group and others have shown that pancreas-after-islet (PAI) and islet-after-pancreas (IAP) transplantation provide additional multimodal pathways to maintain long-term insulin independence for patients with Type 1 diabetes [10–12].

Choice of immunosuppression remains a critical factor in maintaining long-term beta cell function and optimizing patient outcomes. Calcineurin inhibitors (CNI) have revolutionized the field of transplantation, but the known beta cell and renal toxicities limit their efficacy for pancreas and islet transplantation. Our group has previously published 5-year outcomes in a cohort of ten consecutive patients receiving CNI-sparing maintenance immunosuppressive regimens based on either the costimulation blocker belatacept (BELA; LEA29Y, BristolMyers Squibb, New York, NY) or the antileukocyte functional antigen-1 antibody efalizumab (EFA; Raptiva, Genentech, Inc., S. San Francisco, CA; JDRF grant #4-2004-372) [13–15]. BELA is a cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) fusion protein which binds CD80 and CD86 on antigen-presenting cells and serves to block the co-stimulation necessary for full T cell activation after antigen-specific T cell receptor binding occurs. By blocking this crucial second-step, T cell activation is prevented, and the T cell instead undergoes anergy and apoptosis. BELA has been shown to be effective in preventing graft rejection, allowing reduced levels of conventional immunosuppressive medications, and has no beta-cell or renal toxicity [14, 16, 17]. EFA is a monoclonal antibody which binds the CD11a subunit of leukocyte function antigen-1 (LFA-1), inhibiting its binding to the intercellular adhesion molecule-1 (ICAM-1), thereby preventing adhesion of leukocytes which is a necessary step for T cell activation and trafficking [18–20]. Despite its efficacy in islet transplantation, several patients in a concurrent trial of EFA for psoriasis developed progressive multifocal leukoencephalopathy (PML) and thus EFA was withdrawn from the market in May 2009 [21].

Here, we describe 10-year outcomes in a cohort of pre-uremic patients with Type 1 diabetes who received islet transplants with BELA- and EFA-based immunosuppression. Our results detail a multi-modal, personalized approach to beta cell replacement including a combination of islet and pancreas transplant in

pursuit of long-term insulin independence for Type 1 diabetes patients while limiting the adverse effects of CNI and preserving renal function. Additionally, we review the clinical course of one islet transplant recipient who continues to maintain operational tolerance 13 years after a single islet infusion, over 9 years after discontinuing all immunosuppression.

MATERIALS AND METHODS

Patients

Patients were consecutively enrolled in this study as previously described [13]. To meet inclusion criteria for islet transplant during this study, patients had a history of Type 1 diabetes mellitus for a minimum of 5 years, baseline C-peptide level less than 0.5 ng/mL, and/or a history of symptomatic severe hypoglycemic episodes. Eligible patients were required to have a body mass index less than 30 kg/m^2 (or weight less than 80 kg), a daily total insulin requirement below 55 units/day, and preserved renal function. Patients meeting inclusion criteria were screened for malignancy before undergoing informed consent in accordance with the institutional review board and clinical protocols registered at clinicaltrials.gov [13, 15].

During the study period, all islet transplants performed at our institution were completed as a part of this clinical trial. All 10 islet transplant were performed consecutively within the institutional experience, and no patients meeting inclusion criteria were excluded from participation in the study or transplanted under alternate immunosuppression protocols. Study enrollment was completed at time of islet offer, with patients undergoing a standardized informed consent process in addition to a research consent as approved by the institutional review board. Enrollment in the study was non-randomized: the first five patients enrolled received efalizumab and the second five patients received belatacept.

Islet Preparation and Transplantation

Pancreatic islet isolation was performed as previously described [13, 15]. Enrolled patients received at minimum 4,000 islet equivalents (IEQ) per kg body weight, with islets having at minimum 70% viability and glucose stimulated insulin secretion index above 1.0. All islet allografts during this study met criteria, and no islet preparations were discarded post-enrollment. Islets were cultured until infusion by percutaneous transhepatic cannulation of the portal vein. Patients received systemic anticoagulation via intravenous heparin infusion for the first 48 h after transplant, followed by an additional 5 days of therapeutic enoxaparin injections subcutaneously. Patients were considered for a second infusion of islets 2–3 months after loss of insulin independence. Patients were selectively considered for subsequent pancreas-after-islet (PAI) transplant if they had returned to insulin use following at least one islet transplant, were deemed acceptable surgical candidates, and expressed willingness to proceed with pancreas transplant.

Immunosuppression

For induction, all patients received 2 mg/kg/day of antithymocyte globulin (thymoglobulin) for 2 days (4 mg/kg total dose) prior to islet transplantation, with one dose of methylprednisolone prior to the first thymoglobulin administration. The BELA and EFA immunosuppression regimens have previously been described [13]. Patients enrolled in the BELA protocol (n = 5) received 10 mg/kg intravenously on days 0, 4, 14, 28, 56, and 75 post-transplant, followed by 5 mg/kg every 4 weeks until 18 months post-transplant, followed by 5 mg/kg every 8 weeks thereafter. Patients receiving the EFA immunosuppression protocol (n = 5) received 1 mg/kg subcutaneously every week from 1 day prior to transplant until 3 months post-transplant, followed by 0.5 mg/kg per week. Immunosuppression was supplemented with sirolimus and mycophenolic acid (MPA) as previously described [13].

Following FDA discontinuation of EFA in May 2009, three of the EFA-treated patients were continued on combination immunosuppression with sirolimus and MPA, one patient was continued on MPA monotherapy, and one patient was continued on combination therapy with tacrolimus and MPA.

Immunosuppression for patients receiving PAI transplantation has been previously described [12]. Briefly, PAI recipients received induction therapy with anti-thymocyte globulin (6 mg/kg) and methylprednisolone. Patients were then started on a three-drug maintenance immunosuppression regimen including tacrolimus (trough target 5–7 ng/mL), mycophenolic acid (360–720 mg twice daily) and prednisone 5 mg per day, with addition of a mammalian target of rapamycin (mTOR) inhibitor (sirolimus or everolimus) at 1-month post-transplant. A full summary of patient immunosuppression is available in **Supplementary Table S1**.

Post-Transplant Patient Monitoring

Patients were asked to perform blood glucose checks three to five times daily, with accurate recording of both dietary intake of carbohydrates and use of insulin. Patients underwent serial laboratories for fasting serum blood glucose, Hemoglobin A1c (HbA1c) levels, and C-peptide levels. Post-transplant HbA1c levels above 6.4% were considered hyperglycemic and defined loss of insulin independence. Partial insulin use was defined as less than 0.5 units/kg/day, with full insulin use defined as greater than 0.5 units/kg/day. Patient renal function was also closely monitored to evaluate for glomerular filtration rate (GFR). All patients undergoing PAI transplantation were subject to protocol pancreas biopsies between 2- and 6-month post-transplant as long as they were medically stable to undergo the procedure.

Immunologic Screening and Alloreactive T cell Frequency Analysis

Patient whole blood samples were collected pre-transplant and at pre-determined post-transplant intervals (days 7, 14, 28, 56, 75, 90, 120, 175, 270, and 365). Specimens were processed by Ficoll gradient to isolate peripheral blood mononuclear cells (PBMC) for immunologic analysis. As previously described, specimens were cryopreserved in liquid nitrogen at −196°C prior to flow

cytometric analysis for phenotypic characterization by expression of CD3, CD4, CD8, CD25, Foxp3, and CD127 (13, 15).

Patient PBMCs were evaluated for alloreactivity *in vitro* as previously described [13]. Patient PBMC, donor splenocytes, and third-party donor splenocytes were thawed from cryopreservation and resuspended in complete RPMI (RPMI 1640 supplemented with human AB serum (heat-inactivated), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM sodium glutamate) [15]. Patient PBMCs were cultured alone, with third-party splenocytes, or with donor splenocytes. *Staphylococcus* enterotoxin B (1 µg/mL) was used as a positive control in separate culture with patient PBMCs. Cultures were maintained for 5 days, at which point brefeldin A (Epicentre, 10 µg/mL) was added to culture for 6 h. Cells were harvested, washed with PBS twice, and FACSPerm Solution II (BD Biosciences) was used to permeabilize cells for 10 min. Cells were labeled with antibodies to CD4, CD8, PerCP, IFN-γ, TNF-α, APC, and IL-4 (BD Biosciences).

Statistical Analysis

Data are presented as means ± standard error except where otherwise stipulated. A student's t-test was used for statistical analysis, with a p-value of less than or equal to 0.05 as significant. For analysis of renal function in patients with preserved islet function, individual patient GFR data was utilized. For data only recorded as creatinine (mg/dL) or normal GFR (>60 mL/min), estimated GFR values were computed using the CKD-EPI Creatinine Equation (2021). Renal function (GFR) for each individual patient was modeled over time using restricted cubic splines, utilizing 4 knots per patient. The model-based values allowed alignment in approximate 1 month intervals so that mean profiles using all subjects in each group could be computed. The mean ± standard error GFR profiles are reported for each group. Computations were carried out using R 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org).

RESULTS

Donor and Recipient Characteristics

Islet and pancreas donor characteristics have previously been described [12, 13], with recipient characteristics summarized in **Table 1**. A total of 14 islet transplants were performed into 10 recipients, with four patients receiving a second infusion of islets. The number of IEQ administered per islet transplant ranged from 402,666 to 691,500 IEQ (mean 575,131 ± 92,923 IEQ), corresponding to a dose of 6,101 to 12,825 IEQ/kg (mean 9,554 ± 1,980 IEQ/kg).

Recipients were between 40 and 62 years old at time of their first islet transplant. Nine out of ten patients included in this study were female, with BMI ranging from 19.1 to 29.3 kg/m^2 (mean 23.4 ± 3.2 kg/m^2). All recipients were confirmed to have diabetes, with pre-transplant HbA1c values of 6.7%–8.7% (mean 7.6% ± 0.8%). Baseline GFR measured from 41 mL/min to 98 mL/min (mean 76.5 ± 23.1 mL/min). Patients were followed for an average of 4,714 ± 399 days following their first islet transplant

TABLE 1 | Donor and recipient characteristics for islet and pancreas-after-islet transplant.

Recipient	Age at Islet Txp	Gender	BMI	Preislet HbA1c	Islet Txp 1			Islet Txp 2			PAI Txp			Total Follow Up (mos.)
					Total IEQ	IEQ/kg	Duration of Insulin Independence (mos.)	Total IEQ	IEQ/kg	Duration of Insulin Independence (mos.)	Age at PAI Txp	Time between Islet and PAI Txp (yr.)	PRA at PAI Txp	
BELA-1	56	F	24.7	8.7	507,660	7,577	143							144
BELA-2	53	F	23.6	6.5	645,500	10,940	144							144
BELA-3	60	F	19.1	7.6	691,500	12,805	54							147
BELA-4	43	M	29.3	8.4	608,400	8,112	19	724,125	9655	13	48	2.9	2%	153
BELA-5	62	F	21.2	7.6	557,500	9,450	10	450,000	8215	62				156
EFA-1	47	F	25.4	6.7	542,777	12,825	1	573,128	9244	82				167
EFA-2	58	F	21.3	8.2	661,409	10,667	169							169
EFA-3	48	F	25.6	7.3	482,050	8,000	27				51	3.2	10%	172
EFA-4	58	F	19.2	8	630,165	11,458	172							172
EFA-5	40	F	24.9	6.7	577,950	8,711	4	402,666	6101	25	44	3	62%	177

(12.9 years; range 4,214–5,286 days). As previously described, patients received EFA for a range of 392–804 days prior to drug removal from market and transition of immunosuppression [13].

Three patients went on to PAI transplant following loss of islet function. Two patients (BELA-4 and EFA-5) had previously received two islet infusions, while one patient (EFA-3) expressed an interest to proceed to pancreas transplant following a single islet transplant. Patients undergoing PAI transplant had a panel reactive antibody (PRA) of 2%–62% with a mean interval of 3.0 ± 0.15 years between final islet infusion and subsequent pancreas transplant.

Post-Transplant Islet and Pancreas-After-Islet (PAI) Graft Function

Four patients maintained long-term insulin independence following a single islet infusion for an average duration of 157 ± 15.3 months (13 years; range 144–172 months); two of these patients received BELA-based immunosuppression (BELA-1 and BELA-2) and two of these patients received EFA-based immunosuppression (EFA-2 and EFA-4). Three of these patients remain insulin independent, while the fourth patient (BELA-1) resumed partial insulin dependence 11.8 years after islet transplant.

Six patients experienced failure of their first islet transplant after an average of 19 ± 19 months (range 1–54 months). Four of these patients (BELA-4, BELA-5, EFA-1, and EFA-5) proceeded with a second islet infusion, one patient (BELA-3) elected to forego a second islet infusion after 54 months of insulin independence and returned to insulin use, and one patient (EFA-3) proceeded to PAI transplant after returning to insulin use 27 months post-islet infusion. For patients undergoing a second islet transplant, the mean duration of insulin independence was 45.5 ± 32.0 months (range 13–82 months), with all four returning to insulin use following a second islet infusion. Two of these four patients (BELA-4 and EFA-5) underwent PAI transplant following failure of their second islet transplant, while the remaining two patients (BELA-5 and EFA-1) remain on exogenous insulin therapy. At time of publication, six out of ten patients continue to experience insulin independence (two of five BELA patients and four of five EFA patients), including the three patients who underwent PAI transplantation. Islet and PAI outcomes are summarized in **Table 2**; **Figure 1**.

Post-Transplant Glycemic Control

Three of five BELA patients and four of five EFA patients remained insulin independent at 10 years, with improvement in HbA1c from 7.1% ± 1.1% to 5.8% ± 0.45% for BELA patients and 6.8% ± 0.6% to 5.5% ± 0.4% for EFA patients (**Figure 2A**). All patients were free from hypoglycemic unawareness, irrespective of insulin independence. Beyond 10 years, one patient (BELA-1) resumed partial insulin use 11.8 years post-transplant, while six patients remain insulin independent with average follow-up of 4,867 ± 384 days from first islet infusion (13.3 ± 1.1 years, range 4,307–5,286). The four patients reliant on exogenous insulin use (BELA-1, BELA-3, BELA-5, and EFA-1) require a range of 6–53 regular insulin unit equivalents per day (**Table 2**).

Preservation of Renal Function

Overall, patients demonstrated preserved renal function with a mild decrease in GFR from 76.5 ± 23.1 mL/min to 50.2 ± 27.1 mL/min over the duration of the study (p = 0.192; **Figure 2B**). Patients receiving BELA demonstrated slight decrease in GFR from 67.6 ± 24.5 mL/min to 45.4 ± 31.4 mL/min (p = 0.077), and patients receiving EFA demonstrated mild downtrend in GFR from 85.4 ± 20.1 mL/min to 55.0 ± 24.7 mL/min (p = 0.073). When analyzing patients with functional islet grafts, renal function was preserved for both BELA (−9.4 ± 9.8 mL/min) and EFA (−17.8 ± 15.1 mL/min) patients, with no significant difference between BELA and EFA cohorts (**Figure 2C**). The three patients undergoing PAI transplant, with initiation of a CNI-based immunosuppression regimen, demonstrated the highest degrees of renal impairment over the study interval, with a mean decrease in GFR, from 92.6 ± 16.4 mL/min at time of PAI transplant to 39 ± 11.3 mL/min (−56% ± 18.7%) at most recent follow-up. Overall, eight of ten patients maintained renal function over the course of the study, with two patients progressing to stage 4 or stage 5 CKD (**Figure 2D**). One patient (BELA-4) progressed to stage 4 chronic kidney disease (CKD) following PAI transplant, while one patient (BELA-5) progressed from stage 3B to stage 5 CKD following islet transplant failure.

Adverse Events

A total of 19 complications occurred in 10 patients over the study period and are summarized in **Table 2**. Following islet transplant, one patient (EFA-2) experienced post-infusion bleeding while another (EFA-5) experienced a partial portal vein thrombus which resolved following a course of oral anticoagulation. Three patients had complications related to sirolimus administration while additional infectious complications included bronchitis, oral thrush, and urinary tract infection. Complications following PAI included peri-pancreatic abscess requiring drainage and antibiotic therapy (EFA-3), squamous cell carcinoma requiring local resection (EFA-5), and bronchitis which resolved following antibiotic therapy (BELA-3). Two patients (BELA-5 and EFA-4) developed severe opportunistic infections, including CMV esophagitis, cryptococcal meningitis, pulmonary aspergillosis, and post-transplant lymphoproliferative disorder (PTLD). Infectious complications responded to antiviral and antifungal medications, and immunosuppression was completely withdrawn with rituximab administration for treatment of PTLD in patient EFA-4.

Post-Transplant Immunologic Screening

The early effects of BELA and EFA on circulating levels of $CD4^+$ FoxP3+ regulatory T cells (Treg) in the first year following islet transplant have previously been reported along with medium-term outcomes in this cohort, but remain relevant to patient outcomes (**Figure 3A**) [13, 15]. Briefly, patients receiving BELA demonstrated stable levels of Treg as a percentage of total circulating T cells in the first year post-transplant, with Tregs comprising 3.3% ± 1.8% of circulating T cells at time of transplant,

TABLE 2 | Recipient outcomes, immunosuppression, and adverse events following islet and pancreas-after-islet transplant.

Recipient	Initial HbA1c	Current HbA1c	Current Insulin	Initial GFR	Current GFR	Duration of EFA (days)	Current IS	Complications	Management	PAI Transplant	
										Rejection	Treatment
BELA-1	7.4	7.3	6 U Tresiba morning	98	91		Sirolimus/ myfortic/ belacept	Oral mucosal lesions			
BELA-2	5.9	6	None	44	48		Myfortic/ belacept	EBV viremia, subnephrotic proteinuria	stopped sirolimus, proteinuria improved		
BELA-3	6.9	7.4	10 U Tresiba morning, Aspart 2-3 U with meals (~16–19 U/day)	80	54		None	Proteinuria, nonhealing leg wound in 2013	Stop sirolimus, increase myfortic		
BELA-4	8	5.7	None	75	26		Tac/Everolimus/ Myfortic/ Prednisone	Bronchitis	Antibiotic therapy	No	N/A
BELA-5	7.1	6.1	14 U determir at night, 2–3 U insulin regular with meals (~20–23 U/day)	41	8		None	CMV esophagitis; cryptococcal meningitis	valgancyclovir, amphotericin/ flucytosine -> chronic fluconazole		
EFA-1	6.9	6.7	Lantus 10–12 U BID, humalog 28 –30 U (~ 49–53 U/day)	91	99	504	None	None	N/A		
EFA-2	6.8	6.1	None	50	41	568	Sirolimus/ myfortic	Intraperiotneal bleed; Thrush; UTI	None; antifungal therapy; antibiotic therapy		
EFA-3	7.6	5.6	None	91	45	583	Tac/Myfortic/ Prednisone	Peripancreatic abscess (PAI); Gastrointestinal distress, insomnia (PAI)	Percutaneous drainage, antibiotic therapy; dicsontinuation of mTOR inhibitor	Grade 1 ACR	Thymoglobulin, methylprednisolone
EFA-4	6.1	5.9	None	100	44	392	None	Angioinvasive aspergillosis of the lung; CMV viremia; PTLD	Voriconazole, withdrawal of mTOR; valganyclovir and IS dose reduction; complete IS withdrawal, rituximab		
EFA-5	6.7	5.1	None	95	46	804	Tac/Myfortic/ Prednisone	Portal vein thrombus (islet); Facial squamous cell carcinoma (PAI)	Anticoagulation; Local resection	No	N/A

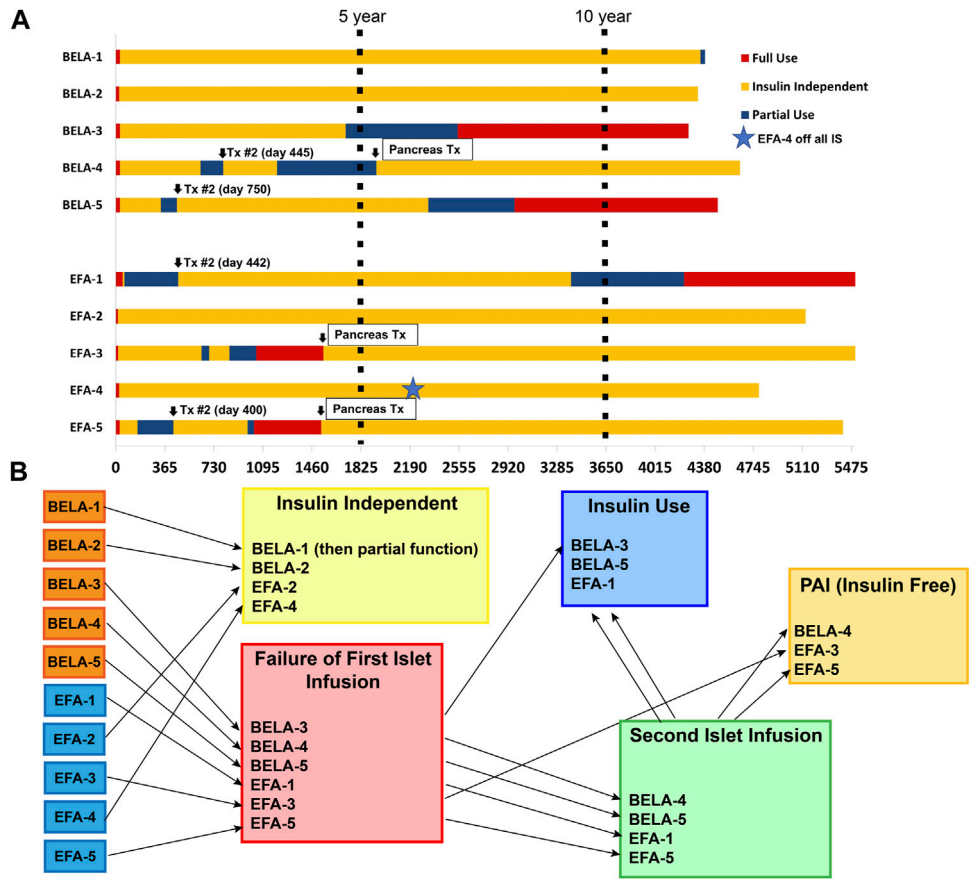


FIGURE 1 | Islet and Pancreas-After-Islet (PAI) outcomes for patients receiving CNI-sparing immunosuppression based on Belatacept (BELA) or Efalizumab (EFA). **(A)** Summary of beta cell replacement outcomes from time of transplant; **(B)** Summary of beta cell replacement outcomes by graft function status.

a peak level of 4.4% ± 1.8% of circulating T cells, and 3.1% ± 1.6% at 1 year post-transplant. EFA patients demonstrated increased levels of circulating Treg in the first year following islet transplant, with Treg prevalence of 5.6% ± 1.8% at time of transplant ($p = 0.076$), peak Treg percentages of 35.6% ± 19.5% ($p = 0.0074$), and Tregs comprising 22.6% ± 10.4% of the circulating T cell population at 1 year post-transplant ($p = 0.0032$).

A Case of Operational Tolerance in Islet Transplant Recipient

As described above, patient EFA-4 demonstrated evidence of profound over-immunosuppression, characterized by multiple post-transplant adverse events (**Figure 3B**). At 17 months post-transplant, the patient was diagnosed with angioinvasive aspergillosis of the lung, prompting treatment with voriconazole and reduction of immunosuppression to MPA monotherapy. The patient developed CMV viremia at 53 months post-transplant which was cleared following valganciclovir and MPA dose reduction to 360 mg BID. However, biopsy done at 56 months post-transplant for fatigue and gingival soreness confirmed diagnosis of post-transplant lymphoproliferative disorder (PTLD). All immunosuppression was withdrawn and the patient was treated with eight cycles of rituximab with complete remission. Since being removed from all immunosuppression, the patient continues to maintain insulin independence following a single islet infusion, demonstrating a case of operational tolerance in an islet transplant recipient.

Patient EFA-4 showed profound expansion of Tregs from a normal level of 5% and reaching a peak of 68% of all circulating T cells at 30 days post-transplant. The percentage of Tregs in the peripheral blood remained high for the first year post-transplant, with Tregs comprising 38% of circulating $CD4^+$ T cells at 1 year. Further functional analysis by alloreactive T cell frequency assay demonstrated lack of *in vitro* proliferation with absence of interferon-gamma (IFNg) or tumor necrosis factor-alpha (TNFa) production on exposure to donor-specific and third-party antigen for the first 12 months post-transplant (**Figure 3C**). Following withdrawal of EFA, the patient demonstrated a return of normal cytokine production at 24 months post-transplant in response to donor-specific and third-party antigen, as compared to positive controls. Despite the return of responsiveness to allo-antigen and withdrawal of all immunosuppression for 10 years, the recipient continues to experience insulin independence without clinically significant immune response to her islet allograft, consistent with operational tolerance.

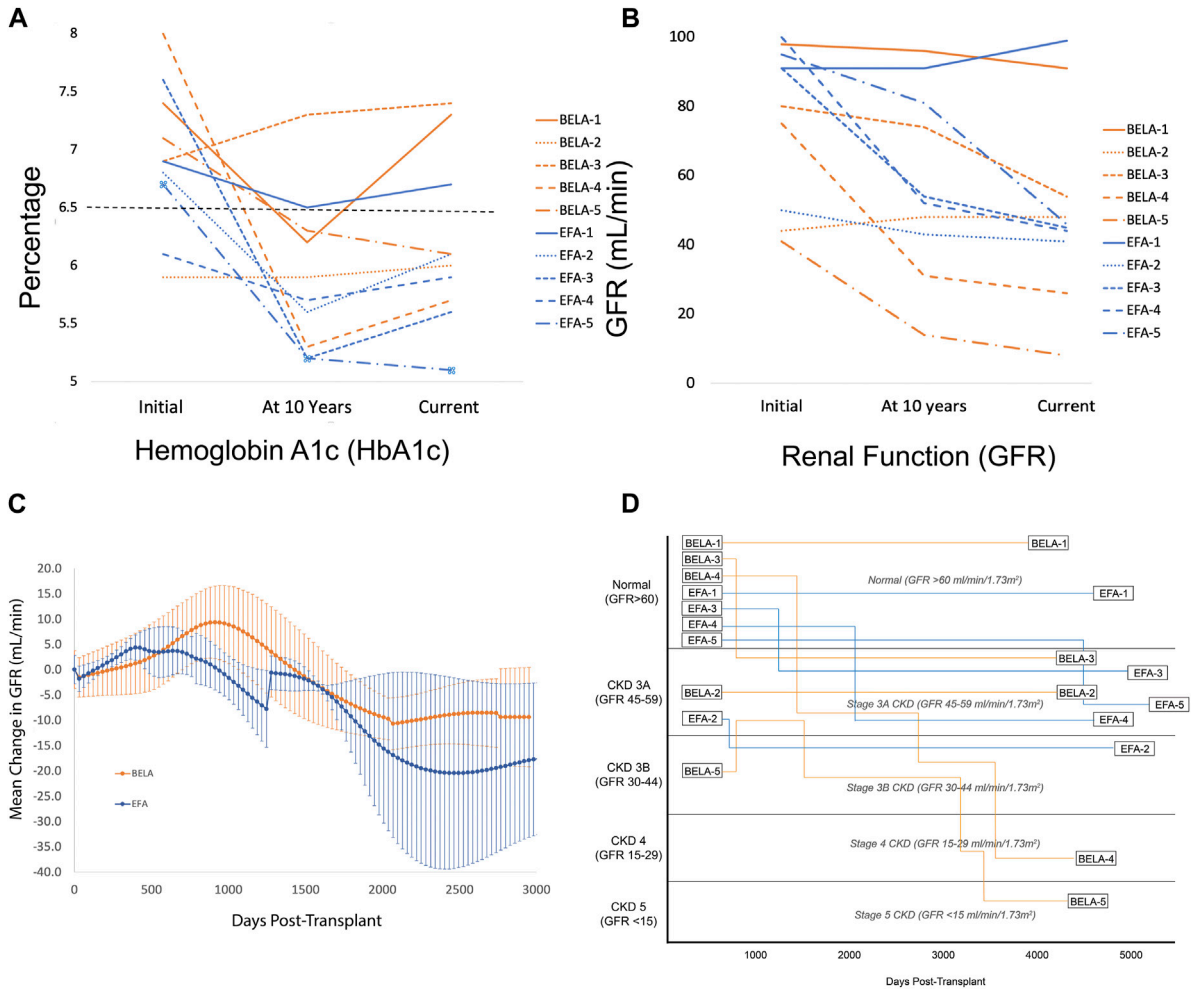


FIGURE 2 | (A) Glycemic control measured by HbA1c (%); **(B)** Renal function measured by GFR (mL/min); **(C)** Change in GFR for patients with preserved islet function; **(D)** Renal function by GFR and stage of kidney disease from time of transplant.

DISCUSSION

Beta cell transplantation requires weighing the surgical risk of operative intervention with solid organ pancreas transplant against the limited long-term durability previously demonstrated in islet transplant outcomes. Furthermore, both islet and pancreas transplantation have been limited by selection of immunosuppression, with CNI use contributing to both beta cell deterioration and chronic renal impairment [9, 22, 23]. Here, we describe a multi-modal approach using a patient-centered combination of islet and pancreas transplantation with CNI-sparing immunosuppression regimens based on BELA and EFA to achieve long-term insulin independence. Seven of ten patients maintained insulin independence at 10 years post-transplant, and six of ten patients remain insulin independent up to 14 years following a first islet infusion, with three of these patients pursuing PAI transplant as a path to insulin independence following failed islet transplant. Importantly, this series represents 10 consecutive patients, reinforcing the real-world applicability of this data.

Medium-term results previously published on this cohort of patients demonstrated insulin independence for all ten patients at 1–3 years post-islet infusion [15]. Here, we demonstrate that islet transplantation using CNI-sparing immunosuppression regimens based on BELA or EFA result in long-term insulin independence for 40% of the patients after a single islet infusion. Part of the long-term success experienced in this cohort may be attributable to the avoidance of CNI-based immunosuppression. Avoidance of CNI provides the dual benefit for this patient population of avoiding both the nephrotoxic and the beta-cell toxic effects of CNI [9, 22, 23]. Patients who maintained islet function showed no significant decrease in GFR over the course of the study, with no difference in outcomes for BELA and EFA cohorts. Two patients did experience significant decline in GFR over the course of this study. One patient progressed to stage 5 CKD following early islet failure in the setting of full insulin dependence and progression of Type 1 diabetes. A second patient progressed to stage 4 CKD following initiation of CNI at time of PAI transplant. Indeed, all three patients undergoing PAI demonstrated decline in GFR following initiation of CNI-based

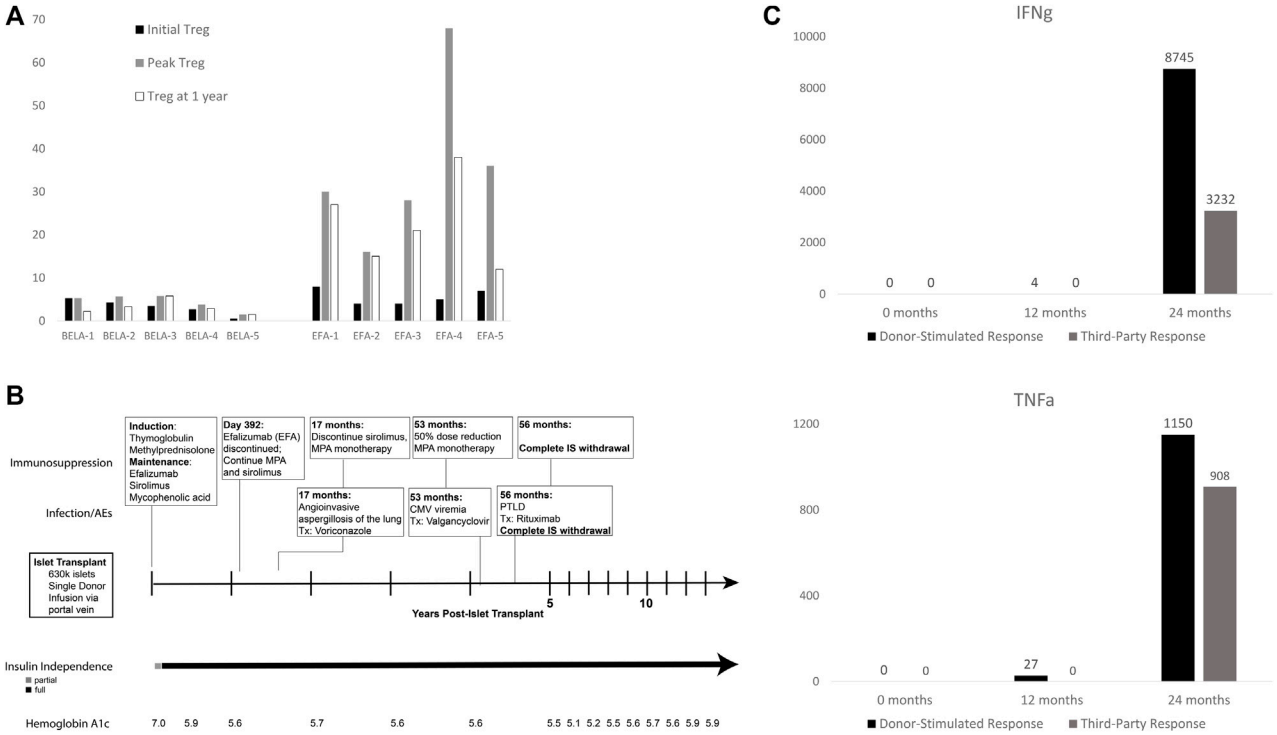


FIGURE 3 | (A) Treg expansion in first year post-transplant (adapted from Posselt, et. al., 2010); **(B)** Operational tolerance in patient EFA-4 following withdrawal of immunosuppression; **(C)** EFA4 cytokine production in mixed lymphocyte reaction to donor-specific (black) and third-party (gray) antigen. EFA4 demonstrated no reactivity to donor or third-party antigen in the first 12 months post-transplant, which returned by 24 months.

immunosuppression, but remain off dialysis with preserved renal function. For non-uremic patients pursuing PAI as a pathway to insulin independence, the risk of subsequent renal impairment should be emphasized, along with utilization of a multi-drug immunosuppression regimen that limits dependence on CNI.

In this study, patients initiated on both BELA and EFA demonstrated excellent long-term results for insulin independence. For patients in the EFA cohort, it is important to acknowledge that EFA was removed from market per the manufacturer's preference following several episodes of PML identified in a simultaneous trial for psoriasis at much higher doses. Although patients received EFA for the first 392–804 days following their first islet infusion, EFA remains of interest in islet transplantation because of its early and dramatic upregulation of circulating peripheral Tregs as a percentage of total $CD4^+$ cells. In one patient, operational tolerance was achieved following a profound expansion of Tregs, at one point comprising 68% of all circulating $CD4^+$ T cells. This patient was ultimately discontinued off all immunosuppression due to infectious complications and PTLD, suggesting that such high levels of Tregs may predispose to an anergic state. However, the predominance of Tregs induced by EFA also may have contributed to a tolerogenic milieu, as this patient subsequently achieved operational tolerance. Pre-clinical studies have likewise demonstrated that anti-LFA monoclonal antibodies induce donor-specific tolerance in a murine model of cardiac transplant [24] and suppression of both $CD4^+$ and $CD8^+$ activity [25]. These findings support a re-evaluation of EFA at lower doses for use in islet transplant recipients and emphasize the significance of early EFA use at the time of transplant. In the absence of EFA, BELA remains an excellent immunosuppressive choice for islet transplant, as it shares the benefit of avoiding nephrotoxicity and beta-cell toxicity.

When considering options for beta cell replacement, the results of this cohort suggest that islet transplant may be considered as an initial transplant option with selection of an appropriate immunosuppressive agent. Once an islet transplant has failed, the next steps to re-establish insulin independence should be individualized to the patient. Importantly, all ten patients included in this study were free of hypoglycemic episodes for the duration of follow-up. The four patients who underwent a second islet infusion experienced medium-term loss of insulin independence, at an average of 1–6 years post islet transplant. In comparison, all three patients who underwent PAI transplant remain insulin independent 7–11 years post-PAI. PAI transplantation has been previously explored by our group as a pathway to re-establish insulin independence with excellent graft survival and preservation of renal function, without effect of prior immunologic sensitization on outcomes [12]. Following a failed islet transplant, we recommend PAI transplant for patients who desire a return to insulin independence if the recipient's surgical and cardiovascular risks are deemed acceptable. For patients who cannot tolerate a major abdominal operation, additional islet infusions remain the sole route to insulin independence.

Although the rates of infectious and malignant complications related to excess immunosuppression were overall low in this

study, close post-transplant infectious surveillance for all beta cell replacement patients remains essential to long-term outcomes. In addition to the adverse events described here, our series of PAI patients were at higher risk for subsequent BK virus infections [12]. Patients undergoing beta cell replacement—particularly PAI patients—are exposed to high levels of immunosuppression to maintain graft function. Given this requirement, patients should undergo vigilant post-transplant monitoring to minimize both infectious and neoplastic events.

This study is limited by the small cohort size and the non-randomized assignment of immunosuppression regimens. However, the long-term results for these patients suggest several routes of further investigation in islet transplantation. Future directions will be defined by the availability of EFA as a choice for immunosuppression, and progress on FDA approval of islet transplant as a therapeutic modality for Type 1 diabetes. Ultimately, a multi-modal approach incorporating islet and pancreas transplant with all available immunosuppressive options will maximize the benefits of beta cell replacement for patients with Type 1 diabetes mellitus.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of California, San Francisco Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PS, AP, JG, GS, and SW participated in research design. SW, MN, YK, CW, KS-P, and PS participated in writing the manuscript. PS, AP, GW, GR, SS, GS, MT, KJ, and UM conducted the research. 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.2023.11367/full#supplementary-material

Supplementary Table S1 | Full summary of immunosuppression regimens for EFA and BELA patients.

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ORIGINAL RESEARCH
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Pasireotide Versus Octreotide in Preventing Complications After Simultaneous Pancreas-Kidney Transplantation

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In elective pancreatic surgery, somatostatin-analogues pasireotide and octreotide are variably used to reduce postoperative complications, but knowledge on their role in pancreas transplantation is limited. This study compared pasireotide and octreotide for their association with complications after simultaneous pancreas-kidney transplantation (SPK). This retrospective study included consecutive patients undergoing SPK's from July 2013 to July 2022. Between July 2013 and April 2020, octreotide was administered 0.1 mg s.c. once daily and between May 2020 and July 2022 pasireotide was administered 0.9 mg twice daily, both until third postoperative day. Complications within 90 days postoperatively were collected, and reoperation rate and Comprehensive Complication index (CCI) ≥ 33.7 (morbidity equal to one reoperation) were used as primary outcomes. Of the 213 patients undergoing SPK, 150 patients received octreotide and 63 pasireotide. Baseline characteristics were comparable. Reoperation rate was 25.3% (n = 38) and 17.5% (n = 11) (p = 0.213) and rate of CCI ≥ 33.7 was 40.7% (n = 61) and 30.2% (n = 19) (p = 0.148) in octreotide and pasireotide groups, respectively. When adjusted with donor BMI, pancreas donor risk index, and donor sex, receiving pasireotide translated into OR 0.49 (95% CI: 0.25–0.96 p = 0.037) for CCI ≥ 33.7. Pasireotide was independently associated with lower postoperative morbidity within 90 days of SPK compared to octreotide.

Keywords: pancreas, postoperative complications, pancreas transplant, somatostatin, pasireotide

Abbreviations: ARR, absolute risk reduction; BMI, body mass index; CCI, comprehensive complication index; CRP, C-reactive protein; DBD, donor after brain death; ESKD, end-stage kidney disease; IQR, interquartile range; NNT, number needed to treat; POD, postoperative day; RCT, randomized controlled trial; SPK, simultaneous pancreas-kidney transplantation; TID, Type 1 diabetes mellitus; ULN, upper limit of normal.

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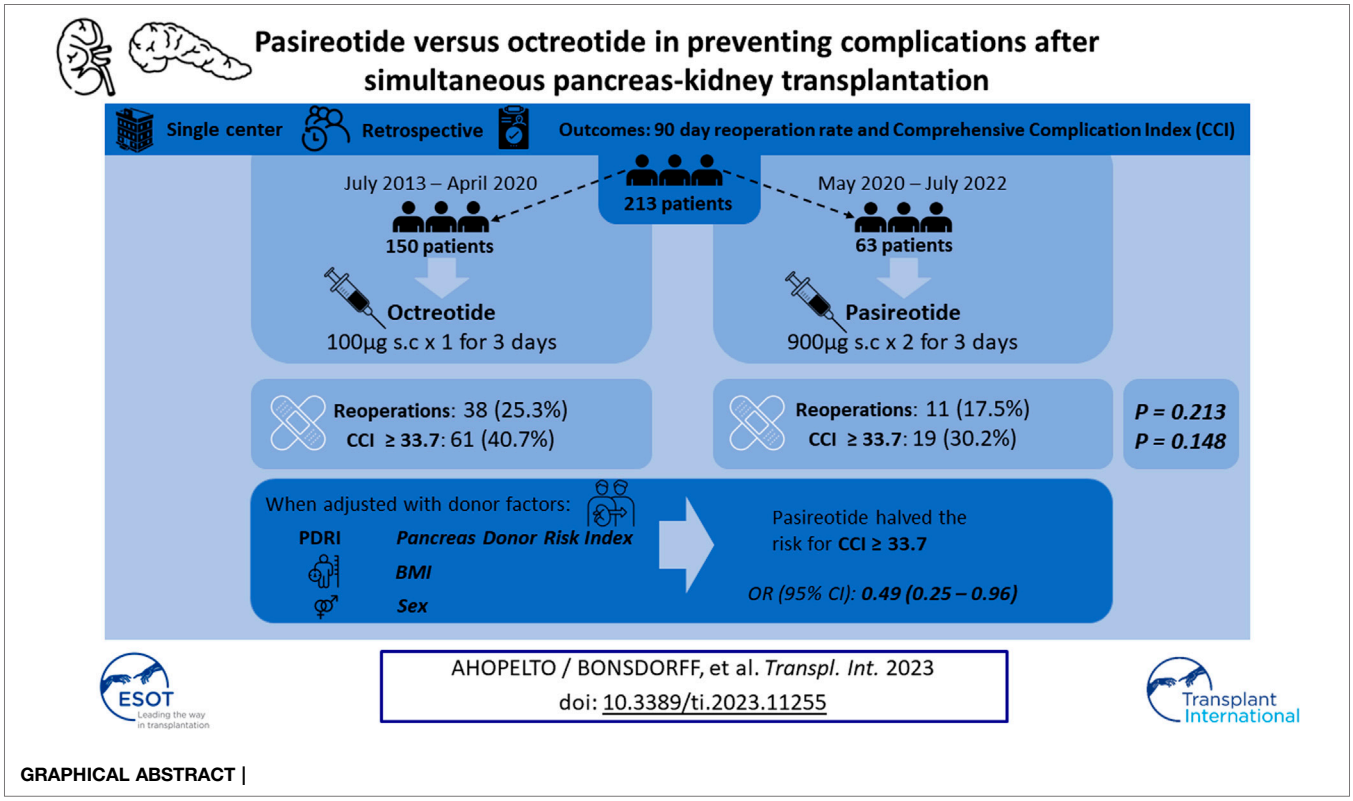

GRAPHICAL ABSTRACT |

INTRODUCTION

Simultaneous pancreas-kidney transplantation (SPK) offers superior survival over kidney transplantation only (or pancreas after kidney transplantation) to patients with type one diabetes (T1D) and end-stage kidney disease (ESKD) [1, 2]. It improves the quality of life and life-expectancy compared to patients remaining on dialysis [3]. The results of pancreas transplantation have improved over the years [4]. However, the complication burden is still high with as many as 25% of SPK patients undergoing reoperation within 90 days of the transplantation [5]. World consensus guidelines for pancreas transplantation do not discuss the use of somatostatin-analogues [3]. While thrombotic complications are a major cause for graft loss, intra-abdominal infections including pancreatitis and pancreatic fistulas also cause postoperative morbidity.

In elective pancreatic surgery, somatostatin-analogue octreotide has been variably used to prevent pancreatic fistula, but, according to current best evidence, octreotide has no effect on reducing complications, including pancreatic fistula after pancreatic resection [6]. Octreotide has been studied in pancreas transplantation setting, but all the studies are over 15 years old, and its routine use has not gained wide acceptance [7–9].

Pasireotide, another somatostatin-analogue with higher somatostatin receptor affinity and longer half-life, reduced the number of clinically significant pancreatic fistulas compared to placebo after pancreatic resections [10], as well as overall postoperative complications and rate of pancreatic fistulas compared to hydrocortisone in distal pancreatectomies [11] in randomized controlled trials. As noted previously by our group, hyperamylasemia after SPK on postoperative day 1 is a significant risk factor for subsequent morbidity [5]. This finding has been reproduced recently by another study [12], and recent findings have also demonstrated hyperamylasemia and postoperative pancreatitis to have clinical relevance after pancreatic resections [13, 14]. Thus, drugs targeting pancreatic exocrine suppression—such as somatostatin-analogues—may offer a potential mitigation strategy for pancreas graft related complications after pancreas transplantation.

Perioperative octreotide had been routinely used at our center in all SPK since the beginning of our pancreas transplantation program in 2010. Our institutional policy was recently changed to substitute octreotide with pasireotide as of May 2020.

The aim of this study was to compare octreotide and pasireotide for their association with postoperative morbidity after SPK, as well as to assess their association with early postoperative laboratory value trends.

MATERIALS AND METHODS

Patients

This was a retrospective cohort study comparing the association of pasireotide and octreotide with postoperative complications after

SPK. Consecutive patients suffering from T1D and ESKD undergoing SPK's at Helsinki University Hospital, Helsinki, Finland, between 8th July 2013 and 12th July 2022 were included in the study cohort. On 1st of May 2020, our institutional policy was changed from routine perioperative administration of octreotide to pasireotide. Patients undergoing SPK during 8th July 2013 and 30th April 2020 received octreotide 100 ug once daily starting at induction and up to at least 3rd postoperative day (POD). After 1st of May 2020, patients received pasireotide 900 ug twice daily starting at induction and up to the 3rd POD. All the grafts were from donors after brain death (DBD). Immunosuppression, surgical technique, and postoperative care remained similar throughout the study period, and have been described in detail elsewhere [5]. Institutional review board of Helsinki University Hospital approved the study (HUS/155/2021). No ethical board approval was required due to the observational nature of this study.

Variables Collected

Basic patient and donor demographics were collected. Donor age, donor sex, donor BMI, donor height, donor reason of death, pancreas cold-ischeamia time (CIT), and donor ethnicity were used to calculate pancreas donor risk index (PDRI) for every patient [15]. The PDRI is a continuous risk index where value 1.0 represents an average donor, and higher values represent a higher risk donor.

All postoperative complications occurring before the 90th POD were collected retrospectively from electronic patient records and graded according to the Clavien-Dindo classification [16]. The Comprehensive Complication Index (CCI) was used as an outcome to assess and compare the total cumulative morbidity of the patients [17]. In CCI, raw points are allocated according to the grade of the complication, summed together, and then scaled from 0 to 100. It allows for a much more sensitive comparison of patient outcomes since the cumulative effect of all postoperative complications are captured in the final score. In addition to using CCI as a continuous outcome, a cutoff of 33.7 points, which represents the burden of one reoperation, was used as an outcome in multivariable logistic regression to identify variables associated with higher postoperative morbidity. Pancreas graft associated complications comprised graft pancreatitis, pancreatic fistula/leakage from enteroanastomosis, and peripancreatic fluid collections. Length of hospital stay (LOS) was defined as time in days from the index operation to discharge.

Values of postoperative laboratory tests reflecting pancreatic secretions and inflammation—plasma amylase, drain fluid amylase, and C-reactive protein (CRP) - on each morning up to 7th POD, and laboratory test values reflecting graft function—fasting c-peptide levels, estimated glomerular filtration ratio (eGFR) and HbA1c—up to 180th POD were collected. Plasma amylase values are reported as a multiplication of our institutional upper limit of normal (ULN) to allow for better comparability between centers using different assays. Trends of laboratory values stratified by the type of somatostatin were analyzed to assess for possible differences in exocrine/endocrine suppression. Some cases had missing laboratory test values and multiple imputation (with 10 iterations) was performed with basic patient demographics as dummy variables to account for these missing values. Multiply imputing missing values is associated with smaller bias than excluding cases with missing values [18]. 52/2,130 (2.4%) of c-peptide, 99/1,141 (8.7%) of plasma amylase, 32/852 (3.8%) of HbA1c, 25/852 (2.9%) of eGFR, and 12/213 (5.6%) of donor creatinine values were missing and thus imputed.

Statistics

Continuous variables are reported as median and interquartile range (IQR) due to nonparametric distribution. Categorical variables are reported as frequencies and percentages. Differences in the distribution of continuous variables between the groups were assessed with Mann-Whitney-U -test and for categorical variables with Chi-squared test. Pre- and intraoperative risk factors for CCI $\geq$ 33.7 were assessed with logistic regression and a multivariable analysis was performed by including variables with strong univariable association ($p < 0.15$) to a multivariable model constructed with backwards stepwise logistic regression. Somatostatin-analogue variable was forced in to the multivariable model regardless of its univariable association as the aim was to control for the case mix between the cohorts. Variance inflation factors (VIF) were used to assess possible multicollinearity between variables in multivariable analyses. VIF -values under 2.5 are generally interpreted as insignificant correlation between the variables. Odds ratios (OR) with 95% confidence intervals are reported for the uni- and multivariable analyses. In general, a two-sided p-value of < 0.05 was considered statistically significant. All analyses were performed with IBM SPSS v28.

RESULTS

During the study period, 214 patients underwent SPK, of which one from the pasireotide group was excluded due to not receiving the correct drug. The final cohort included 150 patients receiving octreotide and 63 patients receiving pasireotide. The pasireotide and octreotide groups were comparable regarding recipient and donor baseline characteristics, excluding pancreas and kidney cold-ischeamia times (CIT), which were on average 1 h shorter in the pasireotide group, and duration of diabetes, which was on average 8 years longer in the octreotide group (**Table 1**). PDRI was comparable between the groups (**Table 1**). Median PDRI levels per 2-year intervals during the study period are illustrated in **Figure 1**.

Postoperative Complications and Outcomes

The frequency of individual postoperative complications is presented in **Table 2**. Hemorrhagic complications (Clavien-Dindo grade IIIa or worse) were the most common, occurring in 38 (17.8%) individual patients, followed by intra-abdominal fluid collections, which occurred in 30 (14.1%) individual patients. Only two pancreas graft thromboses were observed, both of them partial and successfully treated with anticoagulants.

The reoperation rate up to 90th POD was 25.3% ($n = 38$) in the octreotide group, and 17.5% ($n = 11$) in the pasireotide group, but

TABLE 1 | Basic demographics of 213 patients undergoing simultaneous pancreas-kidney transplantation, stratified by the type of somatostatin-analogue received perioperatively.

Variable	Octreotide (*n* = 150)	Pasireotide (*n* = 63)	*P* (Chi-squared or Mann-Whitney U)
Age, years, median (IQR)	43 (37–49)	40 (33–48)	0.063
Male sex, n (%)	101 (67.3)	37 (58.7)	0.363
BMI, median (IQR)	24.1 (21.5–27.1)	23.9 (21.7–26.9)	0.769
Donor age, years, median (IQR)	41 (29–50)	45 (28–54)	0.179
Donor, male sex, n (%)	75 (50.0)	31 (49.2)	0.916
Donor BMI, median (IQR)	23.7 (21.9–25.7)	24.5 (21.6–27.3)	0.089
Donor, reason of death, n (%)			0.811
CVA	98 (65.3)	45 (71.4)	
Anoxia	17 (11.3)	7 (11.1)	
Trauma	32 (21.3)	10 (15.9)	
Other	3 (2.0)	1 (1.6)	
> 2 HLA-AB mismatch (*n* = 138)	93 (62.0%)	45 (71.4%)	0.217
> 1 HLA-DR mismatch (*n* = 120)	84 (56.0%)	36 (57.1%)	0.754
Cold ischeamia time, pancreas (hours)	7.78 (6.27–8.77)	6.75 (5.42–7.92)	0.001
Cold ischeamia time, kidney (hours)	9.74 (7.74–10.77)	8.27 (5.70–9.75)	<0.001
Duration of diabetes in years, median (IQR)	34 (28–40)	26 (23–36)	<0.001
Duration of dialysis before transplantation, months, median (IQR)	13 (8–19)	13 (8–21)	0.604
PDRI, median (IQR)	1.48 (1.00–1.89)	1.74 (0.96–2.26)	0.097

Abbreviations: IQR, inter-quartile-range; BMI, body-mass-index; CVA, cerebrovascular attack; HLA, human leukocyte antigen; PDRI, pancreas donor risk index.

this difference was not statistically significant ($p = 0.213$). These results would translate into absolute risk reduction (ARR) of 7.8% and a number needed to treat (NNT) of 13 to avoid one reoperation. The most prevalent reason for reoperation in the whole cohort was hemorrhage [25/49 (51.0%)], followed by pancreas graft associated complications [13/49 (26.5%)], and postoperative ileus [5/49 (10.2%)]. No significant differences were observed for these reasons of reoperation between the groups (**Table 3**). Four (1.9%) pancreas grafts were lost during the 90-day postoperative period due to persistent intra-abdominal infections, and all occurred in the octreotide group.

The median (IQR) CCI was similar between the groups at both 30th and 90th POD timepoint with little difference between the timepoints demonstrating that most of the severe complications occurred during the first 30 postoperative days (**Table 3**). The 90-day CCI distribution stratified by somatostatin-analogue received is presented in **Figure 2**. The length of initial hospital stay was statistically significantly longer in the octreotide group, median 16 (IQR: 13–24) vs. median 14 (IQR: 10–19), $p = 0.009$ (**Table 3**). In addition, the incidence of intra-abdominal fluid collections requiring radiological intervention was significantly higher in the octreotide group, 26 (17.3%) vs. 3 (4.8%), $p = 0.015$. Other studied outcomes were similar between the groups (**Table 3**).

Pre- and intraoperative risk factors for the morbidity of one reoperation (CCI ≥ 33.7, $n = 80$, 37.6%) are presented in **Table 4**. Recipient age, BMI, estimated blood loss, duration of diabetes, and pancreas CIT had *p*-values over 0.150 in univariable analyses with morbidity, and were omitted from the backwards stepwise logistic regression. According to the final multivariable analysis, the probability for CCI ≥ 33.7 was lower for patients receiving pasireotide when adjusted with the identified risk factors PDRI, donor BMI, and donor sex, 0.49 (95% CI: 0.25–0.96), $p = 0.037$. VIF -values for somatostatin, PDRI, donor BMI and donor sex in this multivariable model were 1.08, 1.15, 1.11, and 1.03, respectively, showing neglibile multicollinearity between the variables.

Laboratory Test Trends

To assess the association of pasireotide and octreotide on pancreatic secretions, trend lines stratified by the type of somatostatin received were drawn for plasma amylase, drain fluid amylase, and CRP (**Figure 3A**). In general, the trend curves were declining in nature with the highest values occurring on the earliest POD's. No statistically significant differences were observed for amylases or CRP. Other laboratory variable trends reflecting graft function (eGFR, HbA1c, and c-peptide) are presented in **Figure 3B**. Interestingly, while significant difference in c-peptide levels during the first postoperative week was observed, this difference leveled off at the 180-day timepoint.

DISCUSSION

SPK predisposes patients to high risk for postoperative morbidity with a reoperation rate close to 25% [5, 19]. While graft thrombosis is commonly reported to account for the majority of pancreas graft loss, other complications more related to the exocrine pancreas function—like graft pancreatitis, anastomotic leaks, pancreatic fistulas and intra-abdominal infections—seem to contribute significantly to the overall morbidity [20, 21]. In this present study, overall reoperation rate was 23%, with postoperative hemorrhagic complications accounting for roughly half of the reoperations, followed by graft pancreatitis/infection in one-fourth of the cases. Postoperative graft loss rate was 1.9%, and all were due to persistent intra-abdominal infections. Additionally, in this retrospective study, pasireotide was independently associated with lower postoperative morbidity after SPK compared to octreotide.

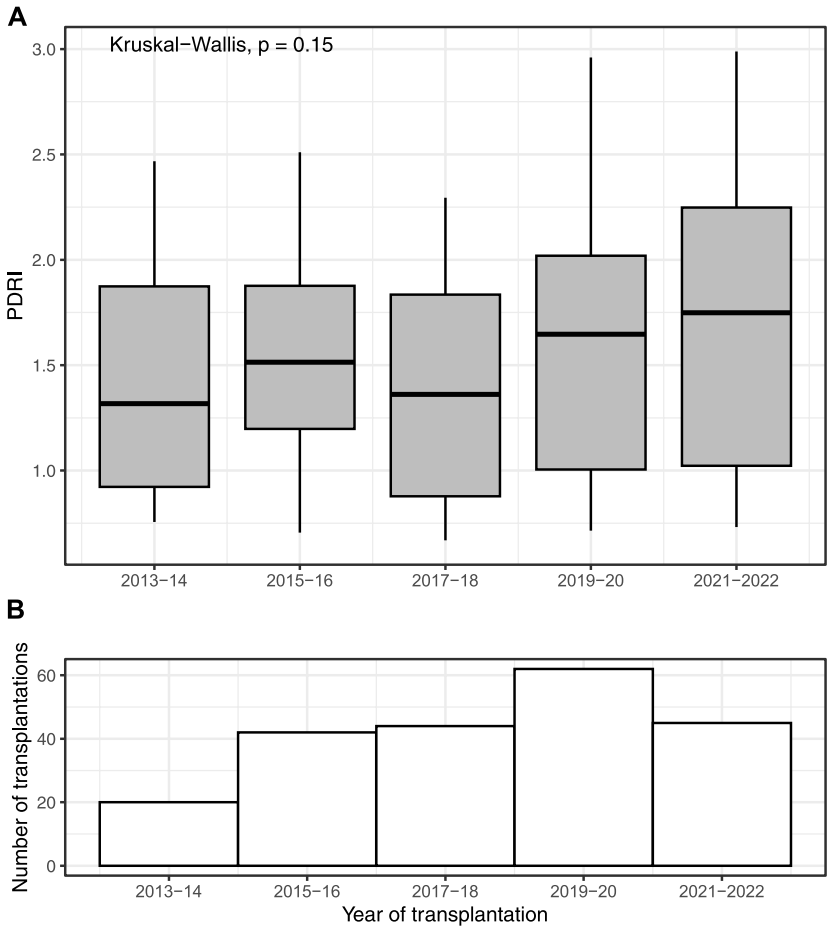

FIGURE 1 | Median (IQR) pancreas donor risk index (PDRI) of 213 patients undergoing simultaneous pancreas-kidney transplantation, reported per year during the study period from 2013 to 7/2022 **(A)**. Number of transplantations per year is reported below **(B)**.

TABLE 2 | Frequency of postoperative complications in the whole cohort and the somatostatin -groups of 213 patients undergoing simultaneous pancreas-kidney transplantation.

Complication	All (*n* = 213)	Octreotide (*n* = 150)	Pasireotide (*n* = 63)	*p*-value
Hemorrhage, region of graft pancreas, CD IIIa or worse	29 (13.6%)	20 (13.3%)	9 (14.3%)	0.853
Hemorrhage, region of graft kidney, CD IIIa or worse	10 (4.7%)	6 (4.0%)	4 (6.3%)	0.459
Pancreas graft thrombosis, any grade	2 (0.9%)	1 (0.7%)	1 (1.6%)	0.525
Graft pancreatitis, any grade	19 (8.9%)	17 (11.3%)	2 (3.2%)	0.057
Pancreatic fistula, CD IIIa or worse	3 (1.4%)	3 (2.0%)	0	0.258
Ileus, any grade	15 (7.0%)	11 (7.3%)	4 (6.3%)	0.798
Bowel perforation, CD IIIb or worse	2 (0.9%)	1 (0.7%)	1 (1.6%)	0.525
Wound dehiscence	13 (6.1%)	10 (6.7%)	3 (4.8%)	0.596
Peripancreatic fluid collection, CD IIIa or worse	9 (4.2%)	7 (4.7%)	2 (3.2%)	0.621
Perirenal fluid collection, CD IIIa or worse	21 (9.9%)	20 (13.3%)	1 (1.6%)	0.009
Hydronephrosis, CD IIIa or worse	16 (7.5%)	15 (10.0%)	1 (1.6%)	0.034
Unexplained fever, CD II or worse	47 (22.1%)	30 (20.0%)	17 (27.0%)	0.262

p-*values calculated for octreotide vs. pasireotide with chi-squared test. CD, Clavien-Dindo (grade).*

While great efforts have been made to reduce complications in elective pancreas surgery, few trials include patients undergoing pancreas transplantation. Somatostatin-analogues have been used in pancreatic surgery to reduce the risk of complications, especially pancreatic fistulas [10, 11, 22]. While several RCTs assessing the efficacy of octreotide exists, and meta-analysis of these RCTs show no effect [6], only two RCTs assessing pasireotide exists, both of which show benefit.

TABLE 3 | Postoperative outcomes up to 90th postoperative day of 213 patients undergoing simultaneous pancreas-kidney transplantation, stratified by the type of somatostatin-analogue received perioperatively.

Variable	Octreotide (*n* = 150)	Pasireotide (*n* = 63)	*P* (Chi-squared or Mann-Whitney U)
Length of hospital stay (d), median (IQR)	16 (13–24)	14 (10–19)	0.009
Reoperation, n (%)	38 (25.3)	11 (17.5)	0.213
Pancreas graft loss, 90 days, n (%)	4 (2.7)	0	0.191
Reoperation due to hemorrhage, n (%)	18 (12.0)	7 (11.1)	0.854
Reoperation due to pancreas graft associated complication, n (%)	10 (6.7)	3 (4.8)	0.759
Reoperation due to postoperative ileus, n (%)	3 (2.0)	2 (3.2)	0.605
Comprehensive Complication Index, median (IQR), 30 days	29.6 (20.9–42.6)	29.6 (20.9–33.7)	0.833
Comprehensive Complication Index, median (IQR), 90 days	29.6 (20.9–43.4)	29.6 (20.9–34.8)	0.434
CCI ≥ 33.7, n (%)[a]	61 (40.7)	19 (30.2)	0.148
CCI ≥ 47.7, n (%)[a]	21 (14.0)	6 (9.5)	0.370
Drainage of intra-abdominal fluid collection, n (%)	26 (17.3)	3 (4.8)	0.015
Organ space or deep SSI, n (%)	17 (11.3)	3 (4.8)	0.133
Pancreas associated complication, Clavien-Dindo II or worse, n (%)	40 (26.7)	12 (19.0)	0.259
PONV, DGE, ileus	24 (16.0)	7 (11.1)	0.265

CCI, comprehensive complication index; SSI, surgical site infection; PONV, postoperative nausea and vomiting; DGE, delayed gastric emptying.
[a]*CCI ≥ 33.7 equals cumulative morbidity of one reoperation, ≥47.7 of two reoperations.*

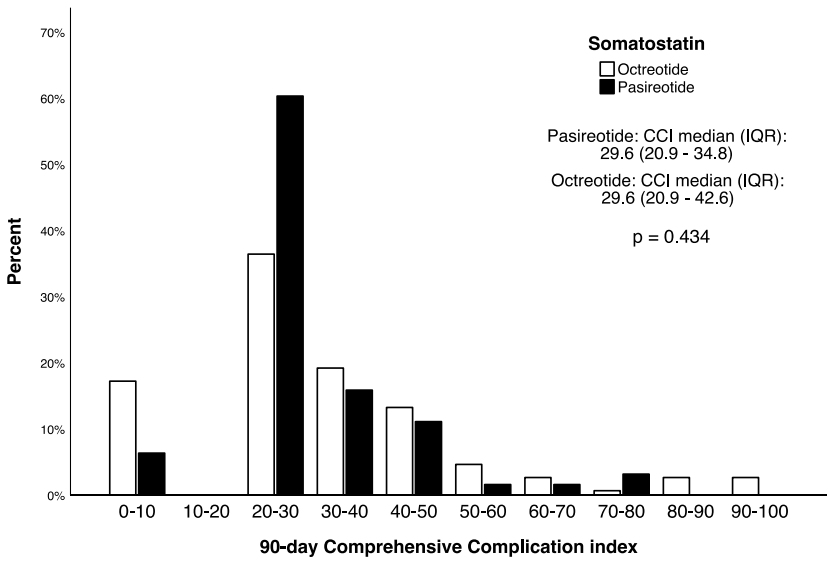

FIGURE 2 | The distribution of Comprehensive Complication index in 213 patients undergoing simultaneous pancreas-kidney transplantation, stratified by the type of perioperative somatostatin-analogue received.

There are three randomized controlled trials comparing octreotide to no treatment in pancreas transplantation setting [8, 9, 23], but all of them are over 15 years old and significantly underpowered due to small sample sizes.

The first randomized study of a somatostatin-analogue in pancreas transplant setting, by Stratta et al. in 1993, compared 13 patients that received octreotide 100 μg twice daily to 12 patients that received no somatostatin treatment [7]. Octreotide was initiated after transplantation and continued for 8 (±4) days. Octreotide reduced drain fluid amylase output, but there were no significant differences between the groups in patient or graft survival, infection, or surgical complications. In 1998, a study with 10 patients receiving perioperative octreotide 100 μg three times daily and seven patients receiving no treatment was conducted [8]. The patients in the octreotide group had no complications compared to the group receiving no somatostatin-analogue where one patient had a bladder leak and two developed intra-abdominal infections. Patient and graft survival were similar in both groups. In 2005, Hesse et al. reported no difference between 20 patients receiving perioperative octreotide 100 μg three times dailys compared to 20 patients receiving no treatment in terms of formation of pancreatic fistula (2 vs. 0). As octreotide interferes cyclosporine metabolism and possibly other immunosuppressive therapy as well and is costly, the study concluded that prophylactic treatment with octreotide cannot be recommended.

TABLE 4 | Univariable and multivariable analysis of pre- and intraoperative risk factors for one reoperation's morbidity (CCI ≥ 33.7) (*n* = 80, 37.6%).

Variable	Univariable		Multivariable	
	OR (95%CI)	*p*	OR (95%CI)	*p*
Age (y)	1.02 (0.98–1.05) per increase of 1 year	0.367		
BMI (kg/m^2)	0.97 (0.88–1.06) per increase of 1 kg/m^2	0.469		
Duration of diabetes, years	1.00 (0.97–1.03) per increase of 1 year	0.954		
Male sex	1.02 (0.57–1.81)	0.960		
PDRI	1.92 (1.17–3.15) per increase of 1	0.010	1.92 (1.11–3.33) per increase of 1	0.020
Donor age (y)	1.03 (1.01–1.05) per increase of 1 year	0.017		
Donor BMI (kg/m^2)	1.13 (1.03–1.24) per increase of 1 kg/m^2	0.009	1.11 (1.00–1.23) per increase of 1 kg/m^2	0.049
Donor male sex	1.65 (0.94–2.88)	0.081	1.80 (0.99–3.24)	0.051
Estimated blood loss (mL)	1.03 (0.94–1.14) per increase of 100 mL	0.499		
Nontraumatic donor death	1.32 (0.65–2.67)	0.449		
Cold ischeamia time, pancreas	1.09 (0.92–1.29) per increase of 1 h	0.310		
Cold ischeamia time, kidney	1.10 (0.97–1.24) per increase of 1 h	0.140		
Pasireotide (compared to octreotide)	0.63 (0.34–1.18)	0.150	0.49 (0.25–0.96)	0.037

Variable was entered into multivariable analysis if univariable association p < *0.15, backwards stepwise method was used.*
Abbreviations: BMI, body-mass-index; CCI, comprehensive complication index; PDRI, pancreas donor risk index.

An obvious limitation of these existing studies is small sample size, the largest study recruiting 20 patients per arm, leading to underpowered results and difficulties in drawing conclusions. Of note, the first two studies from the last century used bladder drainage technique instead of enteric drainage, and as such the results might not be generalizable to the contemporary era.

The levels of serum and intra-abdominal amylase was shown to be lower in the group receiving octreotide in these previous studies [8]. In our study, serum and drain fluid amylase levels were comparable between the groups, and receiving pasireotide did not seem to translate into stronger exocrine suppression. As no control group was available, it is difficult to assess the exocrine suppressive effect of these somatostatins. As noted in previous studies [5, 12], early hyperamylasemia is a significant risk factor for subsequent morbidity after SPK, and interventions mitigating it—such as somatostatin-analogues—could be of interest [5]. Interestingly, c-peptide levels were significantly lower throughout the first 7 POD's in the pasireotide group but leveled off during the 180-day follow up, and did not seem to associate with adverse events.

To the best of our knowledge, there are no other studies assessing pasireotide in pancreas transplantation setting. Pasireotide seems to be a safe alternative for octreotide and was independenctly associated with reduction of severe postoperative complications when compared to octreotide. Reoperation rate was 17.5% in the pasireotide group compared to the 25.3% in the octreotide group. Patients in the pasireotide group had a significantly lower incidence of intra-abdominal collections requiring radiological intervention (17.3% vs. 4.8%) and spent on average 2 days less in the hospital. The shorter hospital stay could be confounded by an overall trend to shorter hospital stays over the years, as the patients in the pasireotide group were operated later during the study period. In addition, pancreas CIT was statistically significantly shorter in the pasireotide-group, but this finding did not translate into an association with morbidity. This may be explained by the fact that median CIT was relatively short in both cohorts (7.8 h in the octreotide-group, 6.8 h in the pasireotide-group), and previous studies have identified CIT exceeding 12 h to associate with heightened morbidity [24]. When adjusted with PDRI, donor BMI, and donor sex to control for case-mix, receiving pasireotide translated into OR 0.49 for high postoperative morbidity compared to octreotide. On another note, no clinically meaningful outcomes favored octreotide in the comparisons. No significant difference in early amylase and CRP, or post-transplant eGFR or HbA1c levels was observed between the groups.

Limitations

There are several limitations to our study. Use of octreotide was introduced at the beginning of our SPK-program in 2010, and it was adapted and modified from other existing protocols. Partly due to the lack of evidence supporting octreotide use and the promising results from the pancreatic surgery RCTs by Allen et al. in 2013 and Tarvainen et al. in 2020 our protocol was changed [10, 11]. This is a retrospective analysis of the short-term results of this change. This was not planned as a study and thus lacks a control group. All our patients received a somatostatin-analogue and based on these results, we do not know the incidence of pancreas graft related complications if a somatostatin-analogue had not been used. The patients in the octreotide treatment group received a significantly smaller dose than in all other studies and one might argue that octreotide 100 μg daily is not comparable to pasireotide 900 μg twice a day, rather closer to no treatment. While generally unadvisable, CCI was dichotomized due to a relatively small sample size and its discrete distribution, and this might introduce optimism to the multivariable estimate of pasireotide effect size. Due to the retrospective setting, controlling for confounders is subpar and no causality can be concluded. Finally, while the study cohort is relatively large for a cohort of pancreas transplantations, the statistical analyses suffer from lack of power and most likely type 2 error is present. In order to have a 80% chance of detecting the reduction in reoperation rate reported in this study (from 25.3% to 17.5%), as significant at the 5% level, 862 patients would have been required.

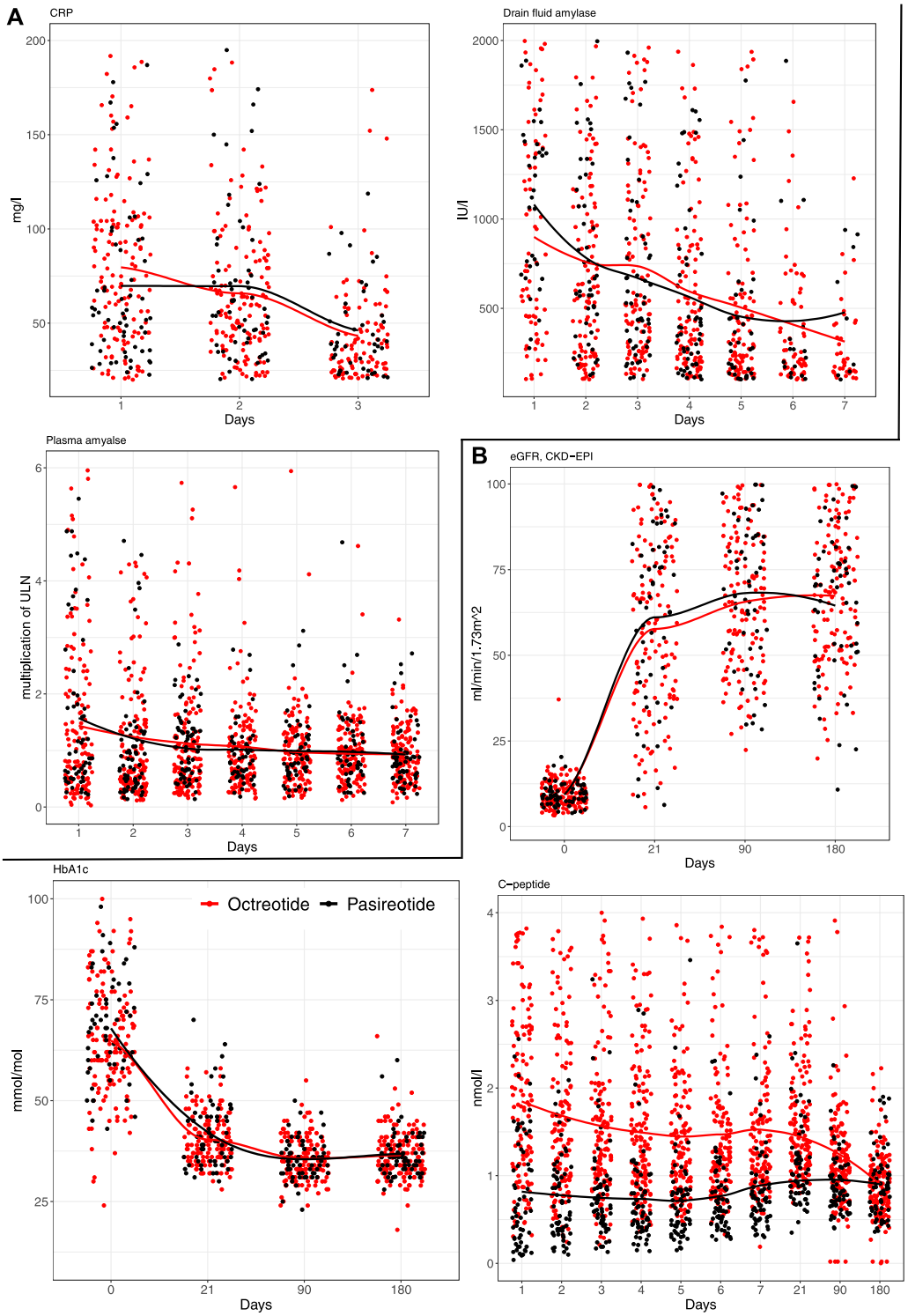

FIGURE 3 | Trend curves for **(A)** early and **(B)** late postoperative laboratory values after 213 simultaneous pancreas-kidney transplants, stratified by the type of somatostatin-analogue received.

CONCLUSION

Pasireotide is safe to use for patients receiving SPK transplant and its use was independently associated with reduced severe complications up to 90 days post-transplantation. Further prospective randomized study in larger cohorts is warranted but may be difficult to carry out due to relatively large number of patients required for statistical power.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data is not available due to the regional legislation. Requests to access the datasets should be directed to ville.sallinen@helsinki.fi.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

KA: participated in research design, data accrual, data-analyses, and writing of the paper. AB: participated in research design, data accrual, data-analyses, and writing of the paper. JG: participated in research design, data accrual, and writing of the paper. ML: participated in research design, and writing of the paper. AN: participated in research design, and writing of the paper. IH: participated in research design, data interpretation, and writing of the paper. VS: participated in research design, data interpretation, and writing of the paper. 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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Peripheral Blood Immune Cell Composition After Autologous MSC Infusion in Kidney Transplantation Recipients

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Tacrolimus is the backbone of immunosuppressive agents to prevent transplant rejection. Paradoxically, tacrolimus is nephrotoxic, causing irreversible tubulointerstitial damage. Therefore, infusion of mesenchymal stromal cells (MSC) 6 and 7 weeks post-transplantation was assessed to facilitate withdrawal of tacrolimus in the randomized phase II TRITON trial. Here, we performed detailed analysis of the peripheral blood immune composition using mass cytometry to assess potential effects of MSC therapy on the immune system. We developed two metal-conjugated antibody panels containing 40 antibodies each. PBMC samples from 21 MSC-treated patients and 13 controls, obtained pre-transplant and at 24 and 52 weeks post-transplantation, were analyzed. In the MSC group at 24 weeks, 17 $CD4^+$ T cell clusters were increased of which 14 Th2-like clusters and three Th1/Th2-like clusters, as well as CD4+FoxP3+ Tregs. Additionally, five B cell clusters were increased, representing either class switched memory B cells or proliferating B cells. At 52 weeks, $CCR7^+CD38^+$ mature B cells were decreased. Finally, eight Tc1 (effector) memory cytotoxic T cell clusters were increased. Our work provides a comprehensive account of the peripheral blood immune cell composition in kidney transplant recipients after MSC therapy and tacrolimus withdrawal. These results may help improving therapeutic strategies using MSCs with the aim to reduce the use of calcineurin inhibitors.

Clinical Trial Registration: ClinicalTrials.gov, identifier NCT02057965.

Keywords: kidney transplantation, immunosuppression, mesenchymal stromal cells, immune regulation, mass cytometry

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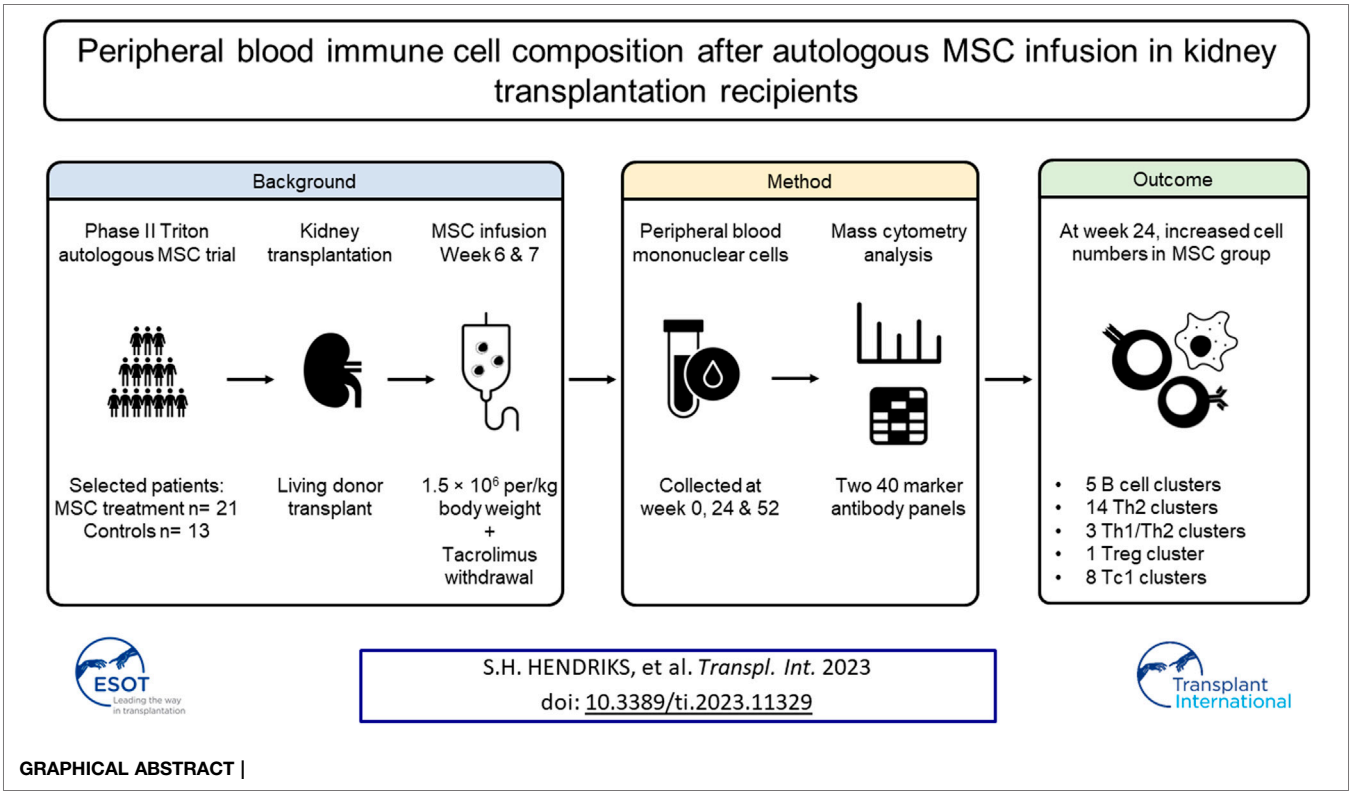
Abbreviations: B2M, β-2-microglobulin; dnDSA, *de novo* donor-specific antibodies; ILC, innate lymphoid cell; mAbs, monoclonal antibodies; MSC, mesenchymal stromal cells; NFAT, nuclear factor of activated T lymphocytes; NK cell, Natural killer cell; PBMC, Peripheral blood mononuclear cell; RT, room temperature; Tc, cytotoxic T cell; Th, helper T cell; Treg, regulatory T cell.



GRAPHICAL ABSTRACT |

INTRODUCTION

Kidney transplantation remains the preferred treatment for end-stage renal disease [1]. Tacrolimus, a calcineurin inhibitor, is the backbone of immunosuppressive protocols after kidney transplantation. Together with other immunosuppressive agents tacrolimus has vastly improved short-term allograft survival. However, despite these significant improvements, long-term kidney graft survival has not improved accordingly, partly due to long-term toxicity of immunosuppressive drugs [2–4]. Notably, tacrolimus is nephrotoxic, causing irreversible tubulointerstitial damage, which has led to numerous attempts to wean tacrolimus from the immunosuppressive regimen [5]. However, several studies have shown that tacrolimus withdrawal led to acute rejection episodes even in long-term stable patients [6–8]. Therefore, novel therapies are necessary to improve long term graft survival and minimize side effects of the current regiments.

One such new strategy that may allow cessation of tacrolimus use is mesenchymal stromal cell (MSC) therapy. MSCs have been shown to exert anti-inflammatory, immune-regulatory and tissue repair properties [9, 10]. They can interact both directly and indirectly with various immune cells [9, 10]. However, due to the observed short lifespan of MSCs *in vivo* [11], indirect effects through the release of extracellular vesicles, membrane particles and by undergoing apoptosis are thought to be most prominent. As such, MSC-derived vesicles may trigger monocytes and phagocytes to induce tolerogenic dendritic cells and regulatory T cells (Tregs) [11, 12]. This makes MSCs a promising new option to allow for tacrolimus weaning after kidney transplantation and possibly even the induction of immunological tolerance.

In the randomized phase II TRITON trial, administration of autologous bone marrow derived MSCs with concomitant early tacrolimus withdrawal was compared to standard tacrolimus dosing in living-donor kidney transplant recipients [13, 14]. The MSC group received MSC infusion at week 6 and 7, after which tacrolimus was reduced by half at week 7 and completely withdrawn at week 8. The control group remained on standard tacrolimus dosing and the study was performed using alemtuzumab as induction and an mTOR inhibitor as maintenance therapy. In our previous work, using flow cytometry on freshly obtained samples, we showed an increase in absolute number of peripheral blood Tregs in the MSC group compared to controls at 24 and 52 weeks after transplantation [13].

In this study we applied mass cytometry to perform in-depth characterization of the peripheral blood immune composition of patients included in the TRITON trial. We developed and validated two metal-conjugated mass cytometry antibody panels containing 40 antibodies each for the staining of bio-banked PBMCs and studied the influence of MSC therapy on immune cell subsets at 24 and 52 weeks after transplantation.

MATERIALS AND METHODS

Study Design

The TRITON clinical trial was a randomized phase II, prospective, single-center, open-label study in living-donor

kidney transplant recipients in which autologous bone marrow derived MSC therapy, with concomitant early tacrolimus withdrawal, was compared to standard tacrolimus dosing. The study was performed at the Leiden University Medical Center (LUMC), the Netherlands. The trial design and trial protocol have been previously described and were approved by the local ethics committee at the LUMC, Leiden, and by the Central Committee on Research involving Human Subjects in the Netherlands [13, 14]. The trial was performed in accordance with the principles of the Declaration of Helsinki. Inclusion and exclusion criteria were described in the trial protocol [14]. Written informed consent was obtained from all participants.

In short, patients in the MSC group received two doses of autologous bone marrow derived MSCs, intravenously at weeks 6 and 7 after transplantation. Bone marrow was aspirated from the posterior iliac crest of all patients in the MSC group during the renal transplantation. Processing of the MSCs took place at the GMP Facility of the LUMC. The MSC product was infused at week 6 and week 7 via peripheral infusion within 30 min with a target dose of 1.5×106 per/kg body weight intra venously (range $1–2 \times 10^6$ cells).

During the trial, protocol blood samples were obtained before transplantation (week 0), at weeks 6, 12, 24 and 52 after transplantation. Of the 70 subjects, 34 were selected for the mass cytometry study of which 21 had received MSC treatment and 13 were control patients. Selection was based on the availability of sufficient PBMCs, a 3:2 ratio between the MSC group and control group and similar age distribution (control; 26–66 years, mean: 50 years, MSC; 31–70 years, mean: 51 years), **Supplementary Table S1**.

All patients received their allocated treatment. In the control group one patient had not enough PBMCs stored at 24 weeks and another patient lacked the 52 weeks timepoint. Limited immune phenotyping by flow cytometry was already performed on fresh PBMCs of these patients and showed several differences at 24 and 52 weeks [13]. Therefore, we selected week 0, week 24 and week 52 for high dimensional analysis by mass cytometry.

Mass Cytometry Staining and Data Acquisition

PBMCs were isolated by Ficoll-Paque density-gradient centrifugation and cryopreserved in liquid nitrogen until time of analysis in RPMI, 20%FCS, 10%DMSO. Two metal conjugated 40-antibody panels for mass cytometry were developed, panel 1 focusing on B cell, NK cell and T cell markers and panel 2 focusing on myeloid and NK cell markers. Heavy metal isotope-tagged monoclonal antibodies (mAbs) for mass cytometry are listed in **Supplementary Tables S2, S3**. Samples were live-cell barcoded, stained and measured in batches of nine patient samples and one reference sample (total of 11 batches, samples of each patient were kept within one batch). Barcoding of live cell samples was performed with α-B2M (anti-β-2-microglobulin) and α-CD298 mAbs using a protocol adapted from Mei et al [15]. In brief, both mAbs were conjugated to Pd104, Pd105, Pd106, Pd108 or Pd110 using isothiocyanobenzyl-EDTA. Next, 10 barcode mixes were made, each containing both α-B2M and α-CD298 conjugated to their respective Pd isotopes, aliquoted and stored at −80°C until time of staining. For staining, purified mAbs were pre-conjugated by Fluidigm or conjugated with heavy metals in-house using the MaxPar X8 Antibody Labeling Kitaccording to the manufacturer's instructions (Fluidigm). All mAbs were titrated to determine the optimal labelling concentration. Antibody mixes for barcoding and extracellular staining of panel 1 and panel 2 were aliquoted in maxpar cell staining buffer, the intracellular mix of panel 1 was aliquoted in Perm Buffer (eBiosciences), all stored at −80°C until time of staining.

PBMCs were thawed, washed with RPMI, 50%FCS, and incubated with 0.04 mg/mL DNase in IMDM, 10% FCS in at room temperature (RT) for 30 min. Cells were washed with IMDM, 10% FCS, counted and for each panel $2.5*10^6$ cells/sample were washed with cell staining buffer. Next, the cells were incubated with 1 mL cell staining buffer containing 1 μM Cell-ID intercalator-103Rh (Fluidigm) for 15 min at RT. Cells were washed and incubated with human Fc receptor block (BioLegend) for 10 min at RT and stained with thawed barcode antibody mixes for 45 min at RT. After washing twice, 10 samples were pooled, washed and incubated for 45 min at RT with the extracellular antibody mix. After washing, for panel 1 intracellular staining was performed, for panel 2 we continued with DNA staining. Intracellular staining was performed using the Foxp3/transcription factor staining buffer set (eBiosciences). Cells were incubated with Fix/Perm working solution for 45 min at 4°C, cells were washed with Perm Buffer and incubated with thawed intracellular antibody mix for 30 min at RT in a final volume of 200 μL. For the DNA stain, the cells were washed, incubated with 1 mL Maxpar Fix and Perm buffer (Fluidigm) containing 0.125 μM Cell-ID intercalator-Ir (Fluidigm) overnight at 4°C.

Cells were acquired within 48 h of staining on a Helios mass cytometer (Fluidigm) at an event rate of <250 events/sec in Cell Acquisition Solution containing ×10 diluted EQ Four Element Calibration Beads (Fluidigm). For the compensation matrix, staining beads (eComp) were individually stained with the conjugated mAbs and incubated for 45 min. After washing, the beads were pooled, washed and acquired in cell staining buffer. Experiments and acquisition were performed in a period of 81 days.

Mass Cytometry Data Analysis

Data were normalized with EQ-normalization passport for each experiment. Subsequently, the data were gated using Flowjo v10.6.1, using channels 89Y_CD45, 193Ir_DNA, Residual, 103Rh_DNA (live/dead) and 140Ce_bead, removing debris, dead cells and doublets. Next, the data were compensated and debarcoded in R v4.1.1 using the CATALYST package and automatic cutoffs. The data were arcsin 5 transformed in Cytosplore. Using the reference sample, the data were corrected for batch effects using R after which the data were downsampled to a maximum of 50.000 cells/sample. For the discovery analysis the FlowSOM package was used [16]. The downsampled cells were used for the first overview FlowSOM, containing 100 clusters gathered in 30 metaclusters. Next, metaclusters sharing similar phenotypes were merged,

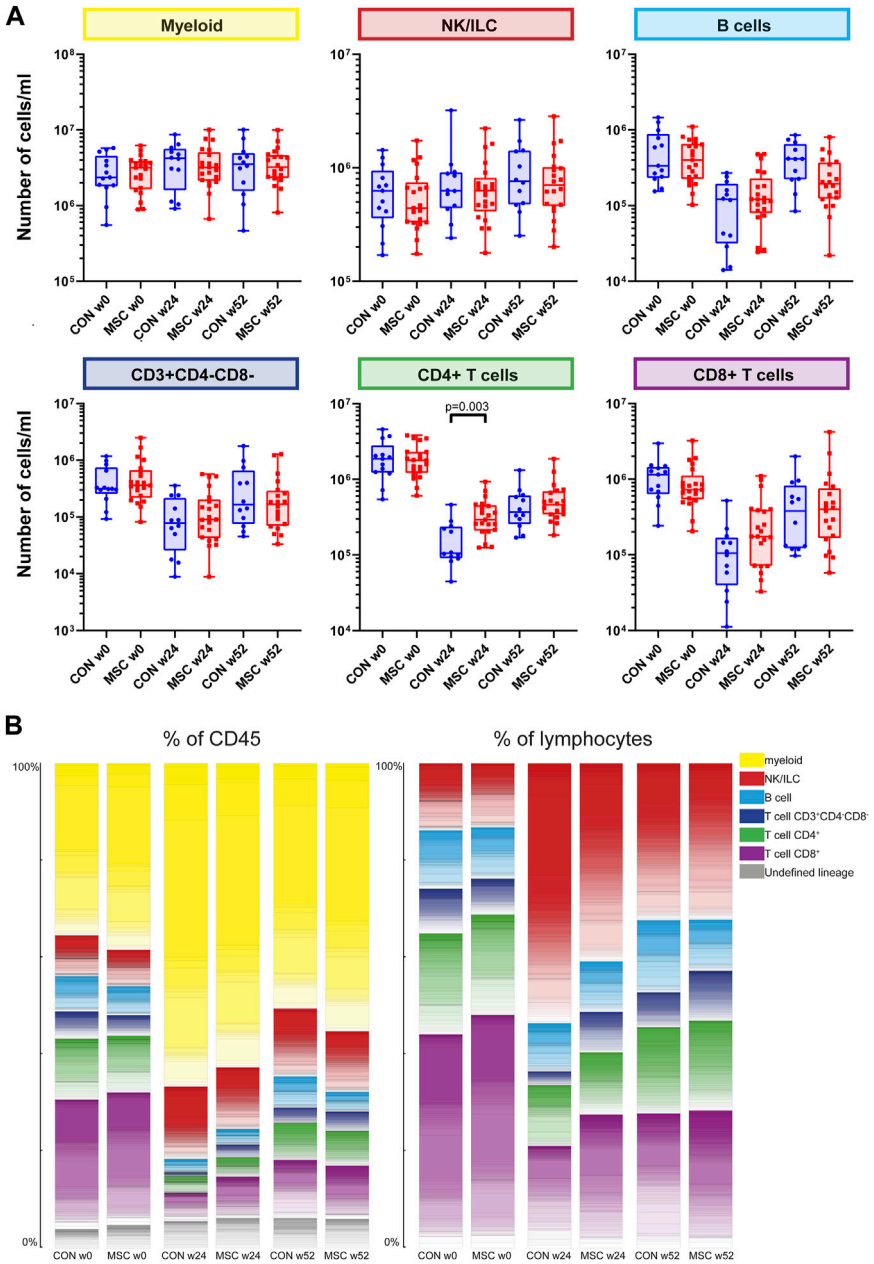


FIGURE 1 | Major immune lineages. **(A)** Graphs showing the number of cells/mL in the control group and the MSC therapy group at timepoint w0, w24, and w52, for the five major immune lineages, Myeloid, NK/ILC, B cells, $CD4^+$ T cells and $CD8^+$ T cells. Each dot represents an individual patient within that timepoint. CON, control group (Blue); MSC, MSC therapy group (Red). P-values were calculated with the Mann-Whitney U test and corrected within each cluster with Bonferroni. **(B)** The contribution of the different cell clusters and major lineages as percentage of $CD45^+$ cells (left panel) and as percentage of lymphocytes (right panel). Both for the control group and the MSC therapy group at timepoint w0, w24, and w52.

resulting in four groups (panel 1) and three groups (panel 2). A separate FlowSOM was performed for each group, allowing for in-depth analysis. For panel 1, group 1, 2 and 3, and panel 2, group 1, 2 and 3, a FlowSOM was created with 121 clusters and 100 metaclusters and for panel 1 group 4, a FlowSOM with 225 clusters and 200 metaclusters was made. Metaclusters with similar phenotypes were merged, resulting clusters contained all >500 cells and originated from different samples. Doublet clusters were removed. Using absolute cell counts obtained on fresh blood samples using the BD Multitest kit (BD Biosciences) the absolute number of cells per cluster were calculated. Finally, for each cluster, the MSC therapy group was compared to the control group and graphs were made using Graphpad prism v8.4.2. Measurements with value 0 are depicted as a dot on the X-axis. For validation purposes selected subsets were gated using Flowjo v10.6.1.

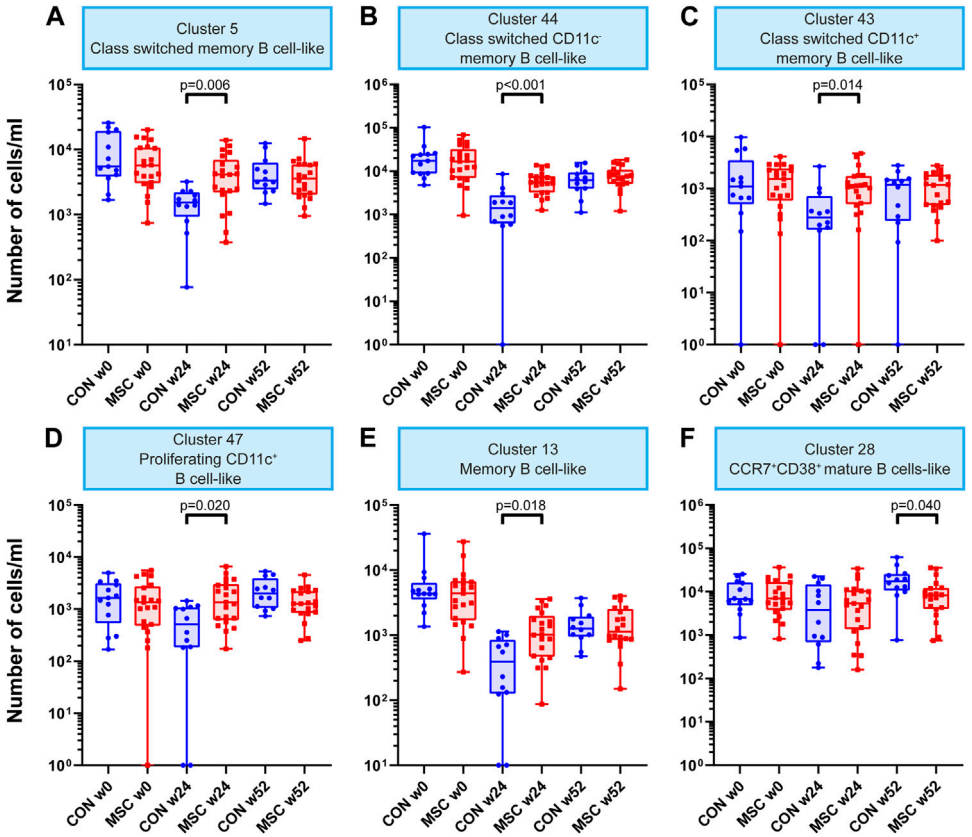


FIGURE 2 | B cell clusters. Graphs showing the number of cells/mL at timepoint w0, w24, and w52 for both the control and the MSC therapy group. **(A)** Cluster 5; Class switched memory B cell-like. **(B)** Cluster 44; Class switched CD11c memory B cell-like. **(C)** Cluster 43; Class switched CD11c$^+$ memory B cell-like. **(D)** Cluster 47; Proliferating CD11c$^+$ B cell-like. **(E)** Cluster 13; Memory B cell-like. **(F)** Cluster 28; CCR7$^+$CD38$^+$ mature B cells-like. CON, control group (Blue); MSC, MSC therapy group (Red). P-values were calculated with the Mann-Whitney U test and corrected within each cluster with Bonferroni.

Statistical Analysis

For the discovery analysis, the comparisons of the control group versus the MSC therapy group within each cluster were performed with the Mann-Whitney U test in Graphpad prism version 8.4.2, corrected with Bonferroni.

RESULTS

CD4$^+$ T Cells are Increased in MSC-Treated Patients at 24 Weeks

Data were analyzed using the FlowSOM clustering method. First, we performed a highly detailed analysis of panel 1 at the single cell level which resulted in the identification of 346 phenotypically distinct cell clusters. For each cluster we determined the major lineage (B cells, myeloid cells, CD4$^+$ T cells, CD8$^+$ T cells and NK/ILCs, **Supplementary Figure S1**) based on all markers in the panel, and compared the number of cells of each lineage in the control and the MSC therapy group at weeks 0, 24 and 52 (**Figure 1A**).

As a consequence of the alemtuzumab-induced lymphodepletion, B cells and T cells were still repopulating at week 24 and did not reach baseline levels at week 52, reflected in the data by a rising number of B cells and T cells between week 24 and week 52 in both groups. There was no difference in absolute cell numbers between the control and the MSC group for B cells, myeloid cells, NK/ILCs, CD3$^+$CD4$^-$CD8$^-$ T cells and CD8$^+$ T cells. However, at week 24 CD4$^+$ T cells were increased in the MSC group compared to the control group (p = 0.003). To validate this finding, traditional two-dimensional manual gating was used for analysis of the major immune cell lineages. This likewise identified a significant increase of the CD4$^+$ T cells in the MSC patients at week 24 (p = 0.038, **Supplementary Figure S2**).

Next, the contribution of each lineage as a percentage of the total CD45 population at each of the timepoints was assessed (**Figure 1B**). While pre-transplantation (week 0) lymphocytes make up 56%–60% and myeloid cells 35%–38% of the total CD45$^+$ population, at week 24 this distribution was skewed towards a dominance of myeloid cells due to the alemtuzumab-induced lymphodepletion. As expected, at week 52 the myeloid cells still made up the majority of CD45$^+$ cells, however the proportion of lymphoid cells was increasing. At 24 weeks, both the percentage of CD3$^+$CD4$^-$CD8$^-$ and CD8$^+$ cells were increased in the MSC group compared to the controls. While absolute cell numbers CD4$^+$ T cells were increased in the MSC group compared to the controls, as a percentage of both

total CD45$^+$ cells and total lymphoid cells the numbers of CD4$^+$ cells were similar in both groups (**Figure 1B**).

Investigating the 346 phenotypically distinct cell clusters individually, 33 (of which 32 assigneable to a lineage) showed a statistically significant difference in absolute cell numbers between the control and the MSC-treated group. The 32 lineage defined clusters will be further discussed below (**Supplementary Table S4**).

Changes Within the B Cell Compartment Upon MSC Treatment

Within the B cell clusters ($n = 47$), five were increased in absolute cell numbers in the MSC therapy group compared to the control group at week 24 (**Supplementary Table S4**). These B cell clusters included class switched memory B cells, class switched CD11c$^-$ and CD11c$^+$ memory B cell-like clusters, a proliferating (Ki-67$^+$) CD11c$^+$ B cell-like cluster and a memory B cell cluster (**Figures 2A–E**). While these clusters were increased in MSC-treated patients at 24 weeks, they were similar to controls at 52 weeks. The sixth B cell cluster of CCR7$^+$CD38$^+$ mature B cells showed a decrease in absolute number of cells at week 52 in the MSC group compared to the control group (**Figure 2F**).

Tc1-Like and Tc1/Tc2-Like Clusters are Enriched in MSC-Treated Patients at Week 24

Investigating the CD8$^+$ T cells clusters ($n = 73$), eight clusters showed a statistically significant increase at week 24 in the MSC group compared to the control group (**Supplementary Table S4**). These were a CD57$^+$CD45RA$^+$CD45RO$^+$ Tc1-like cytotoxic T cell cluster, three memory Tc1-like cytotoxic T cell clusters (CD27$^+$CD127$^+$, CD39$^+$CD27$^+$CD127$^+$ and CD27$^+$CD57$^+$CD127$^+$), two proliferating memory Tc1-like cytotoxic T cell clusters (CD27$^+$CD57$^+$CD127$^+$ and CD27$^+$CD57$^+$CD127$^-$PD-1$^+$Tigit$^+$), an CD57$^+$CD127$^+$ effector memory Tc1-like cytotoxic T cell cluster and finally an CD57$^-$CD127$^+$ effector memory Tc1/Tc2 cytotoxic T cell cluster (**Figures 3A–H**).

Increased Numbers of CD11c$^+$CD127$^+$ NK Cells at 52 Weeks in the MSC Treated Patients

One NK cell cluster was increased in absolute cell numbers in the MSC therapy group compared to the control group at 52 weeks. This cluster is a CD11c$^+$CD127$^+$ NK cell cluster (**Supplementary Table S4** and **Figure 3I**). NK cells are dominant in the early repopulation phase after alemtuzumab. Whereas the first antibody panel was able to discriminate between the major lineages, it did not contain highly detailed information about the different subsets of myeloid and NK cells. To get a more detailed insight into the NK cells and myeloid cells, we developed a second antibody panel. The discovery FlowSOM analysis of this panel resulted in a total of 85 phenotypically distinct myeloid and NK/ILC clusters of which only cluster 15 was significantly different between the control group and MSC treated patients. However, this is unlikely to be due to the treatment, since this difference was already present pre-transplantation (**Supplementary Figure S3**).

Cell Numbers of Th2-Like and Th1/Th2-Like Clusters are Elevated in MSC-Treated Patients at Week 24

Exploring the CD4$^+$ T cell clusters ($n = 57$), 17 showed a statistically significant increase in absolute cell numbers at week 24 in the MSC group compared to the control group (**Table 1**, **Supplementary Table S4**). These were a CD7$^+$CD27$^+$CD127$^+$ central memory Th2-like cluster, seven effector memory Th2-like clusters, six activated effector memory Th2-like clusers and three effector memory Th1/Th2-like clusters.

Mass Cytometry Analysis Confirms Transient Increase in Treg Numbers in MSC-Treated Patients

In our analysis we could identify four FoxP3$^+$ Treg clusters (clusters 130, 131, 132, and 133) which combined likely reflect the total pool of Tregs in the samples. We observed that the total Tregs were increased at 24 weeks for the MSC group ($p = 0.038$, **Figure 4A**). Also when evaluating the four Treg clusters individually, a similar increase in cell numbers at week 24 was observed for the MSC treated patients, reaching significance for cluster 133 (**Figures 4B–E**). Cluster 133, FoxP3$^+$CD7$^-$TIGIT$^-$ CTLA-4$^-$CD39$^+$, differed from the other 3 FoxP3+ Treg clusters by lack of CD7 and Tigit.

Previously, as part of the original TRITON study protocol, fresh PBMCs were measured with the ONE-Study flow cytometry panel [13, 17]. In these analyses absolute cell numbers of CD4$^+$CD25^{hi}CD127^{lo} and CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^-$ Tregs were increased in MSC treated patients at week 24 and week 52. Therefore, in the current mass cytometry study, we used manual gating to identify and quantify these CD4$^+$CD25^{hi}CD127^{lo} and CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^-$ subsets. This revealed a similar trend at week 24, although not reaching statistical significance (**Figures 4F, G**).

DISCUSSION

In this study we used mass cytometry to investigate the effect of the application of MSC therapy at week 6 and 7 after kidney transplantation with concomitant tacrolimus withdrawal on a background of alemtuzumab and mTOR inhibitor. In previous work we described successful tacrolimus withdrawal after MSC infusion [13]. Furthermore, we observed an increase of peripheral blood Tregs in the MSC group compared to controls at 24 and 52 weeks after transplantation [13]. In the current study we aimed to better understand the influence of MSCs on the immune system and on facilitating tacrolimus withdrawal. Two mass cytometry panels were developed, together covering 69 immune cell markers and used to determine the

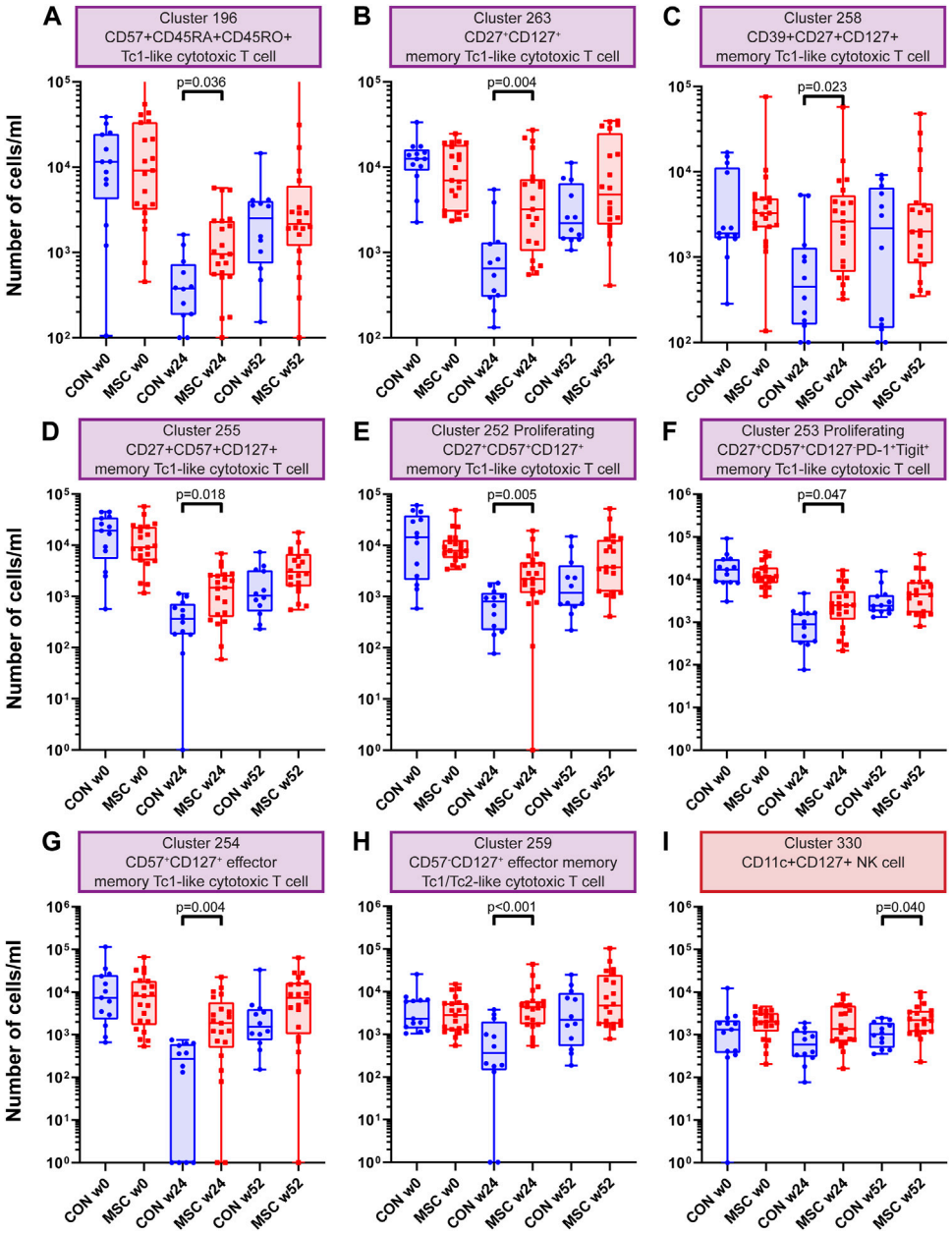


FIGURE 3 | CD8 T cell and NK cell clusters. Graphs showing the number of cells/ml at timepoint w0, w24 and w52 for both the control and the MSC therapy group. **(A)** Cluster 196; $CD57^+CD45RA^+CD45RO^+$ Tc1-like cytotoxic T cell. **(B)** Cluster 263; $CD27^+CD127^+$ memory Tc1-like cytotoxic T cell. **(C)** Cluster 258; $CD39^+CD27^+CD127^+$ memory Tc1-like cytotoxic T cell. **(D)** Cluster 255; $CD27^+CD57^+CD127^+$ memory Tc1-like cytotoxic T cell. **(E)** Cluster 252; Proliferating $CD27^+CD57^+CD127^+$ memory Tc1-like cytotoxic T cell. **(F)** Cluster 253; Proliferating $CD27^+CD57^+CD127^-PD\text{-}1^+Tigit^+$ memory Tc1-like cytotoxic T cell. **(G)** Cluster 254; $CD57^+CD127^+$ effector memory Tc1-like cytotoxic T cell. **(H)** Cluster 259; $CD57^-CD127^+$ effector memory Tc1/Tc2-like cytotoxic T cell. **(I)** Cluster 330; $CD11c^+CD127^+$ NK cell. CON: control group (Blue), MSC: MSC therapy group (Red). *p*-values were calculated with the Mann-Whitney U test and corrected within each cluster with Bonferroni.

composition of the peripheral blood immune cell compartment of control patients and patients receiving MSC therapy.

MSCs can affect many types of immune cells including dendritic cells, monocytes, macrophages, B cells, T cells (Treg/Th1/Th2 and Th17 helper cells), NK cells and NKT cells, ILCs, myeloid-derived suppressor cells, neutrophils, and mast cells through a combination of direct cell-cell contact and soluble factors (reviewed by Weiss et al. and Jiang et al. [18, 19]). MSCs are incapable of passing narrow capillary networks due to their size and thus often accumulate in the lungs upon intravenous infusion [11, 20]. The additional short-life span makes direct cell-cell interaction in other tissues, such as the kidneys, unlikely in the context of a therapeutic setting. Immune modulatory and organ regenerative effects of MSCs can also be mediated by their secretome, as shown in various immune and injury models [21, 22]. Part

TABLE 1 | CD4 clusters.

Cluster	Cell type	Cells/μL median (range) week 0			Cells/μL median (range) week 24			Cells/μL median (range) week 52		
		Control	MSC	*p*-value	Control	MSC	*p*-value	Control	MSC	*p*-value
	CD4 central memory Th2									
141	$CD7^+CD27^+CD127^+$ central memory Th2-like	412 (122–1,091)	371 (147–881)	ns	36 (13–158)	84 (27–230)	0.047	109 (33–291)	92 (34–236)	ns
	CD4 effector memory Th2									
149	$CD7^+CD27^+CD127^+$ effector memory Th2-like	9 (1–34)	7 (3–51)	ns	1 (0–6)	3 (1–26)	0.005	1 (3–33)	1 (6–73)	ns
151	$CD7^{lo}CD27^+CD127^+$ effector memory Th2-like	44 (20–122)	53 (21–151)	ns	2 (1–7)	5 (1–17)	0.038	7 (3–21)	9 (2–16)	ns
228	$CD7^-CD127^+$ effector memory Th2-like	15 (5–126)	16 (6–113)	ns	1 (0–6)	2 (0–14)	0.023	3 (1–44)	7 (1–39)	ns
224	$CD127^+CD161^+$ effector memory Th2-like	11 (3–43)	12 (2–27)	ns	1 (0–4)	2 (0–15)	0.030	3 (1–23)	5 (2–72)	ns
220	$CD39^+CD7^-CD127^+$ effector memory Th2-like	4 (1–17)	3 (1–55)	ns	0 (0–8)	3 (1–49)	0.009	2 (1–61)	4 (0–87)	ns
152	$CD27^+CD127^-PD\text{-}1^+$ effector memory Th2-like	5 (1–11)	4 (1–20)	ns	2 (1–7)	4 (1–25)	0.023	3 (1–19)	4 (1–27)	ns
96	Proliferating $HLA\text{-}DR^+CD7^-CD127^+$ effector memory Th2-like	5 (1–20)	5 (1–40)	ns	1 (0–14)	3 (0–20)	0.011	4 (0–20)	4 (0–33)	ns
	Activated CD4 effector memory Th2									
229	$CD7^+CD127^+$ activated effector memory Th2-like	15 (3–73)	13 (4–106)	ns	1 (0–7)	3 (0–19)	0.008	4 (0–19)	6 (1–33)	ns
145	$CD7^+CD27^+CD127^+$ activated effector memory Th2-like	10 (4–49)	13 (4–84)	ns	0 (0–5)	2 (0–17)	0.020	3 (0–18)	4 (1–29)	ns
148	$CD7^-CD27^+CD127^+$ activated effector memory Th2-like	11 (3–55)	13 (6–64)	ns	1 (0–3)	3 (0–12)	<0.001	3 (1–14)	6 (1–21)	ns
226	$CD7^-CD127^+$ activated effector memory Th2-like	9 (3–23)	10 (4–37)	ns	1 (0–3)	3 (1–46)	0.014	5 (0–12)	4 (2–36)	ns
223	$CD127^+CD161^+PD\text{-}1^+$ activated effector memory Th2-like	11 (1–27)	11 (2–57)	ns	2 (0–7)	4 (1–27)	0.005	6 (1–18)	5 (2–72)	ns
227	$CD7^+CD27^+$ activated effector memory Th2-like	28 (11–113)	38 (9–138)	ns	1 (0–6)	5 (0–22)	0.008	6 (1–32)	9 (2–50)	ns
	CD4 effector memory Th1/Th2									
217	$CD57^+CD127^+PD\text{-}1^+$ effector memory Th1/Th2-like	2 (0–6)	2 (0–16)	ns	0 (0–4)	3 (0–65)	<0.001	2 (0–56)	7 (1–80)	ns
222	Proliferating $CD57^+CD127^+$ effector memory Th1/Th2-like	7 (1–37)	6 (2–27)	ns	1 (0–4)	3 (0–40)	0.030	4 (1–18)	8 (0–46)	ns
221	Proliferating $HLA\text{-}DR^+CD39^+CD57^+CD127^+$ effector memory Th1/Th2-like	3 (1–9)	2 (0–15)	ns	0 (0–8)	3 (0–13)	0.023	3 (1–41)	4 (0–70)	ns

Table depicting significant CD4 clusters. Control, control group; MSC, MSC therapy group; ns, not significant. P-values were calculated with the Mann-Whitney U test and corrected within each cluster with Bonferroni.

of the secretome of MSCs are extracellular vesicles. These vesicles contain extracellular matrix proteins, cell adhesion proteins and microRNAs, all able to influence the immunological response [21, 23, 24]. To which degree these factors are important after therapeutic MSC infusion is still unclear. Finally, it has been shown that MSC therapy could affect the immune system as a result of apoptosis and subsequent phagocytosis by monocytes/macrophages, neutrophils and dendritic cells [10]. Upon phagocytosis these cells were shown to migrate through the bloodstream to the liver and other organs, with altered phenotype and function, possibly modulating the immune response over an extended period of time [11, 25]. In this study, we therefore extensively investigated changes in the myeloid compartment after MSC infusion. Our results showed no differences in this compartment, we could therefore not confirm (long term) involvement of myeloid cells.

In the current study alemtuzumab was used as induction therapy, resulting in profound depletion of circulating lymphocytes. As expected, we could confirm that repopulation after induction therapy is still ongoing at 52 weeks, as the number of $CD4^+$ T cells and $CD8^+$ T cells was still lower compared to baseline. At 24 weeks, we observed that the absolute numbers of $CD4^+$ T cells were significantly higher in the MSC group compared to the control group, a difference which disappeared at week 52. This is in line with our previous work where we reported a increase of $CD4^+$ T cells in the MSC group at 12 weeks and an a similar trend at 24 weeks [13]. This flow cytometry based approach did not allow for detailed analysis on the underlying T cell subsets, which the current mass cytometry based analysis did.

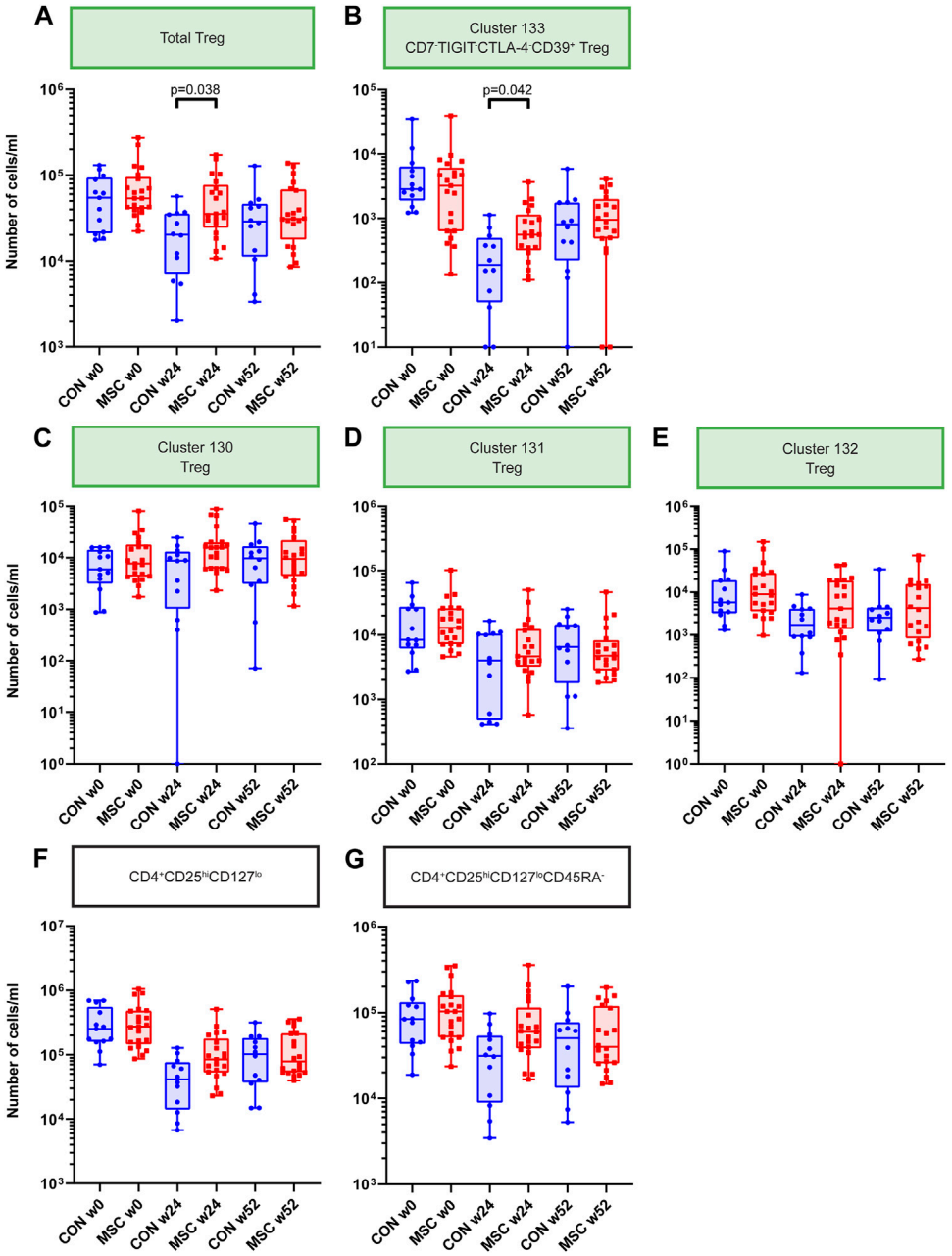


FIGURE 4 | Treg Clusters. Graphs showing the number of cells/mL at timepoint w0, w24, and w52 for both the control and the MSC therapy group. **(A)** Total Treg. **(B)** Cluster 133; CD7-TIGIT-CTLA-4-CD39$^+$ Treg. **(C)** Cluster 130; Treg. **(D)** Cluster 131; Treg. **(E)** Cluster 132; Treg. **(F)** CD4$^+$CD25hiCD127lo subset after manual gating. **(G)** CD4$^+$CD25hiCD127loCD45RA-subset after manual gating. CON, control group (Blue); MSC, MSC therapy group (Red). P-values were calculated with the Mann-Whitney U test and corrected within each cluster with Bonferroni.

Exploring the immune cell clusters in more detail, we found 30 clusters to be increased exclusively at week 24 in MSC treated patients (five B cell, 17 CD4$^+$ T cell and eight CD8$^+$ T cell clusters). In contrast, only three clusters, one NK cell, one lineage undefined and one B cell cluster were different between the treatment groups at 52 weeks. Among the 17 increased CD4$^+$ T cell clusters in MSC treated patients were 14 Th2-like clusters (six activated) and three Th1/Th2-like clusters. Th2 cells can be primed by phagocytotic cells, like monocytes and dendritic cells, after engulfing MSCs. By releasing IL-4 and IL-10, Th2 cells can repress the development of the Th1 cells and subsequently repress an inflammatory environment [26]. We also observed increased cell numbers in eight Tc1 (effector) memory cytotoxic T cell clusters, which are considered to be highly inflammatory and if directed towards the transplanted kidney might play a role in transplant rejection. We also showed five B cell clusters increased in the MSC group at week 24. These

clusters are either class switched memory B cells or proliferating B cells, indicating a possible increase in the capacity to produce antibodies in the MSC group. In line with this, while in the MSC group 7/21 patients developed dnDSA at week 24 none of the control patients (0/13) developed dnDSA. However, when splitting the MSC group into DSA+ and DSA− this did not result in significant differences in any of the 33 clusters discussed. When comparing the two groups at 52 weeks, all the above clusters contained similar cell numbers while cluster 28 ($CCR7^+CD38^+$ mature B cells), was decreased in the MSC group. Finally, Tregs have been proposed to be the mediators of the immune dampening effects of MSCs and were elevated in MSC treated patients when analyzed with flow cytometry on fresh PBMC samples [13]. In line with this, we could confirm the increase of Treg cells at week 24 in the MSC group in this study, indicating a immune dampening environment.

In the TRITON trial, MSCs were administered to facilitate safe tacrolimus withdrawal. Although the MSC patients with tacrolimus withdrawal developed more dnDSA then the control group, their kidney function was not inferior and dnDSA development did not lead to more rejection episodes [13, 27]. In our selection of patients from the TRITON study there was one patient in the control group with a rejection episode (mixed rejection) and two patients in the MSC group with a rejection episode (TCMR) within the first year after transplantation (**Supplementary Table S1**). This number of rejections is too low to correlate them with the discovered subsets. In the Triton study tacrolimus withdrawal was save. We observed that the MSC-treated patients had increased number of cells in multiple immune dampening Th2 subsets as well as an in-creased amount of Tregs. These subsets might play a role in the ability to safely withdraw tacrolimus in combination with MSC infusion. On the other hand, it is important to acknowledge that changes in immune cell subsets might be caused both by the MSC administration as well as by the tacrolimus withdrawal, an effect we cannot dissociate in the current study.

Tacrolimus inhibits the calcineurin pathway preventing dephosphorylation of NFAT (nuclear factor of activated T lymphocytes) and its translocation to the nucleus. This blocks the activation of the IL-2 gene, involved in T cell activation and as a result the initiation of the immune response [28]. In this light, the increased numbers of Th2 and Tc1 cells in MSC treated patients could be the result of the absence of tacrolimus. We therefore cannot determine whether the increased subsets are the result of the infused MSCs or of tacrolimus withdrawal. Also, as the specificity of the B cells and T cells is unknown, it is unclear if the increased subsets are directed against the transplanted kidney or if they are part of ongoing repopulation. Single-cell RNA sequencing including T cell and B cell receptor analysis could shed light on the clonality and specificity of these repopulating cells.

The analysis performed in this study was an unbiased discovery analysis using a large number of immune cell markers. Therefore, many phenotypically different clusters could be identified, and as a result a high number of comparisons were made. In this study we corrected with Bonferroni for the three comparisons made within one cluster. Correcting for false positives within the whole study, with the current statistical options, would result in the need for extremely low *p*-values to remain significant after correction. For this reason we propose that the subsets found in this study should be further investigated for their contributions to MSC therapy in a more focused analysis in future studies.

In conclusion, this study provides a comprehensive description of the PBMC subsets in kidney transplantation patients after MSC therapy and subsequent tacrolimus withdrawal. Our results point towards an active involvement of $CD4^+$ Th2 cells, Tregs, class switched memory B cells and $CD8^+$ Tc1 cells in patients receiving MSCs and the save practice of tacrolimus withdrawal. Future studies are required to validate these findings and investigate the possible functional role of the identified immune cell subsets.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Central Committee on Research involving Human Subjects in the Netherlands. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SHH conceived the study, performed experiments and analyzed the data. JF and MR developed the clinical protocol, provided samples for the study and contributed clinical input. AS contributed to the barcoding. SH, CvK, and FK supervised the study. SHH, SH, CvK, and FK wrote the manuscript in collaboration with all co-authors. 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.2023.11329/full#supplementary-material

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ORIGINAL RESEARCH
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Steroid Sparing Maintenance Immunosuppression in Highly Sensitised Patients Receiving Alemtuzumab Induction

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This analysis reports on the outcomes of two different steroid sparing immunosuppression protocols used in the management of 120 highly sensitised patients (HSPs) with cRF>85% receiving Alemtuzumab induction, 53 maintained on tacrolimus (FK) monotherapy and 67 tacrolimus plus mycophenolate mofetil (FK + MMF). There was no difference in the median cRF or mode of sensitisation between the two groups, although the FK + MMF cohort received more poorly matched grafts. There was no difference in one-year patient or allograft survival, however rejection free survival was inferior with FK monotherapy compared with FK + MMF at 65.4% and 91.4% respectively, $p < 0.01$. DSA-free survival was comparable. Whilst there was no difference in rates of BK between the cohorts, CMV-free survival was inferior in the FK + MMF group at 86.0% compared with 98.1% in the FK group, $p = 0.026$. One-year post-transplant diabetes free survival was 89.6% and 100.0% in the FK and FK + MMF group respectively, $p = 0.027$, the difference attributed to the use of prednisolone to treat rejection in the FK cohort, $p = 0.006$. We report good outcomes in HSPs utilising a steroid sparing protocol with Alemtuzumab induction and FK + MMF maintenance and provide granular data on immunological and infectious complications to inform steroid avoidance in these patient groups.

Keywords: HLA, highly sensitised, Alemtuzumab, calculated reaction frequency, steroid sparing

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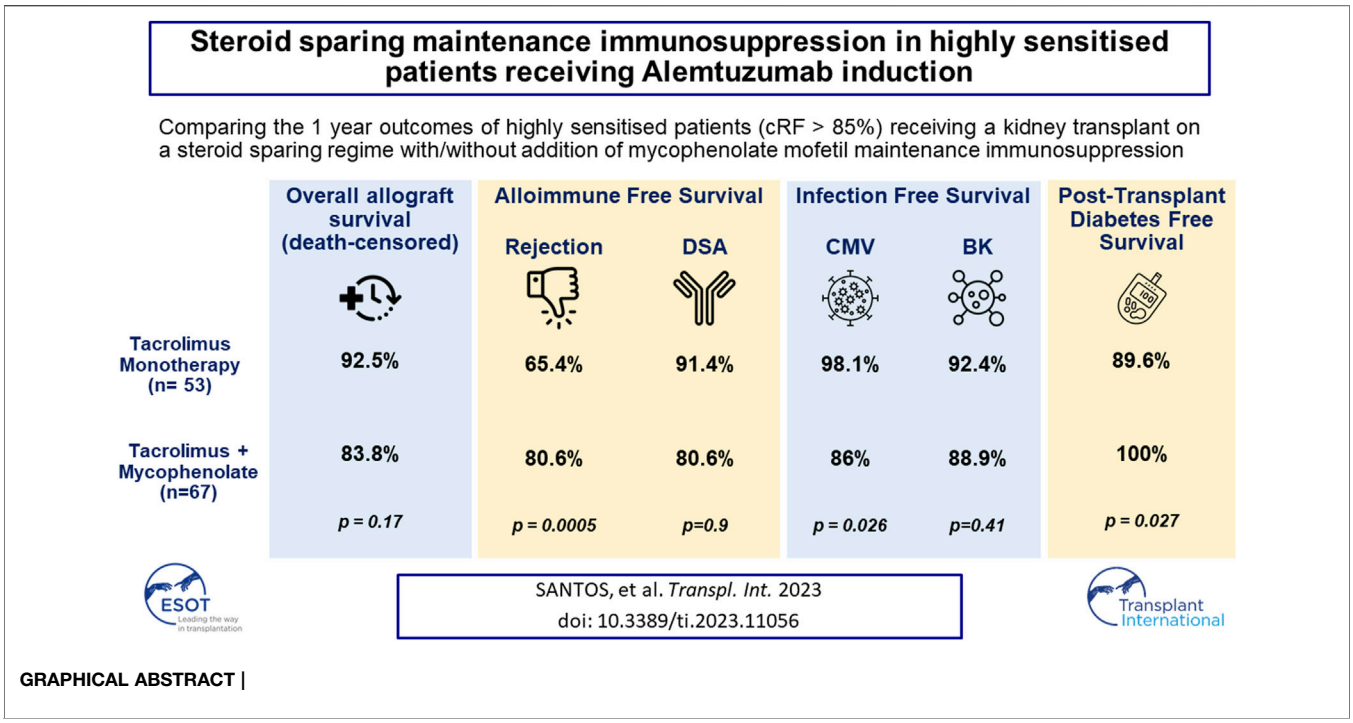
INTRODUCTION

Lymphocyte-depleting induction therapy enables the use of steroid sparing immunosuppression protocols in kidney transplantation. In immunologically high-risk transplant recipients, Alemtuzumab induction has been shown to be equivalent to anti-thymocyte globulin (ATG) in preventing acute rejection in the first-year post-transplant in patients following early steroid withdrawal [1].

In the absence of a positive crossmatch, the definition of immunological risk is unclear, and there remains no uniform consensus on the clinical relevance of preformed DSA detected by single antigen beads (SAB) in the context of a negative crossmatch [2, 3]. Paradoxically, SABs are used to define sensitisation status *via* the calculation reaction frequency (cRF), which is often used to identify



GRAPHICAL ABSTRACT |

"patients at immunological risk" and guide immunosuppression regimens [1, 4–6]. Unquestionably, highly sensitised patients wait longer for an appropriate donor, but in the absence of a detectable DSA by SAB, the contribution of preformed HLA antibodies to post-transplant associated alloimmune injury is not as clear [4, 5, 7].

The 2019 Kidney Offering Scheme (KOS2019) in the UK was implemented to try and improve equity in access to transplantation. Under the scheme, highly sensitised patients (defined as those patients with a cRF≥85%), patients with difficult to match HLA types and long waiters (>7 years) were given allocation priority since its inception in September 2019. In preparation for the increased rates of highly sensitised patients receiving transplants, there was a change to our centre's immunosuppression protocol, which included the addition of mycophenolate mofetil (MMF) to maintenance tacrolimus (FK) following Alemtuzumab induction (FK + MMF), together with early steroid withdrawal in all patients with a cRF≥85%.

The aim of this report is to investigate the clinical outcomes, both immunological and infectious, in transplant recipients with a cRF≥85% receiving Alemtuzumab induction with FK + MMF compared with FK monotherapy.

METHODS AND MATERIALS

Patients

Patients transplanted at a single centre were identified from a prospectively maintained transplant registry. All patients transplanted from 2014 to 2022 were selected if they met the following criteria: cRF≥85% at the time of transplantation, had no identifiable preformed DSA (by SAB or positive crossmatch), received an ABO compatible transplant, had primary function and received Alemtuzumab induction. This study was approved by the West of Scotland Research Ethics Committee (20/WS/0181), and was performed in accordance with the Declaration of Helsinki and UK Data Protection legislation.

UKT Matching Definitions

This paper will refer to HLA mismatch levels and matchability score. The mismatch level defines the antigen mismatches at HLA-A, HLA-B and HLA-DR. It is on a scale of 1-4, as defined by NHS Blood and Transplant [8]: Level 1 representing a 000 antigen match; Level 2 any single HLA mismatch at HLA-B or HLA-DR ([0 DR and 0/1 B] or [1 DR and 0 B]); Level 3 mismatch, a two antigen HLA-B mismatch or single mismatch at HLA-B plus HLA-DR ([0 DR and 2 B] or [1 DR and 1 B]) and a Level 4 mismatch representing either a single HLA-DR plus 2 HLA-B mismatches or two HLA-DR mismatches ([1 DR and 2 B] or [2 DR]). The matchability score is defined by ODT as a measure of how difficult it is to match a patient with an organ donor in the UK, based on comparison with a pool of 10,000 donor HLA types on a national database [8]. Matchability is defined on a scale of 1–10, 1–3 representing easy matchability, 4–7 medium matchability and 8–10 a difficult matchability, in patients with rare HLA types.

Immunosuppression Protocol

The immunosuppression protocol consists of 0.4 mg/kg of alemtuzumab (Campath 1H, Genzyme, Oxford, UK) in the immediate post-operative period. All patients received methylprednisolone 500 mg pre-operative, followed only by a one-week course of corticosteroids. All patients received tacrolimus, with the observed cohort receiving maintenance

mycophenolate mofetil (MMF) in addition. The target trough levels are 6–8 ng/mL for tacrolimus and 1.2–2.4 mg/L for mycophenolate.

HLA Typing and DSA Monitoring

From June 2020, all donors and recipients were typed using high resolution next generation sequencing using GenDx MX6-1 HLA typing kits (HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1). Sequencing reaction was performed using the Illumina iSeq™ platform and results analysed on GenDx NGS engine. All other recipients and transplant donors were routinely typed for HLA-A, HLA-B, HLA-C, HLA-DRB1/B3/B4/B5, and HLA-DQB1 loci using in house PCR-SSP (sequence-specific primers) or the LABType® SSO typing kits (One Lambda ThermoFisher Scientific Inc., Canoga Park, CA, USA). Additional typing, e.g., DPA, DPB and DQA is performed retrospectively in the setting of *de novo* HLA antibodies.

Crossmatching was performed by T and B cell complement dependent cytotoxicity (CDC) and T-cell flow cytometry (FCXM) techniques, together with a single antigen screen. Transplants from donors where the recipient is known to have a preformed DSA detected by SAB are not routinely permissible [9].

Post-transplant, DSA are detected either as part of a screening protocol or at times of allograft dysfunction. Protocolised screening occurs twice in the first week, at 1 month, 3 months and 12 months. Screening is performed using LABScreen mixed beads (One Lambda, Canoga Park, CA) if the patient is non-sensitised and then subsequently or primarily screened using LABScreen single antigen beads if sensitised. Samples were treated with ethylenediaminetetraacetic acid (EDTA) to avoid possible prozone effect and the antibody pattern was interpreted taking into account the patient's own HLA type. A mean fluorescence intensity (MFI) value of >1,000 by single antigen beads on two separate occasions was considered positive for the presence of antibody.

Indications for Biopsy

Patients with a newly detected DSA, are offered a protocol biopsy unless there is a contraindication. Patients are also offered biopsies at times of allograft dysfunction. All rejection episodes were biopsy proven, unless otherwise stated. Biopsy-proven rejection episodes were defined using the 2019 Banff classification for Allograft Pathology, including cases borderline for T-cell mediated rejection (TCMR) and cases of chronic active TCMR. The 2013 Banff definitions were used to also include cases that showed histological features suspicious for active and chronic active antibody-mediated rejection, and cases that were C4d-positive without other features of rejection. Patients receiving tacrolimus monotherapy who had a biopsy for a DSA in the setting of stable allograft function had augmentation in their immunosuppression, with the addition of MMF, even in the absence of rejection. Patients who had subclinical rejection in the context of a DSA were treated with corticosteroids in addition to tacrolimus and MMF. Patients with antibody mediated rejection (ABMR) in the context of a newly detected DSA and allograft dysfunction were treated with plasma exchange and intravenous immunoglobulin, in addition to the introduction of corticosteroids. Patients who have a DSA detected in the first 14 days post-transplant were treated for presumed ABMR in the context of graft dysfunction.

Detection and Diagnosis of Immunosuppression Complications

For the purposes of this analysis, the following definitions were used to define complications associated with immunosuppression. Post-transplant diabetes was defined as the *de novo* need for hypoglycaemic agents (oral or insulin) in the follow up period. A diagnosis of BK viraemia was made on the detection of BK virus DNA in 2 or more blood samples by PCR testing. A diagnosis of CMV viraemia was similarly made *via* PCR testing of 2 separate blood samples.

Statistical Analysis

All analyses were performed using GraphPad Prism version 9.4.0. Comparisons of means and frequencies of normally distributed variables were calculated using t-tests and chi-square/Fisher's exact tests. The Mann–Whitney test was used for nonparametric variables. The Kaplan–Meier estimator was used for survival analysis related to clinical outcome following transplantation; statistical significance was determined by log rank testing. Multivariate analyses were calculated using Cox proportional hazards regression models. A p-value of <0.05 was deemed statistically significant.

RESULTS

One-hundred and sixty-nine highly sensitised patients (HSPs) were identified over the analysis period. Fifty-three (31.4%) patients received Alemtuzumab plus FK monotherapy and 67 (39.6%) received Alemtuzmab plus FK + MMF. In addition, 11 (6.5%) received Basiliximab plus FK + MMF, 27 (16.0%) received either Alemtuzumab or Basiliximab with prednisolone based maintenance therapy, and an additional 11 (6.5%) patients were excluded (1 primary non-function and 10 preformed DSA), **Figure 1**. For comparison of the clinical outcomes for the different immunosuppression regimes see **Supplementary Materials**. From here, we will report on patients receiving Alemtuzumab induction and tacrolimus either with or without MMF, **Table 1**.

Comparison of Baseline Patient Characteristics

There was no gender difference between those recipients who received FK compared with FK + MMF, with 40/53 (75.5%) and 46/67 (68.7%) respectively being female, $p = 0.41$, **Table 1**. There was no difference in median age at the time of transplant, at 54 (46–59) and 54 (45–62) years in the FK and FK + MMF groups respectively, $p = 0.62$. There was also no proportional difference in ethnicity distribution, with 11/53 (20.8%) and 16/67 (23.9%) patients in the FK and FK + MMF groups being from a white

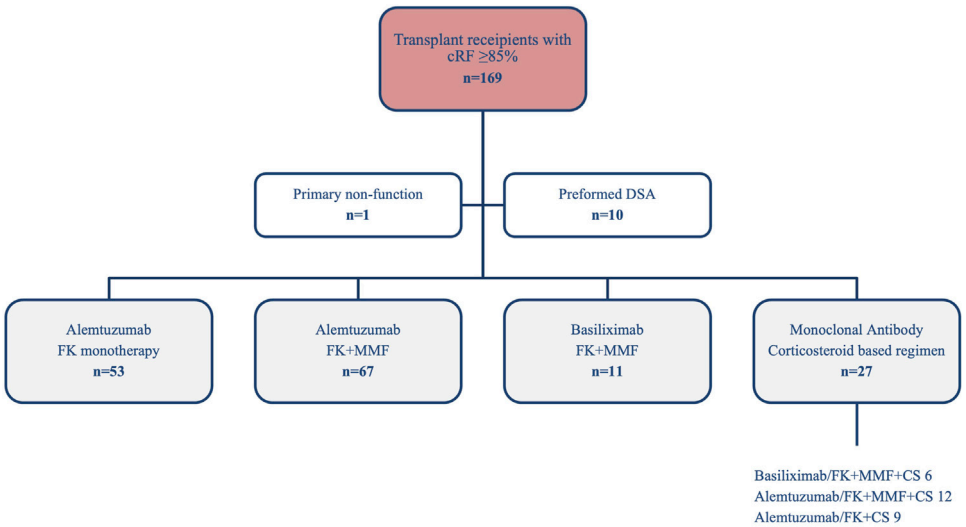


FIGURE 1 | Highly sensitised (cRF≥85%) recipients transplanted between 2014 and 2022 by immunosuppression regimen.

ethnic background respectively, $p = 0.69$. Pre-emptive transplant rates were low overall, with no difference in the FK and FK + MMF groups at 2/53 (3.8%) and 3/67 (4.5%) respectively, $p = 0.085$. The corresponding time on the wait list was long, with a median time to transplant of 1711 (974–2,599) days in patients receiving FK and 1,523 (1,092–2,343) days in patients who received FK + MMF, $p = 0.86$. The proportion of patients who received a living donor kidney was significantly higher in the FK group at 16/53 (30.2%) compared with 4/67 (6.0%) in the patients who received FK + MMF, $p = 0.0004$. There was neither a difference in the median cold ischaemic time, 11.3 (9.3–14.5) hours and 12.0 (9.2–14.0) hours in the FK and FK + MMF groups, $p = 0.57$; or a difference in the deceased donor type, with 7 (18.9%) and 23 (36.5%) patients receiving transplants from donors after cardiac death in the FK and FK + MMF groups respectively, $p = 0.065$. However, the proportion of patients who experience delayed graft function, was significantly lower in the group receiving FK, 5/53 (9.4%), compared with FK + MMF, 27 (40.3%), $p = 0.0002$.

Comparison of Baseline Immunological Characteristics

The median cRF in the FK and FK + MMF groups was 96 (90–99)% and 98 (94–99)%, $p = 0.45$, with 8/53 (15.1%) and 15/67 (22.4%) patients having a cRF of 100% respectively, $p = 0.32$, **Table 2**. Pregnancy was the leading mode of sensitisation in both groups, with 37/53 (69.8%) and 39/67 (58.2%) patients in the FK and FK + MMF cohorts respectively having pregnancy contribute to their highly sensitised status, $p = 0.19$. Whilst in the FK and FK + MMF cohorts, 12/53 (22.6%) and 21/67 (31.3%) respectively were receiving at least a second solid organ transplant, $p = 0.29$; with 2/12 (16.7%) and 3/21 (14.3%) receiving an organ with a repeat HLA mismatch, $p = 0.86$.

Overall, the majority of patients, 96/120 (80.0%), had a HLA type that was recognised as "difficult" to match, with no proportional difference in the FK and FK + MMF groups, $p = 0.15$, **Table 2**. There was no difference in the ABDR mismatch between those who received FK, with a median 3 (IQR 2–4) mismatches, compared with those who received FK + MMF, with a median 3 (IQR 3–4) mismatches, $p = 0.10$. However, patients who received FK monotherapy were less likely to receive an unfavourable Level UKT mismatch compared with those receiving FK + MMF, with 19/53 (35.8%) and 34/67 (50.7%) receiving a Level 4 [2DR or (1DR+1B)] mismatched kidney respectively, $p = 0.03$, **Table 2**. Whilst there was no difference in overall mismatches at HLA-A ($p = 0.59$), HLA-Cw ($p = 0.29$), HLA-DRB1 ($p = 0.62$) and HLA-DQB1 ($p = 0.79$) between the FK and FK + MMF cohorts, patients receiving FK were more likely to receive a kidney matched at HLA-B compared with those patients who received FK + MMF, $p = 0.023$.

Comparison of Clinical Outcomes

The overall median follow up for all patients was 2.8 (1.6–5.1) years, with longer follow up in FK monotherapy group at 5.5 (4.4–6.8) years compared with 1.7 (0.9–2.5) years in the FK + MMF cohort, $p < 0.0001$. Clinical outcomes were therefore restricted to 1 year post-transplant.

All-cause 1 year allograft survival was 92.5% and 83.8% in the FK and FK + MMF groups respectively, $p = 0.17$, **Figure 2**. Five patients died with a functioning graft in the first year post-transplant, one patient in the FK group died of COVID-19 infection, whilst 4 patients in the FK + MMF group (1 COVID-19 infection, 1 sepsis and 2 cardiac). Death censored allograft survival was 94.3% and 89.6% in the FK and FK + MMF groups respectively, $p = 0.40$. There were 3 graft losses in the FK group (1 rejection, 1 transplant renal artery stenosis and 1 BK nephropathy), whilst there were 6 graft losses in the FK + MMF group (2 rejection, 1 transplant renal artery stenosis and 3 donor derived pathology). At 3 and 12 months post-transplant, estimated GFR was not statistically

TABLE 1 | Characteristics of highly sensitised patients receiving Alemtuzumab.

		FK monotherapy	FK + MMF	*p*-value
		n = 53 (%)	*n* = 67 (%)	
Gender	Female	40 (75.5)	46 (68.7)	0.41
	Male	13 (24.5)	21 (31.3)	
Age at Transplant	Years (median)	54 (46–59)	54 (45–62)	0.62
Ethnicity	Black	11 (20.8)	13 (19.4)	0.98
	Caucasian	11 (20.8)	16 (23.9)	
	Indoasian	23 (43.4)	28 (41.8)	
	Other	8 (15.1)	10 (14.9)	
Cause of ESRD	APKD	4 (7.5)	5 (7.5)	0.39
	DM	6 (11.3)	14 (20.9)	
	GN	10 (18.9)	19 (28.4)	
	Other	7 (13.2)	5 (7.5)	
	Unknown	22 (41.5)	19 (28.4)	
	Urological	4 (7.5)	5 (7.5)	
Diabetes	No	40 (75.5)	48 (71.6)	0.64
	Yes	13 (24.5)	19 (28.4)	
Pre-emptive transplant	No	51 (96.2)	64 (95.5)	0.85
	Yes	2 (3.8)	3 (4.5)	
Time on wait list	Days (Median)	1,711 (974–2,599)	1,523 (1,092–2,343)	0.86
Donor Type	LD	16 (30.2)	4 (6.0)	0.0004[a]
	DD	37 (69.8)	63 (94.0)	
	DBD	30 (81.1)	40 (63.5)	0.065
	DCD	7 (18.9)	23 (36.5)	
Cold Ischaemic Time (deceased donors)	Hours (median)	11.3 (9.3–14.5)	12.0 (9.2–14.0)	0.57
Delayed Graft Function	No	48 (90.6)	40 (59.7)	0.0002[a]
	Yes	5 (9.4)	27 (40.3)	

[a]p < *0.05*.

different between the FK and FK + MMF groups, at 54 (IQR 44–67) mLs/min and 46 (34–62) mLs/min respectively, p = 0.09 at 3 months, and 57 (46–66) mLs/min and 51 (37–65) mLs/min, p = 0.16 at 12 months.

One-year rejection free survival was inferior in the FK cohort compared with the FK + MMF cohort at 65.4% and 91.4% respectively, p = 0.0005, **Figure 2**. There were 18 episodes of treated rejection in the FK cohort (12 active ABMR, 4 borderline TCMR, 1 Banff 1 TCMR and 1 case of C4d+ without evidence of rejection in the setting of graft dysfunction), and 5 cases in the FK + MMF cohort (3 active ABMR, 1 borderline TCMR and 1 presumed rejection in the context of a DSA plus acute allograft dysfunction).

One-year DSA free survival was comparable in the two groups, with a reported survival of 80.6% and 80.6%, or 10 and 12 patients with DSA in the FK and FK + MMF cohort respectively, p = 0.90. Fifteen of 22 (68.2%) patients, 7/10 (70.0%) and 8/12 (66.7%) patients in the FK and FK + MMF cohort respectively had the DSA detected in the first 30 days post-transplant, suggesting a likely memory response in the majority of cases. Six of the remaining seven DSA positive patients had the DSA detected between days 31 and the 3-month screen, 3 patients in each of the FK and FK + MMF groups. Only 1 patient developed a DSA between 3 and 12 months, this patient was in the FK + MMF cohort and had MMF stopped following a diagnosis of BK nephropathy. A high proportion of patients had a measured tacrolimus level below target, but there was no difference between the FK and FK + MMF groups; between 1 week and 3 months post-transplant, 33/53 (62.3%) and 36/67 (537%) patients respectively had at least one tacrolimus level below target, p = 0.35. Whilst between 3 months and 1 year, 30/52 (57.7%) of the FK group and 34/64 (53.1%) of the FK + MMF group had at least one tacrolimus level below target, p = 0.62.

Investigating the role of immunological characteristics at the time of transplant with risk of rejection or DSA detection was performed next. On univariate analysis, cRF did not associate with likelihood of rejection in the first year post-transplant, with rejection free survival of 79.8%, 74.7% and 90.6% in the patients with a cRF of 85%–94%, 95%–99% and 100% respectively, p = 0.37, **Figure 3**. Mode of sensitisation also did not associate with rejection, with 1 year rejection free survival of 88.2%, 76.6%, 78.9% and 85.7% in patients with sensitised *via* blood, pregnancy, transplantation or pregnancy and transplantation respectively, p = 0.74. For the 33 patients receiving a ≥2nd transplant, a repeat HLA mismatch was not associated with risk of rejection, with a rejection free survival of 84.4% and 53.3% in those with and without re-exposure to a previous mismatched antigen, p = 0.08. Patients who received a blood product transfusion in the first 28 days post-transplant were at higher risk of rejection, with a one-year rejection free survival of 85.3% and 69.3% in those without and who had received a transfusion respectively, p = 0.045. There was no difference in risk of rejection according to UKT Level mismatch, with a one-year rejection free survival of 80.0%, 85.9%, 78.5% and 75.8% in patients receiving a Level 1,2,3 and 4 mismatch respectively, p = 0.77. One-year rejection free survival in patients with easy, medium and difficult to match HLA types was 100.0%, 76.1% and 79.6% respectively, p = 0.79.

On univariate analysis, cRF did not associate with likelihood of a detectable DSA in the first-year post-transplant, with a DSA free

TABLE 2 | Immunological Characteristics of highly sensitised patients receiving Alemtuzumab.

		FK monotherapy	FK + MMF	*p*-value
		n = 53 (%)	*n* = 67 (%)	
cRF Group	85%–94%	19 (35.8)	18 (26.9)	0.45
	95%–99%	26 (49.1)	34 (50.7)	
	100%	8 (15.1)	15 (22.4)	
cRF	% (Median)	96 (90–99)	98 (94–99)	0.087
Route of sensitisation	Blood	6 (11.3)	12 (17.9)	0.38
	Pregnancy ± Blood	35 (66.0)	34 (50.7)	
	Tx ±Blood	10 (18.9)	16 (23.9)	
	Pregnancy, Tx ±Blood	2 (3.8)	5 (7.5)	
Blood product transfusion in 1st 28 days post-transplant	Yes	22 (41.5)	25 (37.3)	0.64
	No	31 (58.5)	42 (62.7)	
Live Donor-Recipient Relationship	Unrelated	8 (15.1)	2 (3.0)	0.23
	Sibling	4 (7.5)	0	
	Child to Mother	2 (3.8)	0	
	Child to Father	1 (1.9)	0	
	Parent to Child	1 (1.9)	1 (1.5)	
	Partner (Male to Female)	0	1 (1.5)	
Repeat transplant	No	41 (77.4)	46 (68.7)	0.29
	Yes	12 (22.6)	21 (31.3)	
Repeat HLA mismatch	Yes	2 (3.8)	3 (6.4)	0.55
Matchability	Easy	-	2 (3.0)	0.15
	Medium	13 (24.5)	9 (13.4)	
	Difficulty	40 (75.5)	56 (83.6)	
Matchability score	Median	9 (7.75–10)	9 (8–10)	0.16
Total ABDR MM	Median	3 (2–4)	3 (3–4)	0.10
UKT MM Level[a]	1	4 (7.5)	1 (1.5)	0.03
	2	17 (32.1)	15 (22.4)	
	3	13 (24.5)	17 (25.4)	
	4	19 (35.8)	34 (50.7)	
HLA A Mismatch	0	11 (20.8)	12 (17.9)	0.59
	1	26 (49.1)	32 (47.8)	
	2	16 (30.2)	23 (34.3)	
HLA B Mismatch	0	14 (26.4)	7 (10.4)	0.04
	1	24 (45.3)	30 (44.8)	
	2	15 (28.3)	30 (44.8)	
HLA Cw Mismatch	0	13 (24.5)	11 (16.4)	0.29
	1	30 (56.6)	36 (53.7)	
	2	10 (18.9)	20 (26.9)	
HLA DRB1 Mismatch	0	16 (30.2)	17 (25.4)	0.62
	1	28 (52.8)	34 (50.7)	
	2	9 (17.0)	16 (23.9)	
HLA DQB1 Mismatch	0	23 (43.4)	25 (37.3)	0.79
	1	24 (45.3)	34 (50.7)	
	2	6 (11.3)	8 (11.9)	

[a]*Using KOS 2019 mismatch level.*

survival of 81.0%, 75.5% and 95.7% in the patients with a cRF of 85%–94%, 95%–99% and 100% respectively, $p = 0.37$, **Figure 4**. Mode of sensitisation also did not associate with DSA, with 1 year DSA free survival of 88.9%, 80.7%, 84.2% and 71.4% in patients sensitised *via* blood, pregnancy, transplantation or pregnancy and transplantation respectively, $p = 0.82$. For the 33 patients receiving a ≥2nd transplant, a repeat HLA mismatch was not associated with risk of DSA, with a DSA free survival of 60.0% and 80.2% in those with and without re-exposure to a previous mismatched antigen, $p = 0.12$. Patients who received a blood product transfusion in the first 28 days post-transplant were not at statistically higher risk of a DSA, with a one-year DSA free survival of 85.5% and 74.1% in those without and who had received a transfusion respectively, $p = 0.078$. There was no difference in post-transplant DSA according to UKT Level mismatch, with a one-year DSA free survival of 80.0%, 85.9%, 87.3% and 77.2% in patients receiving a Level 1,2,3 and 4 mismatch respectively, $p = 0.85$. One-year DSA free survival in patients with easy, medium and difficult to match HLA types was 100.0%, 81.0% and 79.2% respectively, $p = 0.66$.

Multivariate Analyses of Factors Associated With Allograft Loss, Rejection and DSA

Multivariate analysis of each outcome measure was performed, incorporating variables associated with corresponding outcome on univariate analysis. Factors associated with all-cause allograft loss included delayed graft function, HR 4.85 (1.57–14.98), $p =$

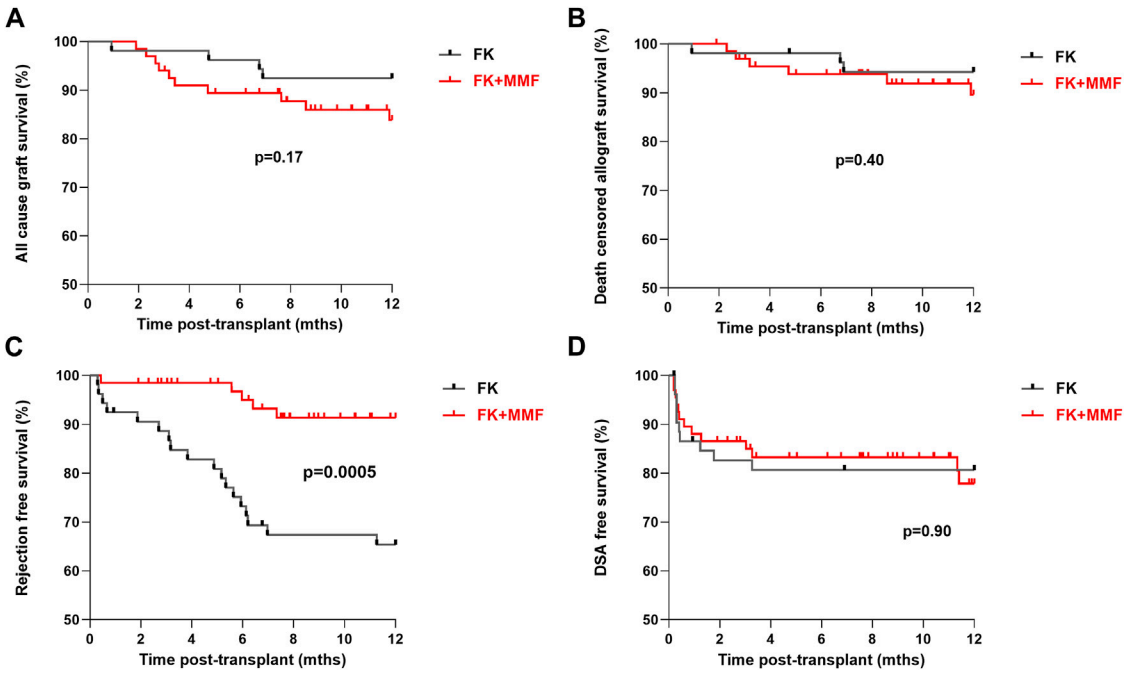


FIGURE 2 | Clinical outcomes associated with and without the use of mycophenolate in highly sensitised patients **(A)** All-cause 1 year allograft survival was 92.5% and 83.8% in the FK and FK + MMF groups respectively, $p = 0.17$ (log-rank) **(B)** Death censored allograft survival was 94.3% and 89.6% in the FK and FK + MMF groups respectively, $p = 0.40$ (log-rank) **(C)** One-year rejection free survival was inferior in the FK cohort compared with the FK + MMF cohort at 65.4% and 91.4% respectively, $p = 0.0005$ (log-rank) **(D)** One-year DSA free survival was 80.6% and 80.6%, in the FK and FK + MMF cohort respectively, $p = 0.90$ (log-rank).

0.006 and total ABDR mismatch, HR 1.93 (1.18–3.14), $p = 0.009$. Whilst factors associated with censored allograft loss included delayed graft function, HR 5.90 (1.40–24.90), $p = 0.016$ and total ABDR mismatch, HR 1.83 (1.00–3.36), $p = 0.049$. Factors associated with rejection included receiving a graft across a repeat HLA mismatch, HR 9.50 (1.92–46.87), $p = 0.006$, and receiving FK monotherapy, HR 10.37 (2.80–38.33), $p = 0.0005$. Whilst no independent variables were found to be associated with risk of DSA at 1 year post-transplant.

Comparison of Rates of the Adverse Effects of Immunosuppression

There was no significant difference in the one-year BK infection free survival in the FK monotherapy compared with the FK + MMF cohorts, at 94.2% and 88.9% respectively, $p = 0.41$, **Figure 5**. However, the one-year CMV free survival was superior in the FK monotherapy compared with the FK + MMF cohorts, at 98.1% and 86.0% respectively, $p = 0.026$. On multivariate analysis, total ABDR HLA mismatch was associated with increased risk of CMV, HR 1.99 (1.08–3.64), $p = 0.03$, whilst FK monotherapy was not significantly associated with a reduced risk statistically, they may be clinically relevant, HR 0.21 (0.04–1.05), $p = 0.057$. Crucially of note CMV risk by serological status of donor and recipient were not included in this model.

For patients not known to have diabetes at the time of transplant a new diagnosis of post-transplant diabetes (PTDM) was more likely in the FK monotherapy compared with the FK + MMF cohort, with a one-year PTDM free survival of 89.6% and 100.0% respectively, $p = 0.027$, **Figure 5**. The one-year prednisolone free survival in the FK monotherapy and FK + MMF cohorts being 69.3% and 88.8% respectively, $p = 0.006$.

DISCUSSION

In the absence of preformed DSA detected by SAB, highly sensitised patients at our centre have historically received our standard immunosuppression protocol of Alemtuzumab induction and tacrolimus monotherapy as maintenance. In this report, we have shown that this strategy results in comparable DSA detection, both memory and *de novo*, when compared with a similar immunosuppression protocol with the addition of MMF. However, overall rejection rates were much reduced in the latter cohort, suggesting under immunosuppression in the absence of MMF. Although we found increased CMV rates in patients receiving MMF, this adverse effect was counterbalanced by a higher incidence of post-transplant diabetes in the FK monotherapy cohort, which may be attributed to the introduction of corticosteroids following rejection in this group. This nicely demonstrates how any benefits of enhanced immunosuppression against rejection, may be offset by their metabolic and infectious complications.

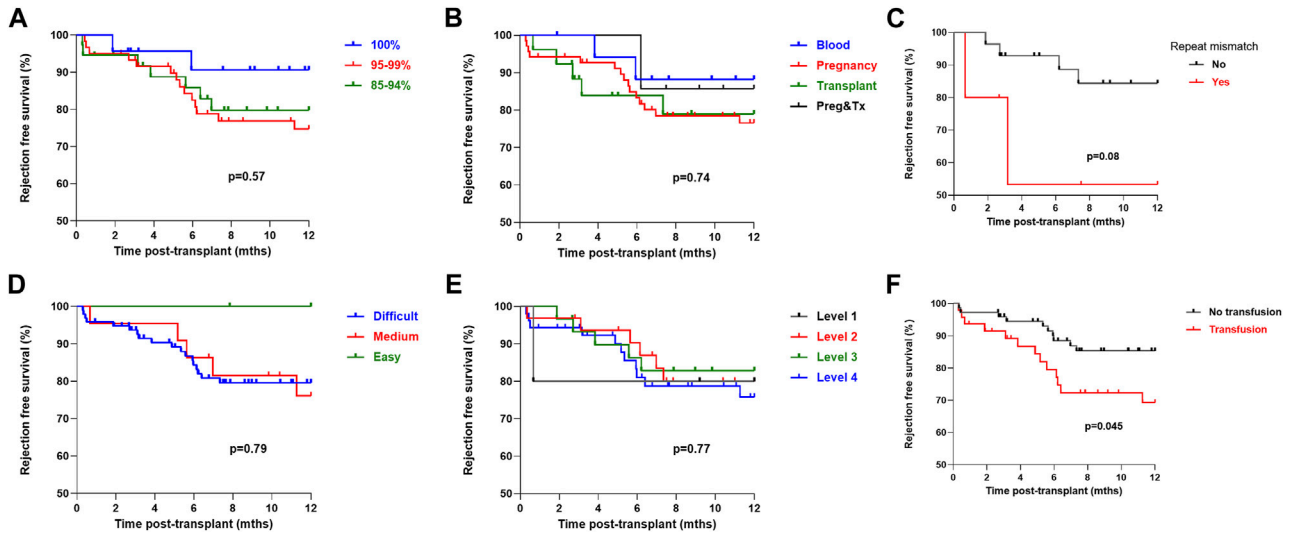


FIGURE 3 | One-year rejection free survival by immunological characteristics (log-rank) One-year rejection free survival (log-rank) was **(A)** No difference by cRF status, $p = 0.57$ **(B)** No difference by mode of sensitisation, $p = 0.74$ **(C)** No difference in patients receiving a >2nd graft by presence or absence of repeat HLA mismatch, $p = 0.08$ **(D)** No difference by HLA matchability, $p = 0.79$ **(E)** No difference by UKT Level Mismatch, $p = 0.77$ **(F)** Inferior in patients who received a post-transplant blood transfusion, $p = 0.045$.

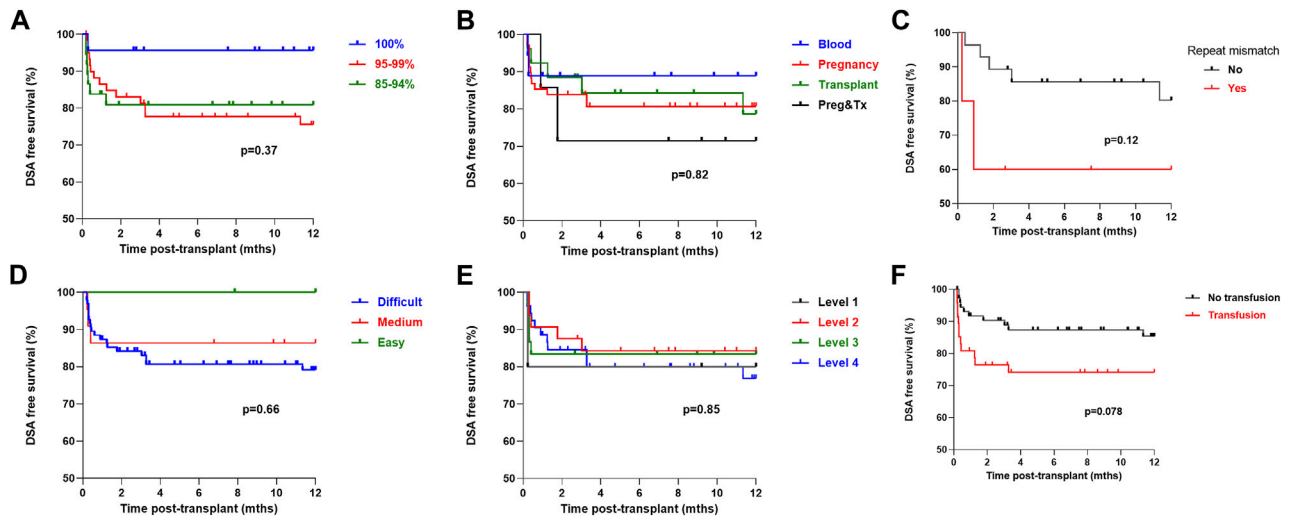


FIGURE 4 | One-year DSA free survival by immunological characteristics One-year DSA free survival (log-rank) was **(A)** No difference by cRF status, $p = 0.37$ **(B)** No difference by mode of sensitisation, $p = 0.82$ **(C)** No difference in patients receiving a >2nd graft by presence or absence of repeat HLA mismatch, $p = 0.12$ **(D)** No difference by HLA matchability, $p = 0.66$ **(E)** No difference by UKT Level Mismatch, $p = 0.85$ **(F)** Inferior in patients who received a post-transplant blood transfusion, $p = 0.078$.

Recent European guidelines on HLA sensitisation have summarised challenges surrounding the management of highly sensitised patients [10]. As highlighted in these guidelines, standardisation of definitions, alignment of allocation policies and harmonisation of treatment strategies are required to provide evidence for optimal management. An increasingly adopted definition of highly sensitised status is a cRF of ≥85%, determined by SAB methods. This cut-off has been utilised by Eurotransplant for many years, and more recently used in the KOS 2019 organ allocation scheme in the UK [8, 10, 11].

A high cRF associates with increasing difficulty in finding a compatible kidney, and hence such patients have to wait a longer time for a transplant. However, the additional immunological risk posed by a high cRF in the absence of preformed DSA and the impact on allograft survival is not clear. To date, there are

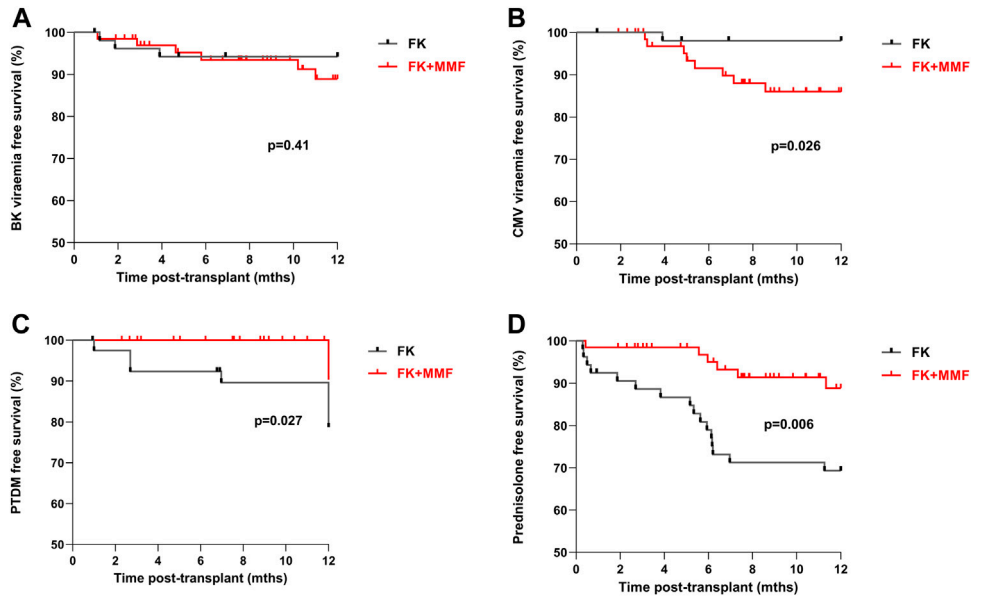


FIGURE 5 | Adverse clinical outcomes associated with and without the use of mycophenolate in highly sensitised patients At one-year post-transplant, there was **(A)** No difference in BK-virus free survival between the FK and FK + MMF groups, $p = 0.41$ **(B)** Superior CMV free survival in the FK group compared with the FK + MMF group, $p = 0.026$ **(C)** Inferior PTDM free survival in the FK group compared with the FK + MMF group, $p = 0.027$ **(D)** Inferior prednisolone free survival in the FK group compared with the FK + MMF group, $p = 0.006$.

conflicting reports. Huber et al. found cRF to be a significant risk factor for long-term graft survival in a study of 726 renal transplant recipients, although their conclusions were limited as they did not specify the absence of preformed DSA [6]. In contrast, Wehmeier et al. in a single centre study, found the broadness of sensitisation not to be an immunological risk factor for ABMR and graft loss [4]. In their study, the main pre-transplant risk factors were the presence or absence of preformed DSA, and the number of donor mismatches to which the recipient can develop post-transplant DSA [4]. This latter study has more recently been supported by registry data from the US, which concluded that cRF was a poor predictor of allograft outcomes, and only patients who were receiving repeat transplants with a cRF≥98% were at risk of death censored graft loss [5].

Despite the lack of consensus of cRF as a prognostic indicator for immunological risk, it is often used to guide induction immunosuppression [1, 10, 12]. This may be considered a pragmatic approach as pre-transplant sensitisation may help identify patients at risk of developing a memory response. Certainly, early DSA detection, suggesting a memory response was more common than *de novo* DSA in this report. In this case, peak or historic cRFs may more accurately correlate with memory, although dynamics may also work the other way, and for some patients, especially those awaiting regrafts, cRF may significantly increase on the wait-list over time [6, 13]. The inclusion of memory response testing as part of pre-transplant risk assessment would certainly be beneficial, although may be difficult to implement. The most common method available until now, HLA-ELISpot, is not suitable for routine use as it is time-consuming and not easily standardised. New Luminex based methods to test cultured B-cell supernatants, could be more promising for routine diagnostics. Although initially the sensitivity was low due to level of IgG, current modifications using concentrated, or IgG isolated supernatants are showing improved detection [14]. As part of wider immunological testing, assessment of pre-transplant T-cell immunity has also been proposed to help risk stratify patients, but is not yet incorporated into clinical use [15, 16].

Surprisingly, we did not find a significant association between matchability or UKT level mismatch on either rejection or DSA development in this highly sensitised cohort. This is in contrast with studies that have looked at the correlation between an increasing number of HLA antigen mismatches and alloimmune outcomes. In addition we have previously shown that higher HLA Level mismatches are associated with *de novo* DSA and ABMR [17, 18]. Our findings in this study, may relate to the possibility that degree of mismatch being less significant in transplants in highly sensitised patients, or it may be a reflection of the relatively small number of patients in our study.

This report supports the potential use of steroid sparing protocols in HSPs, with previous studies assessing efficacy of such protocols including sensitised patients down to a cRF≥20% [1, 19]. Whilst the patient outcomes in our report were impacted by COVID-19, assessing allograft outcomes alone, 1-year rejection free survival was excellent at 91.4% in the group receiving FK + MMF. This is reassuring as steroid sparing protocols are recognised to be associated with increased rates of acute rejection, but there are no reports of this translating into inferior graft survival [20]. Steroid sparing protocols may be of survival benefit in this population who may have accumulated significant co-morbidity whilst awaiting a transplant and are particularly attractive for our

predominantly non-white population who are at increased risk of post-transplant diabetes [19, 21, 22].

This single centre report includes relatively small numbers with short-term follow up, which limit the power of its conclusions. However, larger studies on HSPs lack the granular data we report here on risk factors for memory response and DSA monitoring in HSPs lacking detectable DSA assessed by SAB and crossmatching. As transplant activity in HSPs remain at a high rate, we will continue to prospectively monitor and will report subsequently on patients we maintain on FK + MMF. Whilst the KOS2019 in the UK has led to an increase in the number of highly sensitised patients receiving transplants, as reflected in this report, this should not detract from the need to expand kidney sharing schemes and improve desensitisation protocols [22]. However, as a community we must do more to try and minimise sensitisation, with 2 modifiable areas requiring optimisation including preventing *de novo* sensitisation in patients returning to the wait-list post graft failure and prevention of sensitisation *via* blood product transfusion.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is restricted as it is clinical data. Requests to access these datasets should be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the West of Scotland Research Ethics Committee (20/WS/0181). The approval includes the reporting of anonymised routinely collected clinical data, without individualised informed consent.

AUTHOR CONTRIBUTIONS

ES and MW conceptualised and performed the analysis. KS, NG, ES, MW, and CR all contributed to the data provision. ES and MW wrote the first draft of the manuscript. All authors reviewed, revised, and approved the submitted version of the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11056/full#supplementary-material

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Therapeutic Drug Monitoring of Mycophenolic Acid Identifies Kidney Transplant Recipients Responsive to Two SARS-CoV-2 mRNA Vaccine Doses

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Immune-responsiveness to SARS-CoV-2 mRNA vaccination is reduced in kidney transplant recipients (KTRs). Previous reports point to a role of mycophenolic acid (MPA). Our observational cohort study included all KTRs at University Hospital Zurich receiving two SARS-CoV-2 mRNA vaccine doses more than 6 months post-transplantation, who were assessed by measuring anti-spike immunoglobulin G (IgG). We applied principles of therapeutic drug monitoring (TDM) to correlate MPA exposure and lymphocyte counts with SARS-CoV-2 IgG. MPA trough levels differ largely among KTRs with a median of 3.1 mg/L (range 0.7–9.5 mg/L). 34 of 84 KTRs (40%) developed positive SARS-CoV-2 IgG after two vaccine doses. KTRs who developed positive SARS-CoV-2 IgG showed significantly higher eGFR ($p < 0.001$), lower MPA trough levels ($p < 0.001$) and higher $CD19^+$ lymphocytes ($p < 0.001$). MPA trough levels <2.5 mg/l and $CD19^+$ lymphocytes >40/µl identify KTRs with seroconversion. Upon logistic regression, MPA trough levels <2.5 mg/L were associated with a 7-fold (CI 95%: 1.589–29.934) and ciclosporin use with a 6-fold (CI 95%: 1.148–30.853) increase in the odds of seroconversion. Our study indicates that immune-responsiveness to SARS-CoV-2 mRNA vaccines correlates with MPA exposure measured by MPA trough level but argues against a class effect of MPA. TDM-guided MPA dosing may be a strategy to increase seroconversion rate.

Keywords: kidney transplantation, therapeutic drug monitoring, seroconversion, mycophenolic acid trough level, SARS-CoV-2 vaccination

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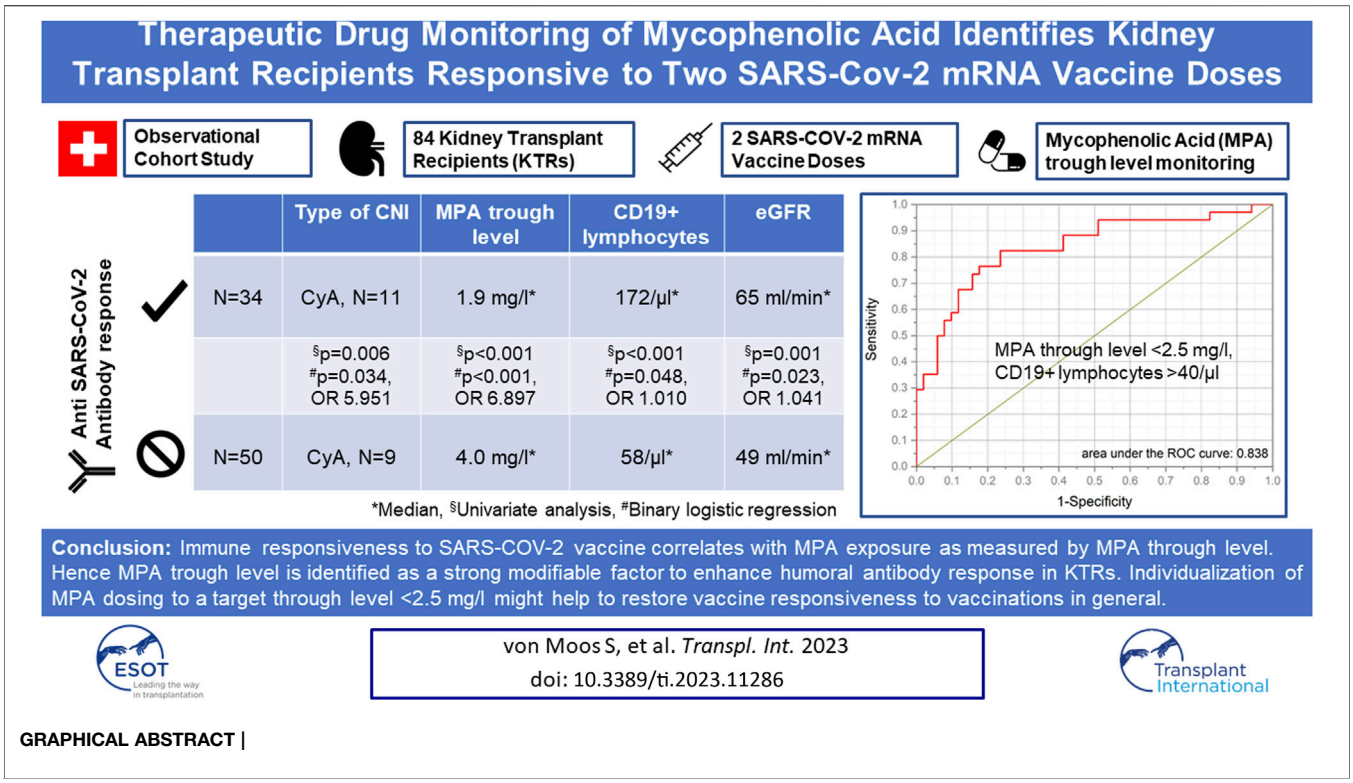
Abbreviations: AUC, Area under the curve; CI, Confidence interval; CNI, Calcineurin inhibitor; DSA, Donor specific antibody; EC-MPS, Enteric-coated mycophenolate sodium; eGFR, Estimated glomerular filtration rate; EHC, Enterohepatic cycling; HPLC, High pressure liquid chromatography; Ig, Immunoglobulin; IMPDH, inosine-5-monophosphate dehydrogenase; KTR, Kidney transplant recipient; MMF, Mycophenolate mofetil; MPA, Mycophenolic acid; NP, Nucleoprotein; mTOR, Mammalian target of rapamycin; OR, Odds ratio; ROC, receiver operating characteristic; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2; TDM, Therapeutic drug monitoring.



GRAPHICAL ABSTRACT |

INTRODUCTION

The appearance of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has changed the world. Marketing BNT162b2 (Pfizer BioNTech) and mRNA-1273 (Moderna) end of 2020 represented a milestone step toward controlling the pandemic by inducing a long-lasting protective immune response [1]. Both vaccines comprise lipid nanoparticles containing nucleoside-modified RNA encoding for the SARS-CoV-2 spike protein. Even though the exact role of cellular and humoral immune responses conferring protection is unknown, the development of neutralizing antibodies has been shown to be an immune correlate of protection against symptomatic SARS-CoV-2; [2, 3]. Yet, humoral immune responsiveness induced by mRNA-based vaccines is significantly reduced in solid organ transplant recipients, with lowest response rates in kidney and heart transplant recipients; [4–6]. Hence after two vaccinations, only 8%–40% of kidney transplant recipients (KTR) show spike-specific IgG and high frequencies of vaccine-specific T helper cells [4, 7, 8]. Also, a third vaccine dose does not substantially improve vaccine effectiveness, with only one-third of previously anti-spike IgG negative patients showing seroconversion [8–10]. A correlation of seroconversion rate with the type of vaccine, type of solid organ transplant, recipient age, years since transplantation, estimated glomerular filtration rate (eGFR), and type of maintenance immunosuppression has been shown [4, 8, 11, 12]. The strongest impairment of humoral immune responses has been reported for the use of B cell-depleting agents and glucocorticoids independent of the dose; [13]. In patients treated with B cell depleting agents, not only time since the last anti-CD20 treatment but also absolute CD19+cell counts and CD4+T-cell helper count were predictive of vaccine efficacy; [14]. Additionally, mycophenolic acid (MPA) as antimetabolite has been associated with an impaired serological response rate [4, 10–12, 15]. MPA is an inhibitor of *de novo* purine synthesis by potently inhibiting the type II isoform of inosine-5-monophosphate dehydrogenase (IMPDH), which is only expressed in activated T- and B lymphocytes [16]. Accordingly, its use has been associated with reduced frequencies of antibody-secreting plasmablasts, lower levels of IgG in the peripheral blood [17], and impeded generation of T follicular helper $CD4^+$ T cells [18]. These immunological observations might explain the reduced immune responsiveness of patients treated with MPA. Based on these observations, it has been proposed by others to suspend MPA in transplant patients in the peri-vaccination period [10]. This strategy, however, can potentially increase the risk of HLA sensitization as a significant association between minimum MPA through level and formation of *de novo* DSA has previously been reported [19]. Hence, caution is recommended when completely withdrawing MPA before vaccination until this issue has been properly investigated by a randomized controlled trial.

We hypothesize that individualizing MPA dosing by applying therapeutic drug monitoring (TDM) [16, 20] might be a promising and safe strategy to improve immune responsiveness to SARS-CoV-2 vaccinations in KTRs. While MPA was initially marketed as a one-dose-suits-all drug,

increasing evidence has accumulated in the past years regarding high interindividual variability of MPA exposure with a fixed dosing strategy [16, 20]. Hence, it has been shown that a fixed dosing strategy leaves a high proportion of patients outside the recommended dose range, which is important to consider in light of the narrow therapeutic window of MPA [20].

In this study, we set out to look for a correlation between MPA exposure guided by TDM and humoral immune responses as measured by spike S1 specific IgG after SARS-CoV-2 vaccination in our cohort of KTRs to find a modifiable surrogate marker with the potential to improve vaccine responsiveness to the administration of future SARS-CoV-2 vaccine doses.

MATERIALS AND METHODS

Patients

Our study was approved by the cantonal ethic commission review board of Zurich, Switzerland (KEK-ZH-Number 2022-00013) and has been conducted in compliance with the declaration of Helsinki.

In this retrospective, observational cohort study, we screened 334 KTRs who underwent kidney transplantation between 1985 and 2020 and were followed at our transplant center after receiving SARS-CoV-2 vaccination. From this cohort, a total of 168 KTRs met the inclusion criteria: i) vaccination with at least two doses of either BNT162b2 (Pfizer BioNTech) or with mRNA-1273 (Moderna) against SARS-CoV-2 more than 6 months post-transplantation. This criterion ensured that none of the included patients received B- or T-cell depleting therapy within the 6 months before SARS-CoV-2 vaccination. ii) available anti-SARS-CoV-2 antibody testing between 3 and 6 months after the second vaccination. Patients who suffered from a SARS-CoV-2 infection before anti-SARS-CoV-2 antibody measurement were excluded, as well as patients with an immunosuppressive drug regimen without MPA or on a regimen with Belatacept. Additionally, patients with incomplete data regarding MPA through level measurement and lymphocyte subset screening were not considered for final analysis. Hence, a total of 84 KTRs were finally analyzed in the current study (**Figure 1**).

Maintenance Immunosuppression

We selected our study for KTRs treated with an immunosuppression regimen comprising MPA together with either a calcineurin inhibitor (CNI) or a mammalian target of rapamycin (mTOR) inhibitor. Target trough levels for CNI at six and after 12 months are 80–120 ng/mL, 60–100 ng/mL and 6–8 ng/mL, 5–7 ng/mL for cyclosporine and tacrolimus, respectively. Target trough levels for mTOR (everolimus) at month six and after 12 months are 6–8 ng/mL and 4–6 ng/mL, respectively, when used in combination with MPA. MPA was either administered as mycophenolate mofetil (MMF, Cellcept®) or enteric-coated mycophenolate sodium (EC-MPS, Myfortic®). According to the immunological risk, KTRs are treated with 5 mg prednisone or steroid is withdrawn.

Assessment of Serologic Response

The serologic response to the SARS-CoV-2 spike (S1) protein receptor-binding domain in human serum and plasma was assessed prospectively as a standard of care in our department by the commercial immunoassay Elecsys® Anti-SARS-CoV-2 S (Roche, Switzerland) as previously described [21]. This assay detects pan-Ig antibody responses and allows for a quantitative assessment of the serological response. The manufacturer states a cutoff of ≥0.8 AE/mL anti spike S1 protein to be considered as threshold for positivity.

Assessment of Lymphocyte Subsets

Total lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, and $CD19^+$ lymphocyte counts were measured at the time of SARS-CoV-2 antibody testing. Flow cytometric determination of T and B cells was performed on a flow cytometer by Beckman Coulter (Navios Ex). The monoclonal antibodies used to identify these cell subsets were anti-CD3, anti-CD4, anti-CD8, anti-CD19, and anti-CD20, with a DuraClone IM Phenotyping BASIC Kit [22].

Assessment of MPA Exposure

MPA trough levels were measured during SARS-CoV-2 antibody testing using high-pressure liquid chromatography (HPLC). Regarding MPA, we additionally collected data to calculate the area under the curve (AUC) using a limited sampling strategy described previously [23, 24] if appropriate data were available. The time points and formulas used for the limited sampling strategy have been shown to correlate well with the full AUC (0–12 h) [23, 24]. For MMF in combination with tacrolimus, the AUC was calculated by measuring MPA predose level (0 min) and levels 30 min and 120 min after drug intake, according to Pawinski et al. [23] For EC-MPS in combination with tacrolimus, the AUC was calculated measuring MPA predose level (0 min) as well as MPA levels 1 h, 2 h, and 4 h after drug intake and calculated according to Sanchez et al. [24].

Statistical Methods

Statistical analysis was performed using IBM-SPSS Version 26 (SPSS, Chicago, IL, USA). Data distribution was evaluated using Shapiro–Wilk normality test and expressed as median and range. For comparisons of study groups, Mann–Whitney U-Test was used for nonparametric independent samples. Clinical characteristics were compared across groups using the Chi-square test for categorical variables. A binary logistic regression model was used to define variables associated with a positive immune response after SARS-CoV-2 vaccine doses. The Spearman's rank correlation coefficient is used to measure the degree of association between two nonparametric continuous variables. Receiver operating characteristic (ROC) analysis was used to establish the optimal cutoff values for MPA trough levels and lymphocyte counts to identify KTRs responsive to two SARS-CoV-2 vaccine doses. Boxplots show median, interquartile range (IQR), and 95th percentile. A p-value of less than 0.05 is considered statistically significant.

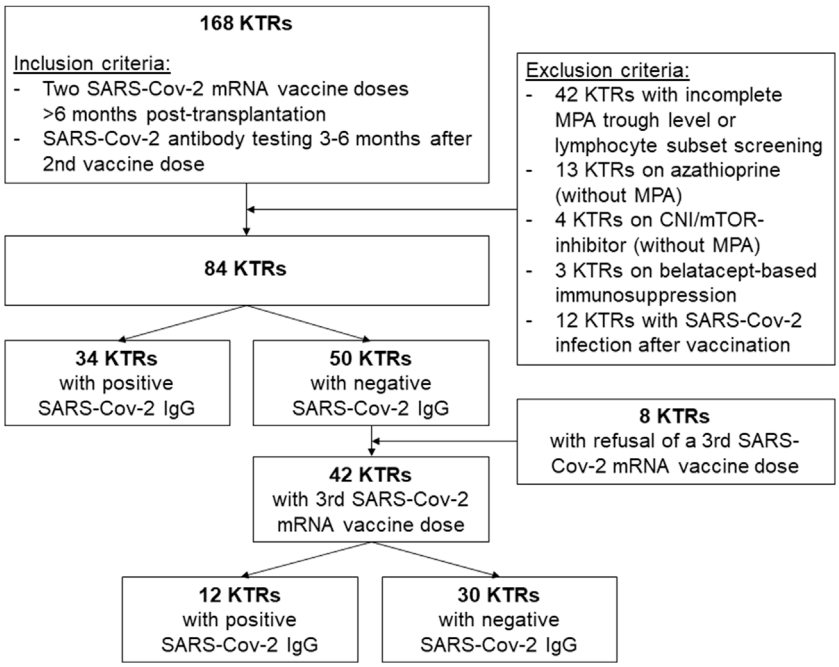


FIGURE 1 | Patient inclusion and exclusion algorithm.

RESULTS

Overall Patient Characteristics

In total, 168 KTRs fulfilled the inclusion criteria of receiving two SARS-CoV-2 mRNA vaccine doses administered at least 6 months post-transplantation with available SARS-Cov-2 S1 IgG between three and 6 months after the second vaccine dose. After excluding KTRs with incomplete MPA or lymphocyte subset measurement, non-MPA-based immunosuppression, and SARS-CoV-2 infection before available SARS-CoV-2 antibody measurement, a total of 84 KTRs were identified for final data analysis (**Figure 1**). Clinical characteristics, detailed information on SARS-CoV-2 vaccination and maintenance immunosuppression, and lymphocyte counts are shown in **Table 1**. In total, 34 of 84 KTRs (40%) responded to two SARS-CoV-2 mRNA vaccine doses with positive SARS-CoV-2 IgG at a median period of 5 months (range 3–5 months) after the second vaccine dose.

Factors Associated With Positive SARS-Cov-2 IgG After Two Doses of a SARS-Cov-2 mRNA Vaccine

Upon univariate analysis, eGFR ($p = 0.001$), MPA trough level ($p < 0.001$, **Figure 2A**), type of calcineurin inhibitor ($p = 0.016$, **Supplementary Figure S1**), total lymphocytes ($p = 0.009$), and $CD19^+$ lymphocytes at the time of SARS-CoV-2 antibody testing ($p < 0.001$, **Figure 2B**) were associated with positive SARS-Cov-2 IgG after two SARS-CoV-2 mRNA vaccine doses (**Table 1**).

MPA trough levels of less than 2.5 mg/L and $CD19^+$ lymphocytes of more than 40/μL identify KTRs with positive SARS-CoV-2 IgG after two doses of SARS-CoV-2 mRNA vaccine with a sensitivity, specificity, positive predictive value, and negative predictive value of 67.6%, 88.0%, 82.1%, and 80.4%, respectively (**Figure 3**, area under the ROC curve of 0.829, $p < 0.001$)). ROC analyses are shown in **Supplementary Figures S2A–C**. MPA trough levels and $CD19^+$ lymphocytes show a weak significant negative correlation (Spearman's correlation coefficient −0.282, $p = 0.009$; **Figure 3**).

Figure 4 shows the distribution of MPA trough levels compared with different MPA doses per day and accompanying immunosuppressive medication. The distribution of MPA-AUC measurements compared among a small subgroup of 14 KTRs with positive and negative SARS-CoV-2 IgG and associated MPA trough levels are shown in **Supplementary Figure S3**.

eGFR showed a weak significant correlation with total lymphocytes (Spearman's correlation coefficient 0.296, $p = 0.006$), a weak significant correlation with $CD3^+$ lymphocytes (Spearman's correlation coefficient 0.224, $p = 0.041$), and a moderate significant correlation with $CD19^+$ lymphocytes (Spearman's correlation coefficient 0.489, $p < 0.001$; **Supplementary Figures S4A–E**).

Upon binary logistic regression, MPA trough levels <2.5 mg/L were associated with a 6.897-fold increase in the odds of seroconversion (CI 95%: 1.589–29.934; $p = 0.010$) and ciclosporin as the calcineurin-inhibitor type was associated with a 5.951-fold increase in the odds of seroconversion (CI 95%: 1.148–30.853). Of note, no collinearity was observed between MPA through level and

TABLE 1 | Clinical characteristics of 84 KTRs with positive/negative SARS-CoV-2 IgG to two doses of an mRNA SARS-Cov-2 vaccine.

	Total (*n* = 84)	Negative SARS-CoV-2 IgG (*n* = 50)	Positive SARS-CoV-2 IgG (*n* = 34)	*p-value*
Recipient characteristics				
Recipient age at transplantation, years[a]	47 (18–71)	47 (18–71)	47 (18–69)	0.834
Recipient age at 1st vaccination, years[a]	59 (19–81)	59 (19–81)	59 (33–80)	0.626
Male sex, n(%)	52 (62)	30 (60)	22 (65)	0.663
eGFR at the time of 1st vaccination, mL/min	52 (15–107)	49 (15–102)	65 (23–107)	**0.001**[a]
Deceased donation, n(%)	63 (75)	36 (72)	27 (79)	0.441
Living donation, n(%)	21 (25)	14 (28)	7 (21)	
Simultaneous kidney/pancreas transplantation, n(%)	3 (4)	2 (4)	1 (3)	0.797
Retransplantation, n(%)	13 (15)	9 (18)	4 (12)	0.438
Primary kidney disease, n(%)				0.286
Diabetic/hypertensive	7 (8)	4 (8)	3 (9)	
Polycystic kidney disease	11 (13)	8 (16)	3 (9)	
Glomerulonephritis	34 (40)	23 (46)	11 (32)	
Others/unknown	32 (38)	15 (30)	17 (50)	
Preformed DSA, n(%)	15 (18)	11 (22)	4 (12)	0.229
de novo DSA, n(%)	19 (23)	12 (24)	7 (21)	0.714
SARS-Cov-2 vaccination				
Type of mRNA SARS-CoV-2 Vaccine, n(%)				0.182
BNT162b2	76 (90)	47 (94)	29 (85)	
mRNA-1273	8 (10)	3 (6)	5 (15)	
Time of 1st vaccination after transplantation, months[a]	93 (7–431)	86 (7–431)	97 (7–430)	0.179
Time of SARS-CoV-2 antibody testing after 2nd vaccination, days[a]	155 (86–196)	158 (86–196)	144 (89–192)	0.251
SARS-CoV-2 S IgG, AE/mL	-	-	21.86 (0.86–869.10)	-
Immunosuppression at the time of SARS-CoV-2 antibody testing				
Mycophenolic acid trough level, mg/L[a]	3.1 (0.7–9.5)	4.0 (1.0–9.5)	1.9 (0.7–6.8)	<**0.001****
Mycophenolic acid dose per day, mg[a]				0.622
360/500[b]	4 (5)	4 (8)	0 (0)	
720/1,000[b]	19 (22)	9 (18)	10 (29)	
1,080/1,500[b]	33 (39)	20 (40)	13 (38)	
1,440/2,000[b]	28 (33)	17 (34)	11 (32)	
Calcineurin inhibitor, (%)	78 (93)	47 (94)	31 (91)	**0.016**
Cyclosporine, n	20	9	11	
Tacrolimus, n	58	38	20	
Everolimus, n (%)	6 (7)	3 (6)	3 (9)	0.682
Cyclosporine trough level, µg/L[a]	69 (30–129)	69 (32–93)	69 (30–129)	0.675
Tacrolimus trough level, µg/L[a]	5.8 (3.7–10.7)	5.9 (3.8–9.0)	5.4 (3.7–10.7)	0.422
Everolimus trough level, µg/L[a]	4.4 (4.1–5.2)	4.5 (4.2–5.2)	4.2 (4.1–4.9)	-
Prednisone, n(%)	43 (51)	28 (56)	15 (44)	0.285
Lymphocyte subsets at the time of SARS-CoV-2 antibody testing				
Total lymphocytes,/µL[a]	1,178 (327–3,450)	1,094 (327–3,450)	1,355 (709–2,828)	**0.009**[a]
$CD3^+$ lymphocytes,/µL[a]	903 (175–3,060)	834 (175–3,060)	1,004 (403–2,328)	0.056
$CD4^+$ lymphocytes,/µL[a]	516 (92–1894)	500 (92–1894)	545 (256–1792)	0.132
$CD8^+$ lymphocytes,/µL[a]	311 (41–4,453)	306 (41–1,159)	317 (53–4,453)	0.298
$CD19^+$ lymphocytes,/µL[a]	89 (1–837)	58 (1–292)	172 (5–837)	<**0.001****

[a]*Median (range).*
[b]*Mycophenolate mofetil (MMF)/enteric-coated mycophenolate sodium (EC-MPS).*
Significant p *values are indicated in bold.*

ciclosporine use indicating independent effects (**Supplementary Table S1**). eGFR at time of vaccination was associated with a 1.041-fold increase in the odds of seroconversion (CI 95%: 1.005–1.078; p = 0.023), and $CD19^+$ lymphocyte counts with a 1.010-fold increase in the odds for seroconversion (CI 95%: 1.000–1.021; p = 0.048, **Table 2**).

DISCUSSION

Even though publications reporting on reduced immune responses after SARS-CoV-2 mRNA vaccination in KTRs are accumulating [4–11, 13, 25], measures for individualized vaccination responsiveness are urgently needed. Despite clear evidence that up to one-third of patients still do not develop

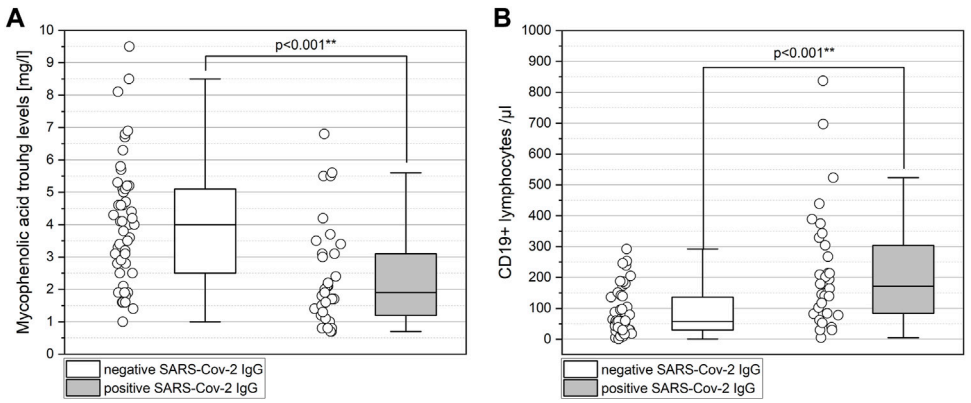


FIGURE 2 | Lower MPA levels (A) and higher CD19$^+$ lymphocytes (B) among KTRs who respond to two doses of the SARS-CoV-2 mRNA vaccine. **(A)** KTRs with negative SARS-CoV-2 IgG show significantly higher MPA trough levels than KTRs with positive SARS-CoV-2 IgG ($p < 0.001$). **(B)** KTRs with negative SARS-CoV-2 IgG show significantly lower CD19$^+$ lymphocytes than KTRs with positive SARS-CoV-2 IgG ($p < 0.001$). Boxplots show median, interquartile range (IQR), and 95th percentile.

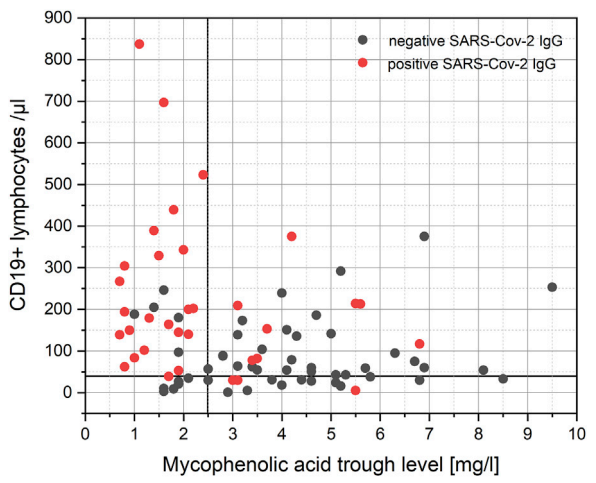


FIGURE 3 | Correlation of MPA trough levels and CD19$^+$ lymphocytes. MPA trough levels of less than 2.5 mg/L and CD19$^+$ lymphocytes of more than 40/µL identify KTRs with positive SARS-CoV-2 IgG after two doses of SARS-CoV-2 mRNA vaccine. MPA trough levels and CD19$^+$ lymphocytes show a significant negative correlation (Spearman's correlation coefficient –0.282, $p = 0.009$).

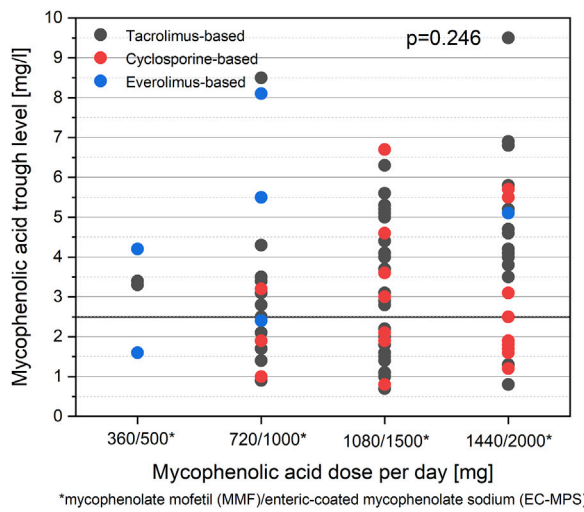


FIGURE 4 | Distribution of MPA trough levels compared with MPA dosing. No differences are observed for MPA trough levels between different categories of MPA dosing per day ($p = 0.246$). No differences are observed in MPA trough levels between a tacrolimus-based and a cyclosporine-based regimen ($p = 0.208$).

seroconversion even after three vaccine doses [8], no clear recommendations have emerged on improving immune responsiveness in this vulnerable cohort, even though evidence for a strong effect of antimetabolite treatment is growing [4, 10, 11, 15, 26]. Our study goes beyond the correlation of MPA dose and vaccine responses but measures MPA through levels, thereby discovering a highly promising and modifiable biomarker predictive of immune responsiveness. We analyzed the humoral immune responses after SARS-CoV-2 mRNA vaccinations in a total of 84 KTRs. In this cohort, 40% (34/84) showed seroconversion after two doses of the SARS-CoV-2 mRNA vaccine, which is in line with the literature [8]. There was no difference between the two groups concerning classically reported risk factors for reduced immune responsiveness, such as recipient age at vaccination or time since transplantation. With respect to immunosuppression, calcineurin inhibitor trough levels were similar between the two groups, as was the use of prednisone. However, upon multivariate analysis, MPA trough levels were significantly lower in KTRs with positive anti-spike IgG response after two vaccine doses and use of ciclosporine as calcineurin inhibitor type was more frequent in responders. Importantly and in contrast to a recently reported study by Kantauskaite et al.; [11], we did not observe a difference in cumulative MPA dose between the groups. This fact underscores the high interindividual variability of MPA exposure with a fixed dosing strategy based on the complex

TABLE 2 | Binary logistic regression model representing factors associated with the development of SARS-CoV-2 IgG after two doses of an mRNA SARS-CoV-2 vaccine.

	β	OR	CI 95%	*p-value*
eGFR at the time of 1st vaccination, mL/min/1.73 m^2	0.040	1.041	1.005–1.078	0.023*
Mycophenolic acid trough level <2.5 mg/L	1.931	6.897	1.589–29.934	0.010*
Total lymphocytes,/μL	0.000	1.000	0.998–1.003	0.763
$CD3^+$ lymphocytes,/μL	–0.002	0.998	0.994–1.002	0.398
$CD19^+$ lymphocytes,/μL	0.010	1.010	1.000–1.021	0.048*
Ciclosporin as calcineurin inhibitor	1.784	5.951	1.148–30.853	0.034*

pharmacodynamic and pharmacokinetics of MPA; [16, 20]. Especially when combined with tacrolimus, drug concentrations in the toxic range are more commonly encountered than in combination with ciclosporin, which inhibits enterohepatic cycling (EHC) [16, 20].

So far, trials investigating the benefit of TDM for MPA primarily focused on the relationship between low MPA exposure as measured by AUC and under-immunosuppression reflected by rejection. Here, a clear correlation between MPA-AUC <30mgxh/L and rejection rates could be observed with the recommendation to target an MPA-AUC of 40mgxh/L [27, 28]. Until now, less attention has been devoted to MPA overexposure [27]. Nevertheless, according to the literature, an MPA-AUC above 60 mgxh/L has been suggested to be associated with adverse events [20]. While MPA-AUC measurement is the gold standard when applying TDM for MPA(16, 20); such practice is cumbersome during daily routine requiring at least three blood samples taken at different time points. Measurement of MPA trough level is much more convenient yet highly debated. Nevertheless, MPA-AUC and MPA trough levels have been shown to correlate with an MPA trough level of approximately 1.4 mg/L, corresponding to an AUC >30 mgxh/L [29]. The OPTICEPT trial, which also showed correlations between MPA-AUC and through levels, targeted MPA throughs of ≥1.3 μg/mL or ≥1.9 μg/mL for ciclosporine and tacrolimus treated patients, respectively [30]. In the present study, we found an MPA trough level of less than 2.5 mg/L being associated with a positive humoral immune response to SARS-CoV-2 mRNA vaccination. In a small subset of KTRs, we could confirm that an MPA trough level of <2.5 mg/L corresponds to MPA-AUC <60 mgxl/h. Hence, higher levels, especially trough levels above 4 mg/L, are likely to correspond to MPA overdosage, reflecting an MPA-AUC >60 mgxh/L. Our data indicate that TDM measuring MPA trough levels is promising for identifying KTRs that respond to SARS-CoV-2 mRNA vaccination. The previously reported observation of reduced vaccine responsiveness in KTRs treated with MPA might therefore not be a class effect but rather a matter of dosing, urgently calling for TDM for this drug with a narrow therapeutic window. We hypothesize that immune responsiveness in KTRs treated with MPA can be restored by individually adapting MPA dosage to a target range.

In addition to MPA trough level <2.5 mg/L, our analysis suggests better responsiveness to two SARS-CoV-2 vaccine doses in KTRs on a ciclosporin-based compared to a tacrolimus-based immunosuppressive regimen independent from MPA trough levels and the well-known impact of ciclosporin on MPA pharmacokinetics. Several independent studies found *in vitro* evidence of ciclosporin-mediated inhibition of SARS-CoV-2 replication, which led to speculation that ciclosporin could be used as the preferred calcineurin inhibitor during SARS-CoV-2 infection [31, 32]. Our findings, however, suggest better virus control and responsiveness to vaccination linked to the lower immunosuppressive potency of ciclosporin compared to tacrolimus. Although ciclosporin and tacrolimus suppress the immune system through the same main mechanism by preventing interleukin-2 production in T cells, ciclosporin and tacrolimus are chemically distinct molecules, and ciclosporin demonstrated weaker immunosuppressive potency compared to tacrolimus [33].

Our analysis further revealed CD19 + lymphocyte counts above 40/μL as a surrogate marker of positive immune responsiveness after two SARS-CoV-2 vaccination, but to a much lesser extent than MPA trough levels and calcineurin inhibitor use. While negatively correlated on the one hand with MPA trough levels, $CD19^+$ lymphocytes and $CD3^+$ lymphocytes positively correlate with eGFR reflecting reduced immune responsiveness with impaired kidney function as previously reported [34]. In line with our observation, $CD19^+$ lymphocytes have previously been reported to be surrogate markers for immune competence [14].

The limitations of our study are the lack of systematic measurement of MPA-AUC, which has been reported to be the gold standard of TDM for MPA. We only had data on MPA-AUC by limited sampling strategy in a small subset of patients. Yet, correlations between MPA-AUC and trough levels have been shown in the literature and are in line with our observation in the limited cohort of patients. Moreover, trough level measurement is much more convenient in the daily routine and, therefore, easily implementable. We acknowledge that MPA dose reductions according to MPA trough levels need to be verified in a second step by using MPA-AUC measurement to ensure drug efficacy and limit the risk of rejections. Ideally, further studies testing the risk of rejections and development of *de novo* DSA following TDM-guided MPA dose reductions would help to support the safety of our recommendations. Even though our data suggest a cutoff of 2.5 mg/l as MPA through level discriminating vaccine responsiveness, we are aware of wide spreading of through levels in both groups underlining the need for larger studies to confirm our results. Yet, our data suggest that a trough level of >4 mg/L is a surrogate marker of drug overexposure, limiting the development of humoral

vaccination responses. A further limitation related to the retrospective study designs is that MPA trough levels were measured when assessing SARS-CoV-2 vaccine responses and not at the time of vaccination. However, as the immunosuppressive regimen was not changed between the two time points, the results are expected to be the same. Additionally, we did not measure the neutralization capacity of anti-Sars-CoV-2 antibodies.

In conclusion, our study underlines the numerous and accumulating previous reports pointing towards an important role of MPA concerning reduced immune responsiveness in KTRs. Yet, it reaches beyond the correlation of MPA doses with immunoglobulin levels but suggests that individualizing MPA drug dosage to an MPA trough level below 2.5 mg/L might restore vaccine responsiveness to future vaccine doses. To ensure drug efficacy and prevent rejections, we recommend verification of dose reductions by MPA-AUC measurement in a second step. Prospective, randomized trials are needed to confirm our hypothesis and prove its safety concerning the risk of rejections and development of *de novo* DSA. Such confirmation of our observation in a larger population could have major implications not only for SARS-CoV-2 vaccination but for vaccinations in general—administered not only to KTRs but also for all other solid organ transplant recipients or patients under MPA therapy for autoimmune diseases.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data is available on request. Requests to access these datasets should be directed to thomas.schachtner@usz.ch.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by cantonal ethic commission review board of Zurich, Switzerland. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SM: Participated in research design—Participated in data collection—Participated in data analysis—Participated in writing of the paper. ER: Participated in data collection. MD: Participated in data collection. SK: Participated in data collection. TM: Participated in writing the paper. TS: Participated in research design—Participated in data collection—Participated in data analysis—Participated in writing of the paper.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11286/full#supplementary-material

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ORIGINAL RESEARCH
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Long-Term Kidney and Maternal Outcomes After Pregnancy in Living Kidney Donors

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For counseling it is important to know if pregnancy after Living Kidney Donation (LKD) affects long-term outcomes of the mono-kidney and the mother. Therefore, we performed a retrospective multicenter study in women ≤45 years who donated their kidney between 1981 and 2017. Data was collected via questionnaires and medical records. eGFR of women with post-LKD pregnancies were compared to women with pre-LKD pregnancies or nulliparous. eGFR before and after pregnancy were compared in women with post-LKD pregnancies. Pregnancy outcomes post-LKD were compared with pre-LKD pregnancy outcomes. 234 women (499 pregnancies) were included, of which 20 with pre- and post-LKD pregnancies (68) and 26 with only post-LKD pregnancies (59). Multilevel analysis demonstrated that eGFR was not different between women with and without post-LKD pregnancies (p = 0.23). Furthermore, eGFR was not different before and after post-LKD pregnancy (p = 0.13). More hypertensive disorders of pregnancy (HDP) occurred in post-LKD pregnancies (p = 0.002). Adverse fetal outcomes did not differ. We conclude that, despite a higher incidence of HDP, eGFR was not affected by post-LKD pregnancy. In line with previous studies, we found an increased risk for HDP after LKD without affecting fetal outcome. Therefore, a pregnancy wish alone should not be a reason to exclude women for LKD.

Keywords: kidney function, living kidney donation, pregnancy, hypertension, BMI

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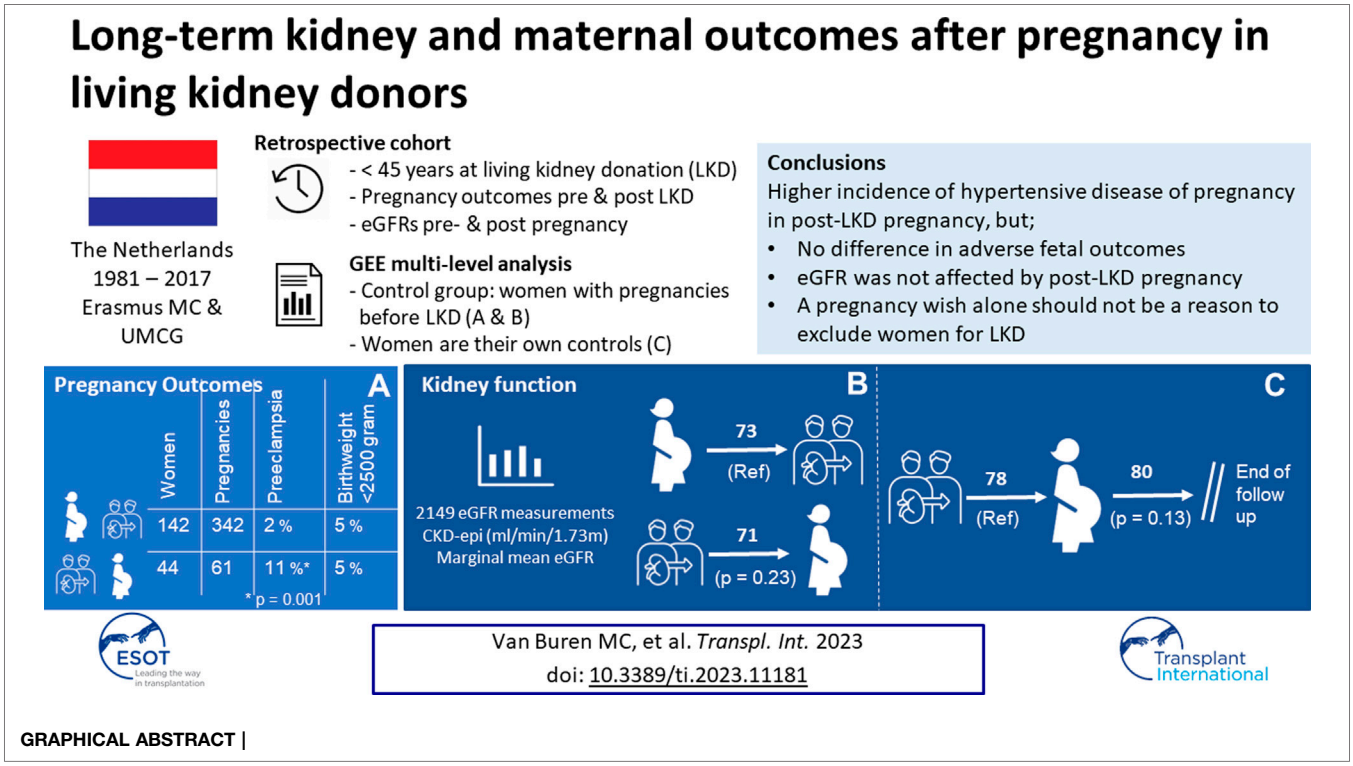
Abbreviations: B, Coefficient estimate; BMI, Body mass index; CI, Confidence interval; CVE, cardiovascular event; eGFR, estimated Glomerular Filtration Rate; GEE, Generalized Estimating Equations; HDP, Hypertensive Disorders of Pregnancy; IUFD, Intra Uterine Fetal Demise; IQR, inter quartile range; LKD, Living Kidney Donation; MAP, mean arterial pressure; SCr, Serum creatinine; SD, Standard deviation; SEM, Standard error of the mean.



GRAPHICAL ABSTRACT |

INTRODUCTION

Living donor kidney transplantation is the best treatment option for patients with end-stage renal disease (ESRD), resulting in better outcomes than dialysis as well as deceased donor kidney transplantation. Less is known about the long-term effects of living kidney donation (LKD) on the health of the donor. Existing research is reassuring and shows no increased cardiovascular risk for donors compared to the control group [1]. However, accurate life-time risk assessment of especially young donors, who spend many years with one kidney, remains uncertain. Current literature reports conflicting results on long-term follow-up, predominantly caused by the difficulty of finding a representative control group [2]. A substantial number of donors are women of fertile age and therefore it is of great importance to know if pregnancy affects long-term outcomes and function of the mono-kidney and if LKD affects pregnancy outcomes.

Previous research shows that LKD reduces pre-donation glomerular filtration rate (GFR) by an average of 30% [3]. The remaining kidney experiences compensatory hypertrophy and hyperfiltration and thereby an increase in GFR [4]. A similar increase in GFR is seen during pregnancy, when GFR and renal plasma flow (RPF) increase by 40%–65% and 50%–85% respectively. A pregnancy potentially adds an additional strain of hyperfiltration on the single kidney after LKD [5, 6]. In the general population, pregnancy with reduced GFR due to kidney disease is associated with adverse pregnancy outcomes [7]. Two studies on pregnancy outcomes of women with a single kidney showed an increase in preterm delivery and preeclampsia compared to women with two kidneys [8, 9].

Research is limited on pregnancy outcomes in otherwise healthy living kidney donors, as was concluded by a recent systematic review by Pippias et al. [10]. They reported an increased risk of hypertensive disorders during pregnancy (hypertension and preeclampsia) post-donation, based on four retrospective cohort studies with limited quality. However, they emphasized that the absolute risk of pregnancy-related complications remains very small. Moreover, fetal and neonatal outcomes were not different when comparing pre-donation pregnancies to post-donation pregnancies [10].

To the best of our knowledge, the effect of pregnancy after LKD on eGFR slope has not been investigated yet. Long-term kidney function post-LKD pregnancy has only been reported by Ibrahim et al. [11]. They reported that the serum creatinine of women with post–LKD pregnancies was not different from women with pre-LKD pregnancies using one time measurements at different time points after LKD without information on eGFR slope before and after LKD [11]. It could be possible that the increased strain of pregnancy on the mono-kidney increases the risk of (accelerated) decline of kidney function in the long-term. Therefore, the aim of this research was to assess if long-term kidney function after LKD is prone to a faster decline after pregnancy. Secondly, we assessed if post-LKD pregnancies have a higher risk of complications than pre-LKD pregnancies in our cohort.

METHODS

Study Design

In this retrospective cohort study, all women who underwent LKD between 1980 and 2017 at the age of 45 years or younger in the University Medical Center Groningen (UMCG) or the Erasmus Medical Center Rotterdam (Erasmus MC) were eligible for inclusion. The study was approved by the medical ethical committees of the UMCG (METc 2014/077) and the Erasmus MC (MEC-2013-585).

Data Collection and Definitions

LKD-specifics and obstetric characteristics of pregnancies pre-LKD and post-LKD were recorded. Check-ups of eGFR, proteinuria, blood pressure, weight and medication-use are (bi-) annually measured as part of the standard care after LKD in the Netherlands. eGFR was calculated using the CKD-EPI formula [12]. Due to the study design, data on pregnancy outcomes were self-reported by questionnaires sent via post or email. Participants who did not respond were contacted twice by telephone to conduct the questionnaire by a direct interview after obtaining direct informed consent from the subject. When this was not possible, the questionnaire was sent again by post or email. If a donor reported gestational hypertension, preeclampsia and/or Intra Uterine Fetal Demise (IUFD) during post-LKD pregnancy in the questionnaire, their medical record was obtained after written informed consent.

Gestational hypertension was defined as ≥140/90 mmHg measured twice or increase in diastolic blood pressure (DBP) >15 mmHg/systolic blood pressure (SBP) >30 mmHg after 20 weeks of pregnancy, without signs of preeclampsia, according to the guideline of the National Institute for Health and Care Excellence (NICE) [13]. Preeclampsia was defined as it was diagnosed by the attending physician according to the guideline in use, defining preeclampsia as the presence of pregnancy induced hypertension at >20 weeks of gestation and proteinuria [14]. Hypertensive disorders of pregnancy (HDP) were defined as a combined endpoint of either gestational hypertension and/or preeclampsia and/or Hemolysis Elevated Liver enzymes Low Platelets syndrome (HELLP). Miscarriage was defined as the spontaneous loss of a pregnancy before 20 weeks and abortion as the deliberate termination of pregnancy before 20 weeks. IUFD was defined as stillbirth after 20 weeks of pregnancy.

During follow-up, hypertension was defined as a SBP ≥140 mmHg, a DBP ≥90 mmHg and/or the use of antihypertensive medication. Proteinuria was defined as urine protein/creatinine ratio >15 g/mol, urine albumin/creatinine ratio >3.5 mg/mmol or when no ratio could be calculated, proteinuria >0.15 g/L. When none of these quantitative urine measurements were available, a positive urine dipstick was defined as proteinuria.

Statistical Analyses

Statistical analysis was performed using SPSS software version 25 and GraphPad Prism version 9.3.1. Continuous variables were reported as mean with standard deviation (SD) in case of a normal distribution and as median with interquartile range (IQR) in case of skewed distribution.

Effect of Pregnancy on eGFR After LKD

This was analyzed at patient level. Firstly, eGFR slope of women with post-LKD pregnancies were compared with women with only pre-LKD pregnancies or nulliparous. This multivariable analysis was performed using Generalized Estimating Equations (GEE) multilevel analysis and an unstructured correlation matrix was used [15]. The number of months after LKD of each individual measurement was used as the within-subject level and as a continuous covariate (transformed to years after LKD). The two groups were adjusted for differences at baseline. For calculating eGFR slope per year, the interaction term "years after LKD*pregnancy after donation" was used.

Furthermore, a sub-analysis was performed of eGFR slopes before and after pregnancy in women with post-LKD pregnancies (>20 weeks). eGFR measurements within 180 days after LKD were excluded since eGFR post-LKD rises in the first period after LKD [16]. eGFR during pregnancy and in the first month after pregnancy was also excluded, since eGFR is physiologically higher during pregnancy [6]. For calculating eGFR slope per year the interaction term "years after LKD*after first pregnancy" was used. In all analyses, a p-value <0.05 was considered statistically significant.

The second part consisted of comparing pregnancy outcomes of pre-LKD pregnancies with pregnancy outcomes of post-LKD pregnancies. Descriptive statistics were used to describe (complicated) pregnancy outcomes. The experimental level in this part of the study were pregnancies instead of women. Descriptive statistics and multi-level analysis with GEE were performed to account for multiple pregnancies in one woman. Analysis was adjusted for differences in baseline between the two groups. An unstructured correlation matrix structure was used for the multilevel univariable analysis, and an exchangeable correlation matrix structure for the multivariable analysis. Odds ratios (OR) were calculated. Predictors that were statistically significant ($p < 0.05$) were added to the multivariable models.

For both parts relevant predictors were selected based on literature: race, parity, age at delivery, age at LKD, BMI before LKD, mean arterial pressure (MAP) before LKD, eGFR before LKD, year of LKD and year of delivery.

RESULTS

We included 234 women with 499 pregnancies and 43 nulliparous (**Figure 1**). Baseline characteristics are shown in **Table 1**. Most of the pregnancies occurred pre-LKD (75%, $n = 372$). 20 women had pre- and post-LKD pregnancies. Women with post-LKD pregnancies (study group) were younger at LKD then women with only pre-LKD pregnancies (30 years vs. 39 years ($p < 0.001$). eGFR before LKD was significantly higher 118 mL/min/1.73 m^2 versus 104 mL/min/1.73 m^2 ($p < 0.001$), respectively. Women with post-LKD pregnancies were older at their first delivery (33 versus 25 years). Years of follow-up

after LKD was not significantly different between the two groups (13 vs. 12 years (p = 0.28).

Comparing eGFR Slopes of Women With Post-LKD Pregnancies to Women With Only Pre-LKD Pregnancies or Nulliparous

We compared post-LKD eGFR of women with post-LKD pregnancies (study group) to women with only pre-LKD pregnancies or nulliparous (control group). 221 Women were included with 2149 eGFR measurements. Five women were excluded for this analysis because no eGFR levels were available. Multilevel analysis was adjusted for age at LKD, eGFR before LKD, years after LKD and maximum follow-up time. Mean adjusted eGFR in the study group was not significantly different compared to the control group: 71 mL/min/1.73 m^2 (SEM 1.32, 95% CI: 68.51–73.70) versus 73 mL/min/1.73 m^2 (SEM 0.57, 95% CI 71.88–74.11, p = 0.23). As shown in **Figure 2**, eGFR increased in both groups the first 4 years after LKD. Adjusted eGFR slope per year in the study group was −0.21 mL/min/1.73 m^2 per year (SEM 0.09, 95% CI: −0.38 to −0.04) versus −0.15 mL/min/1.73 m^2 per year (SEM 0.05, 95% CI −0.25 to −0.05) in the control-group (p = 0.06). When comparing women with and without HDP pre-LKD, no difference was observed in adjusted mean eGFR after LKD (73 mL/min/1.73 m^2 (SEM 2.39, 95% CI 68.10–77.46) versus 71 mL/min/1.73 m^2 (SEM 0.70, 95% CI 69.76–72.51, p = 0.51)). Furthermore, there was no difference in eGFR slope in women with and without HDP pre-LKD (−0.29 mL/min/1.73 m^2 vs. −0.19 mL/1.73 m^2 (SEM 0.12, 95 CI: −0.33–0.13, p = 0.39)).

eGFR Before and After Post-LKD Pregnancy

Furthermore, a sub-analysis of eGFR slope was performed solely in women with post-LKD pregnancies. Before pregnancy, 108 eGFR measurements were collected in 31 women, which results in a median of 4 measurements per women (IQR 3). After the first pregnancy, 275 eGFR measurements were collected in 37 women: 214 after a first post-LKD pregnancy, 49 after a second post-LKD pregnancy and 12 after a third post-LKD pregnancy. In total, a median of 7 measurements per woman (IQR 10) were included. eGFR analysis was adjusted for age at delivery, years after LKD and eGFR before LKD. The course of adjusted mean eGFR before and after first post-LKD delivery is illustrated in **Figure 3**. Adjusted mean eGFR before pregnancy was 78 mL/min/1.73 m^2 (SEM 0.97, 95% CI 76.19–79.99) and after pregnancy 80 mL/min/1.73 m^2 (SEM 0.60, 95% CI 78.50–80.84) (p = 0.13). eGFR slope per year was decreasing with −0.19 mL/min/1.73 m^2 per year (SEM 0.42, 95% CI −1.01–0.62) before pregnancy, and after pregnancy with −0.23 mL/min/1.73 m^2 per year (SEM 0.10, 95% CI −0.42; −0.43 (p < 0.001). Protein-creatinine ratio was not higher after pregnancy than before pregnancy (14 g/mol versus 15 g/mol, p = 0.83). Risk for a protein-creatinine ratio >15 g/mol was not significantly different after pregnancy compared to before pregnancy (OR 0.83, 95% CI 0.44–1.54, p = 0.55). MAP after pregnancy was not significantly different than before pregnancy (93 mmHg versus 89 mmHg, p = 0.09). Longer time between LKD and first delivery was associated with better eGFR (B 0.96, 95% CI 0.66–1.26, p < 0.001).

Risk for Hypertension or Cardiovascular Events After Pregnancy in LKD

Long-term outcomes after LKD are demonstrated in **Table 2**. During our median follow-up time of 12 years after pregnancy, 14% of the women with only pre-LKD pregnancies used anti-hypertensive drugs versus 9% of the women with only post -KD pregnancies. Seven women were affected by a cardiovascular event (CVE) during follow-up. Six of these women had only pre-LKD pregnancies of whom one had a pre-LKD pregnancy with preeclampsia. She had a myocardial infarction 19 years after LKD. The seventh woman was nulliparous. Only two women developed diabetes mellitus type 2, one with only pre-LKD pregnancies and one was nulliparous. Due to the low incidence of these endpoints, we did not perform further statistical analysis.

Pregnancy Outcomes Before LKD Versus Pregnancy Outcomes After LKD

Pregnancy outcomes of pre-LKD pregnancies and post-LKD pregnancies are illustrated in **Table 3**. Miscarriages occurred in n = 23/84 (27%) in post-LKD pregnancies versus vs. 56/413 (14%) in pre-LKD pregnancies. Median year of miscarriage was 1996 (IQR 19) for pre-LKD miscarriages and 2012 (IQR 8) for post-LKD miscarriages. Univariable analysis demonstrated a higher risk for miscarriage in post-LKD pregnancies (OR 2.142, 95% CI 1.12–4.100, p = 0.021). Multivariable analysis adjusted for age and multiple pregnancies per women, showed no significant higher risk of miscarriages in post-LKD pregnancies versus pre-LKD pregnancies (OR 1.19, 95% CI 0.04–40.46, p = 0.92).

Further analysis was performed only in women with pregnancies >20 weeks (n = 186 women, n = 402 pregnancies). Two women had an IUFD: one before LKD (at 33 weeks of pregnancy, unknown cause) and one after LKD (at 26 weeks of pregnancy, probably caused by a placenta infarction). According to the self-reported questionnaires 30/413 (7%) of the pregnancies pre-LKD were complicated by HDP and post-LKD 18/86 (21%) of the pregnancies were complicated by HDP. After studying their medical files, this alleged HDP could be confirmed in 15 pregnancies post-LKD: 7 with preeclampsia and 8 with gestational hypertension. Four pregnancies with preeclampsia had preterm birth and low birthweight. An overview of these women are shown in **Supplementary Appendix SA**.

In **Table 4** we show the results of univariable and multivariable analyses on the risk of the composite outcome HDP, adjusted for multiple pregnancies in one woman. Post-LKD pregnancies had a significant higher risk of HDP, as well as higher MAP before LKD. In univariable and multivariable analysis, the risk of gestational hypertension was not higher in post-LKD pregnancies compared to pre-LKD pregnancies (OR 1.69, 95% CI 0.56–5.08, p = 0.35). However, post-LKD pregnancies did have a significantly higher risk of preeclampsia (OR 14.77, 95% CI 3.07–70.99, p = 0.001). Multivariable analysis identified that women with higher BMI at

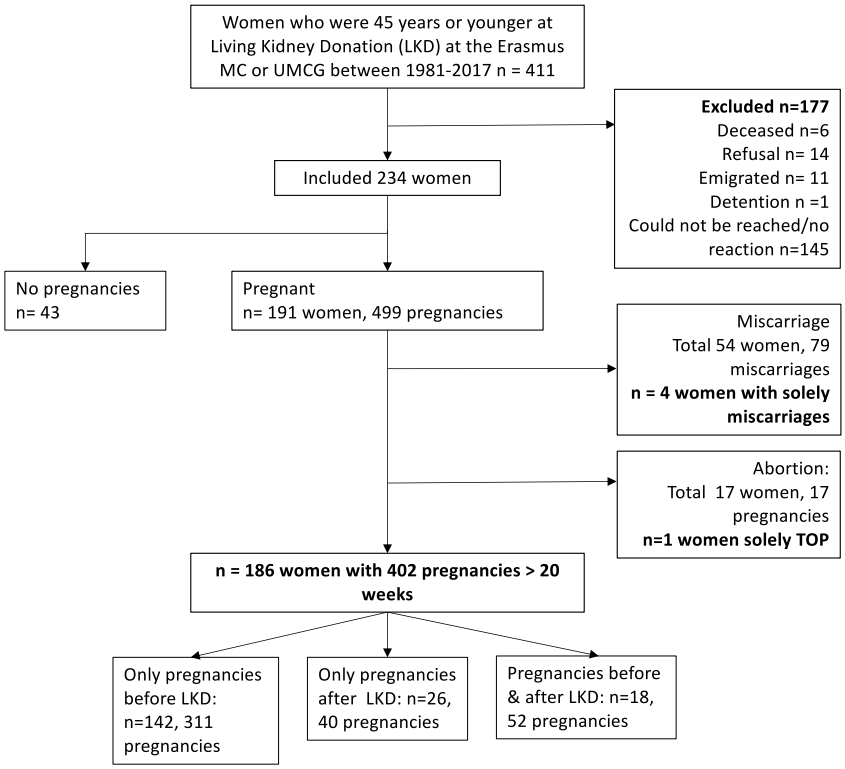


FIGURE 1 | Flowchart.

LKD (OR 1.26, 95% CI 1.08–1.46, $p = 0.003$) and lower parity (OR 0.38, 95% CI 0.18–0.76, $p = 0.007$) had a significantly higher risk of preeclampsia. All univariable analysis are presented in **Supplementary Appendix SB**.

Multilevel univariable analysis on adverse pregnancy outcomes such as birthweight <2,500 g and preterm delivery were not associated with post-LKD pregnancies ($p = 0.58$ and $p = 0.43$) (**Supplementary Appendix SC**). In multilevel multivariable analysis preterm birth was associated with preeclampsia (OR 5.24, 95% CI 1.55–17.70, $p = 0.008$). Birthweight <2,500 g was associated with HDP during pregnancy (OR 4.875, 95% CI 1.607–14.790, $p = 0.005$). Absolute birthweight was significantly lower in pregnancies after LKD ($p = 0.014$, **Supplementary Appendix SC**).

DISCUSSION

To the best of our knowledge, this is the first study focusing on the effect of pregnancy on long-term kidney function and eGFR slope in pregnancies after LKD. Our main finding is that the eGFR slope after LKD is not different in women with or without pregnancy after LKD. Moreover, no difference in mean eGFR before and after post-LKD pregnancy was observed. Our second finding is that post-LKD pregnancies were more often complicated by HDP. However, no differences in adverse fetal outcomes were found when comparing pre- and post-LKD pregnancies. At last, we found that higher BMI and higher blood pressure at LKD were associated with adverse fetal and maternal outcomes during post-LKD pregnancy.

Comparison With the Literature

We demonstrated for the first time that post-LKD pregnancy does not lead to a change in mean eGFR. Interestingly, we did find a small significant difference in eGFR slope before and after post-LKD pregnancy. eGFR decreased with −0.19 mL/min/1.73 m^2 per year before pregnancy, and after pregnancy −0.23 mL/min/1.73 m^2 per year ($p < 0.001$). This might be explained by the fact that eGFR slowly rises the first 4–5 years after LKD, and the median time between LKD and first delivery was 5 years (IQR 4). This phenomenon was recently described in two large prospective studies [17, 18]. In line with the aforementioned findings, we also found that longer time between LKD and first delivery was associated with better eGFR ($p < 0.001$).

Furthermore, none of the women with post-LKD pregnancies developed a CVE. No difference was found in the in incidence of hypertension in the group with only pre-LKD pregnancies compared to the group with only post-LKD pregnancies. It is known that women with a history of preeclampsia are more at risk for hypertension and CVEs later in life [19, 20]. Recent studies demonstrated that the risk of CVE is significantly lower after gestational hypertension and late onset preeclampsia compared to early onset preeclampsia [21, 22]. Most of the preeclampsia in our post-LKD pregnancies were late onset,

TABLE 1 | Baseline characteristics and pregnancy outcomes per woman after living kidney donation (LKD), *n* = 234 women, 499 pregnancies.

	All	Only pregnancies before LKD	Only pregnancies after LKD	Pre- and post-donation pregnancies	Women after LKD with no pregnancies
Number of donors	234	145	26	20	43
Number of pregnancies	499	372	59	68	N/A
Miscarriage, number (%)	79 (16)	48 (13)	19 (32)	12 (18)	N/A
Abortion, number (%)	17 (3)	11 (<1)	1 (2)	5 (24)	N/A
Pregnancies > 20 weeks					
Number of donors	229	142	26	18	43
Number of pregnancies	402	311	40*	52	N/A
Primipara, number (%)	402	142 (46)	26 (65)	18 (100)	N/A
Total pregnancies per donor, number (%)					
1	48 (26)	33 (23)	15 (58)	0	N/A
2	81 (44)	65 (46)	9 (35)	7 (39)	N/A
>3	57 (31)	44 (31)	2 (8)	11 (61)	N/A
IUFD number (%)	2 (<1)	1 (<1)	1 (2)	0	N/A
Race- White/Caucasian, number (%)	199 (85)	124 (87)	22 (85)	12 (67)	37 (86)
Age at LKD (years), mean (±SD)	37 (9)	40 (4)	30 (5)	31 (4)	37 (7)
Age at first delivery (years), mean (±SD)	26 (5)	25 (5)	33 (5)	24 (4)	N/A
BMI at LKD (kg/m^2), median {IQR}	25 (l6)	26 (6)	24 (5)	25 (5)	26 (6)
MAP at LKD (mmHg), median {IQR}	90 (11)	90 (13)	91 (8)	89 (11)	91 (9)
Hypertensive drug use at LKD, number (%)	3 (1)	3 (1)	0	0	0
Creatinine at LKD (umol/L), mean (±SD)	61 (10)	68 (14)	68 (13)	60 (18)	66 (11)
eGFR at LKD (ml/min/1.73 m^2), mean (±SD)	101 (16)	98 (15)	107 (15)	114 (12)	102 (17)

*Miscarriage: spontaneous loss of a pregnancy <20 weeks. Abortion: the deliberate termination of pregnancy <20 weeks. IUFD: intra uterine fetal demise >20 weeks of pregnancy, BMI: Body Mass Index, MAP: Mean Arterial Pressure. eGFR (estimated glomerular filtration rate) calculated using the CKD-epi formula. Hypertension defined as systolic blood pressure ≥140 mmHg and or a diastolic blood pressure ≥90 mmHg and or the use of antihypertensive medication. Proteinuria defined as urine protein/creatinine ratio >15 g/mol creatinine, or urine albumin/creatinine ratio >3.5 mg/mmol or when no ratio could be calculated urine proteinuria >0.15 g/L. When none of these quantitative urine measurements were available a positive urine dipstick was defined as proteinuria. *1 woman had no pregnancies >20 weeks before LKD only an abortion. So, when dividing the group of women with pregnancies >20 weeks she moved to the group of only pregnancies after LKD.*

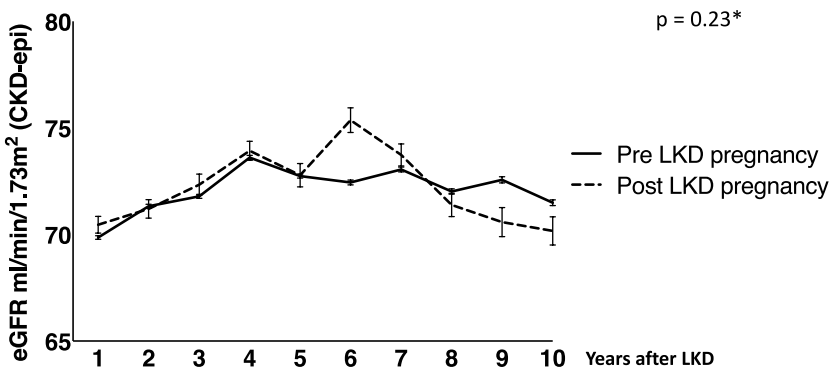


FIGURE 2 | Adjusted mean eGFR after living kidney donation (LKD); women who were pregnant before LKD or nulliparous versus women who got pregnant after LKD (GEE multilevel analysis).

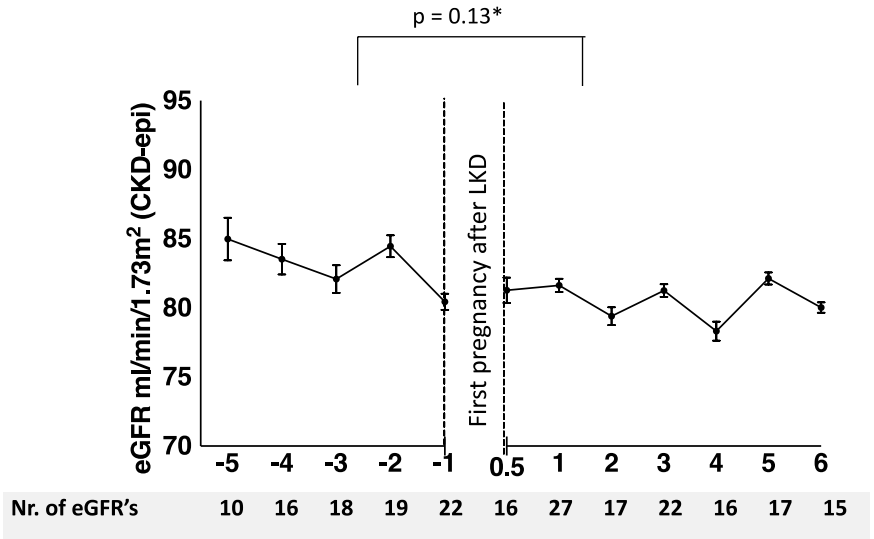


FIGURE 3 | Adjusted mean eGFR before and after first pregnancy after living kidney donation (GEE multilevel analysis).

TABLE 2 | Characteristics during follow-up after Living kidney donation (LKD), *n* = 221, 2,149 visits.

	All	Only pregnant before LKD	Only pregnant after LKD	Pregnant before and after LKD	Woman after LKD with no pregnancies
Number of donors	221	137	23	18	43
Follow-up time after LKD (years) median {IQR}	12 (9)	12 (9)	15 (11)	10 (8)	10 (9)
Follow-up time after first pregnancy (years) median {IQR}	23 (12)	25 (12)	12 (12)	18 (11)	N/A
Nr. of eGFR measurements/donor median {IQR}	13 (10)	13 (11)	16 (7)	11 (7)	13 (11)
eGFR during follow-up (ml/min/1.73 m^2) mean (±SD)	71 (12)	69 (12)	77 (10)	79 (11)	70 (15)
Hypertension during follow-up number (%)	122/ 221 (5)	77/137 (55)	13/23 (57)	7/18 (39)	25/43 (58)
Antihypertensive medication during follow-up number (%),	31/ 221 (14)	19/137 (14)	2/23 (9)	3/18 (17)	7/43 (16)
Proteinuria during follow-up* number (%)	135/ 221 (58)	87/137 (60)	13/23 (50)	8/18 (47)	27/43 (57)
Protein/creatinine ratio, mean (g/mol creatinine)** mean (±SD)	14 (10)	14 (9)	17 (18)	10 (4)	13 (10)
Cardiovascular events during follow-up number (%)	7/221 (3)	6/139 (4)	0	0	1/43 (2)

*Hypertension was defined as systolic bloodpressure ≥140 mmHg and/or diastolic bloodpressure ≥90 mmHg and/or the use of anti-hypertensive drugs. eGFR (estimated glomerular filtration rate) was calculated using the CKD-epi formula. BMI: Body Mass Index, MAP: Mean Arterial Pressure Hypertension was defined as systolic blood pressure ≥140 mmHg and or a diastolic blood pressure ≥90 mmHg and or the use of antihypertensive medication. Proteinuria was defined as urine protein/creatinine ratio >15 g/mol creatinine, or urine albumin/ creatinine ratio >3.5 mg/mmol or when no ratio could be calculated urine proteinuria >0.15 g/L. When none of these quantitative urine measurements were available a positive urine dipstick was defined as proteinuria. *17% missing data ** 66% missing data.*

mild preeclampsia and none of the women who were pregnant after LKD had a CVE. Of note is that these women had a rather short follow-up time (median 11 years after pregnancy) and in literature CVEs occur at longer periods of time after preeclampsia [23]. Women with HDP before LKD had a longer follow-up time (median 22 years) after the first pregnancy. They did not experience more CVEs or hypertension after LKD compared to women who did not experience HDP. We hypothesize that these women also had mild HDP as they still were able to pass the LKD screening and donated their kidney. In an earlier study in the general population, a similar non-negative effect of HDP was shown on kidney function after pregnancy [24].

In line with earlier literature, we also found a higher risk of HDP in post-LKD pregnancies [10]. It is important to discuss

TABLE 3 | Pregnancy outcomes before and after living kidney donation (LKD).

	Before LKD	After LKD
Number of donors with pregnancies	146	45
Number of pregnancies	413	86
Miscarriages, number (%)	56/413 (14)	23/86 (27)
Pregnancy duration at miscarriage (weeks)* median {IQR}	10 (6)	7 (4)
Abortion, number (%)	15 (4)	2 (2)
Pregnancies > 20 weeks		
Number of donors with pregnancies > 20 weeks	142	44
Number of pregnancies > 20 weeks	342	61
Primipara, number (%)	160/342 (47)	26/61 (43)
Follow-up time after first pregnancy (years), median {IQR}	24 (11)	12 (12)
Time between delivery and LKD (years), median {IQR}	−13 (10)	5 (4)
IUFD, number (%)	1 (<1)	1 (<1)
Pregnancy duration (weeks)**, mean (±SD)	39 (2)	39 (3)
Preterm birth with gestation of < 37 weeks, number (%)	31/321 (10)	8/61 (13)
Birthweight (Gram)***, mean (±SD)	3,493 (698)	3,254 (700)
Birthweight < 2,500 G, number (%)	16/324 (5)	3/59 (5)
Gestational hypertension, number (%)	22/341 (6)	8/61 (13)
Preeclampsia, number (%)	6/341 (2)	7/61 (11)
HELLP, number (%)	2/341 (<1)	0

*IUFD: intra uterine fetal demise after 20 weeks of pregnancy, gestational hypertension: ≥140/90 mmHg measured twice after 20 weeks of pregnancy, Preeclampsia: presence of pregnancy induced hypertension >20 weeks of gestation and proteinuria. HELLP: Haemolysis Elevated Liver enzymes and Low Platelets Syndrome. 5 women were excluded of this analysis because of having only miscarriages or abortions. 1 woman with pre LKD abortion and post LKD miscarriage, 1 woman with only miscarriages post LKD and 3 women with only miscarriages before LKD. * 9/56 (16%) pre LKD miscarriages pregnancy duration missing, **21/342 (6%) pre LKD pregnancy duration missing, ***18/342 (5%) pre LKD pregnancies birthweight missing, 2/61 (3%) post LKD pregnancies birthweight missing.*

TABLE 4 | Multilevel risk of hypertensive disorders of pregnancy (HDP) (GEE multilevel logistic regression analysis) *n* = 186 women, 499 pregnancies.

	Univariable analysis			Multivariable analysis		
	Odds ratio	95% Confidence interval	*p*-value	Odds ratio	95% Confidence interval	*p*-value
Afro-American race	0.945	0.335–2.666	0.916			
Pregnancy after LKD	3.053	1.283–7.267	**0.012**	4.192	1.698–10.351	**0.002**
Gravida number	0.819	0.573–1.171	0.273			
Number of pregnancies> 20 weeks	0.595	0.385–0.922	**0.020**	0.539	0.344–0.845	**0.007**
BMI before LKD (kg/m^2)	0.984	0.755–1.284	0.907			
MAP before LKD (mmHg)	1.068	1.011–1.128	**0.019**	1.066	1.032–1.102	< **0.001**
Age at LKD (years)	0.919	0.777–1.087	0.324			
Year of LKD	1.084	0.956–1.184	0.258			
Age at delivery	0.998	0.931–1.071	0.959			
Year of delivery	1.030	0.928–1.145	0.589			

GEE: Generalized estimating equations, exchangeable matrix, Adjusted for multiple pregnancies in one woman. In bold the variables considered significant. Parity: the number of the pregnancy (>20 weeks), Gestational hypertension: ≥140/90 mmHg measured twice after 20 weeks of pregnancy. Hypertensive disorders of pregnancy: gestational hypertension, preeclampsia and/or HELLP, BMI: Body Mass Index, MAP: Mean arterial Pressure.

risks of post-LKD pregnancy with women considering pregnancy before LKD. We report an absolute risk for mild near-term preeclampsia of approximately 7% (versus 2%–4% in the general population). More-over we found a median of 200 g lower birth weight in post-LKD pregnancies (absolute, not corrected for confounders), but no increased risk for birth weight <2,500 g. Comparing outcomes of studies remains difficult, especially since studies use different definitions for gestational hypertension and preeclampsia. This higher risk of HDP in pregnancies post-LKD can also be explained by the higher age of the mothers who were pregnant after LKD [22]. Although in our study pregnancies post-LKD were more often complicated by HDP, in line with previous studies no differences were found in adverse fetal outcomes [10]. Furthermore, no difference was observed in eGFR after LKD with or without HDP, in line with an earlier study in the general population [24].

Higher BMI and blood pressure at LKD were associated with adverse pregnancy outcomes. As was shown earlier, women with a high BMI have smaller rest capacity after LKD and therefore are at higher risk for hypertension and HDP [25]. In studies in the general LKD population, hypertensive donors had no increased risk for reduced eGFR, proteinuria or ESRD compared to donors without hypertension [26]. However, whether obese donors have an increased risk of CVE or ESRD in comparison to the general LKD population has not fully been elucidated yet [27–29]. More research is warranted for counseling overweight women with a future pregnancy wish who want to donate their kidney.

Strengths and Limitations

To date this is the first study that compared eGFR slopes after LKD in women with and without post—LKD pregnancies, and the first study that compared eGFR slopes before and after post-LKD pregnancy. Besides, we provide long-term follow-up data of pregnancies post-LKD up to 12 years after LKD, which has not been reported before. A few limitations should be taken into account, mainly due to the retrospective design of the study. There was some missing data and data on pregnancies was mainly self-reported, except for the complicated pregnancies after LKD. Furthermore, only a small group of women become pregnant after LKD. Due to these small numbers a prospective study on the effect of pregnancy on eGFR after LKD was not feasible. Therefore, the analysis to determine the impact of HDP on GFR decline and long-term CVE is probably not powered enough to detect small risks, but it does show that there is no large effect. There was a significant difference in age at LKD, eGFR before LKD, age at delivery, follow-up after delivery between the two groups, due to the study design. We adjusted for these differences in the model.

Conclusion and Impact for Counseling

We conclude that pregnancy post-LKD does not affect kidney function of the mono-kidney at long-term follow-up, even when HDP occur. In line with existing literature, we report a higher risk of pregnancy complications, but this does not lead to an increase in adverse fetal outcomes. Therefore, our counseling advice is in line with British guidelines and KDIGO recommendations [30, 31]. We conclude that a pregnancy wish alone should not be a reason to exclude women for LKD. However, for women with high BMI or hypertension at the time of screening, a future pregnancy wish might be a reason to postpone or refrain from LKD.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethical Board of the Erasmus Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MB, Participated in research design and performance, data analysis and interpretation, and writing of the article. JM, Participated in data analysis and interpretation, and writing of the article. CO, Participated in research design, data analysis and interpretation. MJ, Participated in data interpretation and review of the article. HG, Participated in data interpretation, statistical analysis and review of the article. TR, Participated in collecting data and review of the article. LM, Participated in collecting data and review of the article. MT, Participated in collecting data and review of the article. MR, Participated in review of the article. AL, Participated in research design and performance, data interpretation, and writing of the article. JW, Participated in research design and performance, data interpretation, and writing of the article. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11181/full#supplementary-material

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Protective Effect of Vaccine Doses and Antibody Titers Against SARS-CoV-2 Infection in Kidney Transplant Recipients

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Patients undergoing kidney transplantation have a poor response to vaccination and a higher risk of disease progression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The effectiveness of vaccine doses and antibody titer tests against the mutant variant in these patients remains unclear. We retrospectively analyzed the risk of SARS-CoV-2 infection in a single medical center according to vaccine doses and immune responses before the outbreak. Among 622 kidney transplant patients, there were 77 patients without vaccination, 26 with one dose, 74 with two doses, 357 with three, and 88 with four doses. The vaccination status and infection rate proportion were similar to the general population. Patients undergoing more than three vaccinations had a lower risk of infection (odds ratio = 0.6527, 95% CI = 0.4324–0.9937) and hospitalization (odds ratio = 0.3161, 95% CI = 0.1311–0.7464). Antibody and cellular responses were measured in 181 patients after vaccination. Anti-spike protein antibody titer of more than 1,689.3 BAU/mL is protective against SARS-CoV-2 infection (odds ratio = 0.4136, 95% CI = 0.1800–0.9043). A cellular response by interferon-γ release assay was not correlated with the disease (odds ratio = 1.001, 95% CI = 0.9995–1.002). In conclusion, despite mutant strain, more than three doses of the first-generation vaccine and high antibody titers provided better protection against the omicron variant for a kidney transplant recipient.

Keywords: kidney transplant, antibody titer, severe acute respiratory syndrome coronavirus 2, vaccine, interferon-γ

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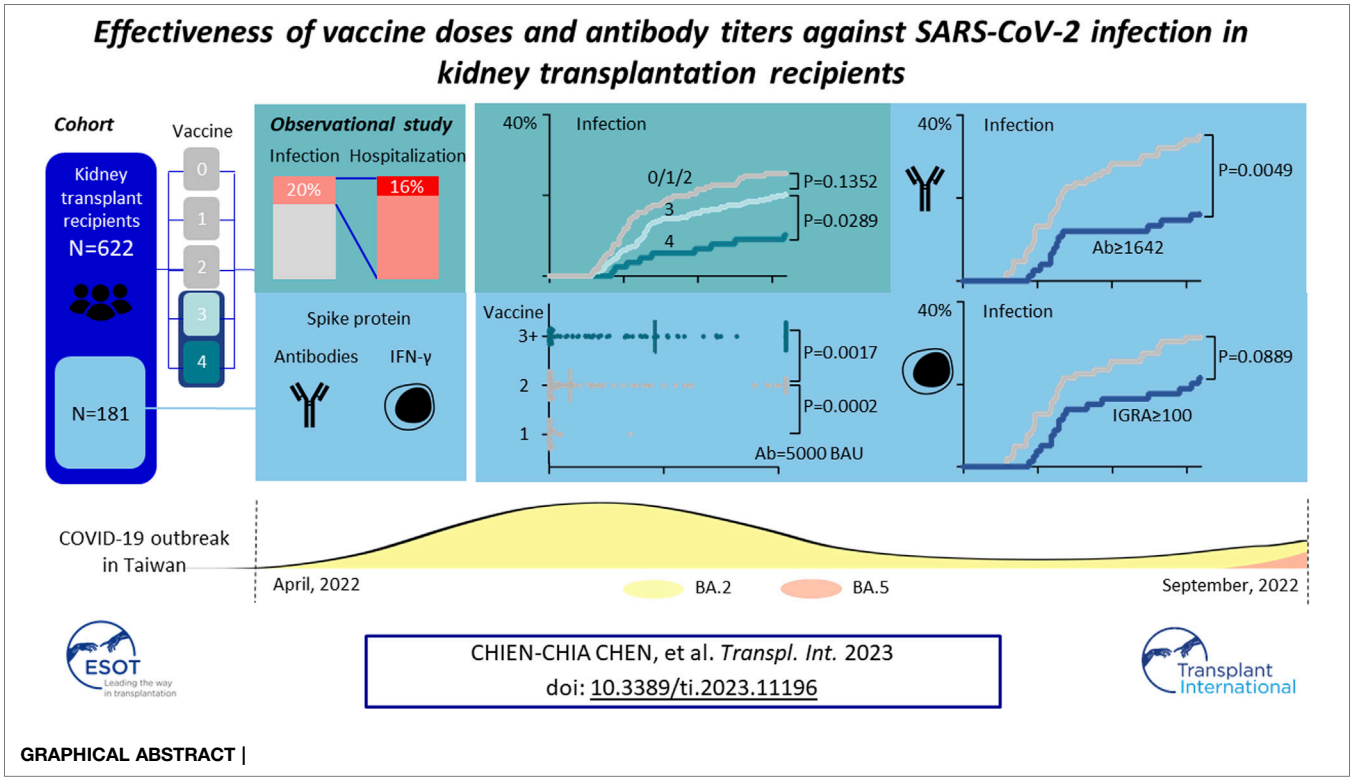
Abbreviations: BAU, binding antibody unit; COVID-19, coronavirus disease 2019; IGRA, interferon-γ release assay; IFN-γ, interferon-γ; KTR, kidney transplant recipient; MMF, mycophenolate mofetil; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SMR, Standardized mortality ratio; SOTR, Solid organ transplant recipient.



GRAPHICAL ABSTRACT |

INTRODUCTION

Despite multiple doses, patients have a poor response to vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) after solid organ transplantation [1–4] compared to the response in immunocompetent population. Moreover, the disease severity is greater in this population. Higher hospitalization and more severe complication rates with significant mortality were reported since the coronavirus disease 2019 (COVID-19) pandemic in early 2020 [5, 6].

Since January 2022, the SARS-CoV-2 Omicron variant has overtaken previous variants and dominated the pandemic. Multiple mutations in the spike protein rendered the Omicron variant a higher affinity for angiotensin-converting enzyme 2 receptor and a lower ability to use the serine protease TMPRSS2 [7, 8]. Compared with the delta variant, these changes made the Omicron variant more transmissible but reduced its severity and risk of mortality [9].

Nevertheless, for Solid organ transplant recipients (SOTR) with an immunodeficient status, there was still a higher risk of hospitalization and mortality than in the general population [10, 11]. Although vaccination against SARS-CoV-2 with repeated boosters is recommended for SOTR, there is a concern for the decreased effect of the first generation of COVID-19 vaccines against the Omicron strain [12]. A higher titer of anti-spike protein antibody is needed to achieve the protection [13], which is usually not fulfilled in SOTR. A cohort study in Canada reported improved effectiveness by the third dose in SOTR [14], but was still lesser than in the general population. Hence, is it necessary to receive a fourth dose or more vaccines in SOTR? Measuring the antibody titer for SOTR may help [15], but there is no consensus on this issue yet [16].

In Taiwan, before the late epidemic outbreak of Omicron variant BA.2 from April to August 2022, there were only scanty COVID-19 cases, and majority of the population received multiple doses of vaccination [17]. We conducted this retrospective study in kidney transplant recipients (KTR) to evaluate the effectiveness of the first-generation vaccine against the Omicron variant. Besides, some patients underwent measurement of antibody and cellular response after vaccination. The relationship between infection risk and laboratory results was also explored.

MATERIALS AND METHODS

This prospective observational study was approved by the Research Ethics Committee of the National Taiwan University Hospital (NTUH) (202106046RINA).

Patients

Taiwan, an island country located in the west Pacific Ocean, with a population of about 24 million, which makes the assessments of immigration and infectious disease control easy. Since late January 2022, strict epidemic prevention policies have been established, including border quarantine for 14 days with

polymerase chain reaction (PCR) tests, mandatory wearing of face masks in public areas, and forbidden large crowd gathering. Confirmed COVID-19 case number was reported daily by the Taiwan Centers for Disease Control (https://www.cdc.gov.tw/). All COVID-19 information was well documented and published by the government.

In Taiwan, SARS-CoV-2 vaccine has been available since June 2021. Some of the KTRs without COVID-19 history at the National Taiwan University Hospital (NTUH) were recruited in July 2021 for an observational vaccination effect study. After obtaining informed consent, blood samples were collected before (if available) and about 28 days after the first dose and 28, 90, and 180 days after the second dose. T and B cell responses after vaccination were analyzed as previously reported [4], which are briefly described in the next paragraph.

All KTRs over 18 years old undergoing regular follow-ups at NTUH outpatient clinic of the surgery department from April to August 2022, without confirmed COVID-19 before April 2022, were recruited in this retrospective study. Of these patients, in those with evidence of vaccination effect, vaccination dosage, clinical data, patient demographic profile, immunosuppressant usage, graft function, comorbidities, T and B cell responses (when available), and COVID-19 status were reviewed.

Quantification of Immune Response After Vaccination

Spike protein-specific T cell response was determined by a SARS-CoV-2 interferon (IFN)-γ release assay (IGRA) kit (Quan-T-Cell SARS-CoV-2, Euroimmun Medizinische Labordiagnostica, Luebeck, Germany). The value of IGRA was considered a positive response if IFN-γ concentration was >100 (mIU/mL), according to the manufacturer's instructions.

B cell response was determined by antibody concentration using an electrochemiluminescence immunoassay kit for spike and nucleocapsid protein (Elecsys Anti- SARS-CoV-2 S and Elecsys Anti- SARS-CoV-2, Roche) using a Cobas 411 analyzer. A value ≥ 0.8 U/mL was considered a positive response according to the manufacturer's instructions. The Elecsys unit (U/mL) for antibody titer can be transformed into a binding antibody unit (BAU/mL) determined by the WHO using equation U = 0.972 × BAU.

Data Analysis

Continuous variables are presented as the mean ± standard deviation for patients' clinical profiles and compared using ordinary one-way ANOVA in three groups or more. The variables included age, transplant duration, serum tacrolimus level, serum creatinine, mycophenolate mofetil (MMF), and daily steroid doses. Student's t-test was used for comparison of continuous variables and antibody and IGRA titers between two groups. Categorical variables, including sex, transplant type (cardaveric or living related transplantation), mTOR inhibitor usage, hypertension, diabetes mellitus, dyslipidemia, and hyperuricemia were analyzed using the chi-square test.

The standardized mortality ratio (SMR) was calculated for comparison between Taiwan's general population and KTRs. Age-and-sex specific COVID-19 rate for the general population was obtained from the website of the Taiwan government, including the Taiwan National Development Council and the Ministry of Health (https://covid-19.nchc.org.tw/) and Welfare (https://www.cdc.gov.tw/).

We compared the cumulative incidence of COVID-19 and hospitalization between different groups of KTRs, defined by different vaccine dosage or antibody and IGRA levels, using the Kaplan-Meier test. The correlation between Ab and IGRA titer was determined by simple linear regression. The risk factors for COVID-19 and hospitalization were determined by simple logistic regression and further by Cox proportional hazards regression analysis.

A two-tailed test with $p < 0.05$ was considered statistically significant between groups. Statistical analysis was performed using GraphPad Prism 9.3.1 (GraphPad Software, LLC, CA, United States).

RESULTS

Patient Demographic Data

During April 2022 to August 2022, 622 KTRs were regularly attending the surgery department of the NTUH. One hundred twenty-six were diagnosed with COVID-19 by a home antigen test or PCR examination in the hospital (**Figure 1**). About 14% (22/126) of infected patients were hospitalized for medical treatment, and there were two mortality cases. Compared to the general population in Taiwan, the infection rate was lower (Standardized mortality ratio 0.80, 95% CI 0.66–0.95, **Figure 2A**), but the mortality rate seemed higher (1.6% vs. 0.18% for the general population) [18]. According to the vaccine doses, 77, 26, 74, 357, and 88 patients were vaccinated before the outbreak of COVID-19 with 0, 1, 2, 3, and 4 doses, respectively, which resembled the general population (**Figure 2B**) [18]. Based on vaccine type and dosage, there were 48 combinations for all KTRs and 24 combinations for KTRs receiving Ab and IGRA test. Among the various combinations, the two most common combinations were three doses of mRNA1273 (Moderna) ($n = 128$, 20.6%) and three doses of BNT162b2 ($n = 51$, 8.2%) (**Supplementary Figures S1, S2**). According to the Taiwan Centers for Disease Control (https://www.cdc.gov.tw/), although variants of the SARS-CoV-2 virus were only sampling tested, during April 1st to June 10th, Omicron BA.2 was the dominant variant (96%) in Taiwan. No BA.4 or BA.5 variant was detected until August 15th. However, the proportion of BA.4 and BA.5 variants increased rapidly to 5% and 40% respectively at the end of August. The patient characteristics regarding sex, age, transplant types, and immunosuppressants were are listed in **Table 1** according to vaccine doses. For KTR with three doses, there were more male patients (53.8%), while KTR without vaccination had higher creatinine levels than of other groups.

Protection Effect According to Vaccine Doses

We retrospectively reviewed COVID-19 in KTR, caused mainly by Omicron BA.2, during the first wave of the outbreak from

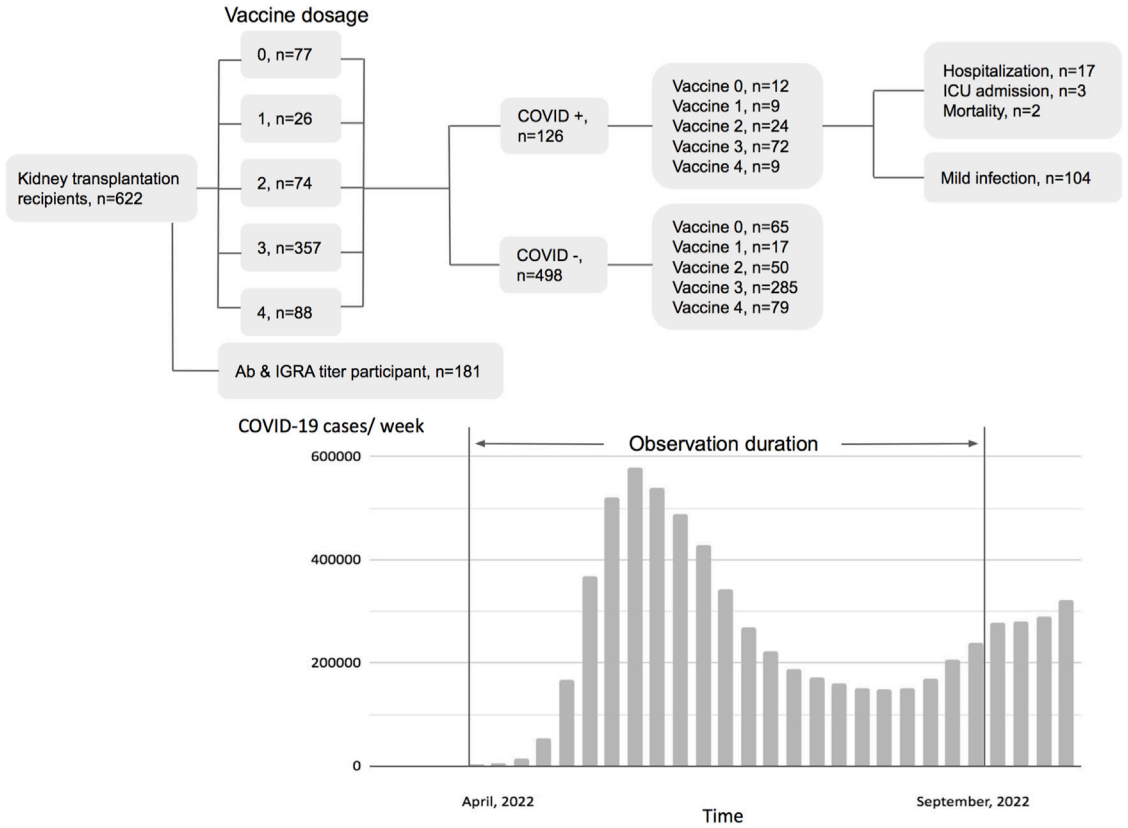


FIGURE 1 | Flow chart of patient distribution by vaccination status and pandemic status of the general population in Taiwan.

April to August 2022 [19, 20] (**Figure 1**), and compared the result with those of the general population according to the information published by Taiwan Centers for Disease Control. After the first wave, KTR with 4 doses had the lowest overall infection rate (10%) compared to 21/30/31% for vaccine doses 3/2/1, respectively (**Supplementary Figure S3A**). Meanwhile, for the risk analysis, KTR with 4 doses had significantly lower infection risk than those with other doses (**Figure 2C**, hazard ratio [HR] 0.48, 95% confidence interval [CI] 0.28–0.81, $p = 0.0289$ for dose 4 vs. dose 3; HR 0.36, 95% CI 0.21–0.63, $p = 0.0036$ for dose 4 vs. dose 0/1/2; HR 0.75, 95% CI 0.51–1.11, $p = 0.1352$ for dose 3 vs. dose 0/1/2). More than 3/4 of infected KTRs were isolated at home and had a smooth recovery. The number of hospitalized patients reduced in each group. We found that more than three doses of vaccine helped to reduce the overall hospitalization rate (**Supplementary Figure S3B**, **Figure 2D**, HR 0.32, 95% CI 0.13–0.82, $p = 0.0055$ for dose 3/4 vs. dose 0/1/2). Other conditions could confound the effect of vaccine dosage. We then performed Cox proportional hazards regression analysis, which showed that more than three doses (HR 0.59, 95% CI 0.40–0.88, $p = 0.0084$) and longer transplantation duration (HR 1.00, 95% CI 0.99–1.00, $p = 0.0101$) were the two protection factors (**Table 2**) for COVID infection. Besides, vaccination with more than three doses was the only protective factor against hospitalization (**Table 3**, HR 0.37, 95% CI 0.15–0.90, $p = 0.0269$).

Measurement of Immune Response

Among the 622 KTRs, there were 181 KTRs undergoing antibody and IFN-γ assay after each dose of vaccine. Patient characteristics are presented in **Table 4**, and 112 KTRs (61.88%) received more than three doses. For antibody measurement, both the positive detection rate and titer increased with the doses (**Figures 3A, B**). For the IFN-γ assay, there was an increasing response after the second dose, but the trend became non-significant after the third dose, both in positive rate and IFN-γ titers (**Figures 3A, C**). The correlation between antibody and IFN-γ titer was more robust in the first two doses than in the last two doses (**Figures 3D, E**).

Infection Risk According to Immune Responses by Vaccination

It has been reported that higher antibody titer provided better protection against SARS-CoV-2 [21]. We performed a receiver characteristics curve (ROC) analysis to determine a cut-off value of 1642 U/mL (1,689.3 BAU/mL) (**Figure 4A**; **Supplementary Table S2**), and KTRs with a titer above this level had a significantly lower risk for infection (HR 0.41, 95% CI 0.23–0.71, $p = 0.0049$, **Figure 4B**) but not hospitalization (HR 0.28, 95% CI 0.05–1.40, $p = 0.2083$, **Figure 4C**). This might be due to low incidence in both groups (1/75 for titer ≥1,689.3 vs. 5/106 for titer <1,689.3). In contrast, a positive IGRA test did not

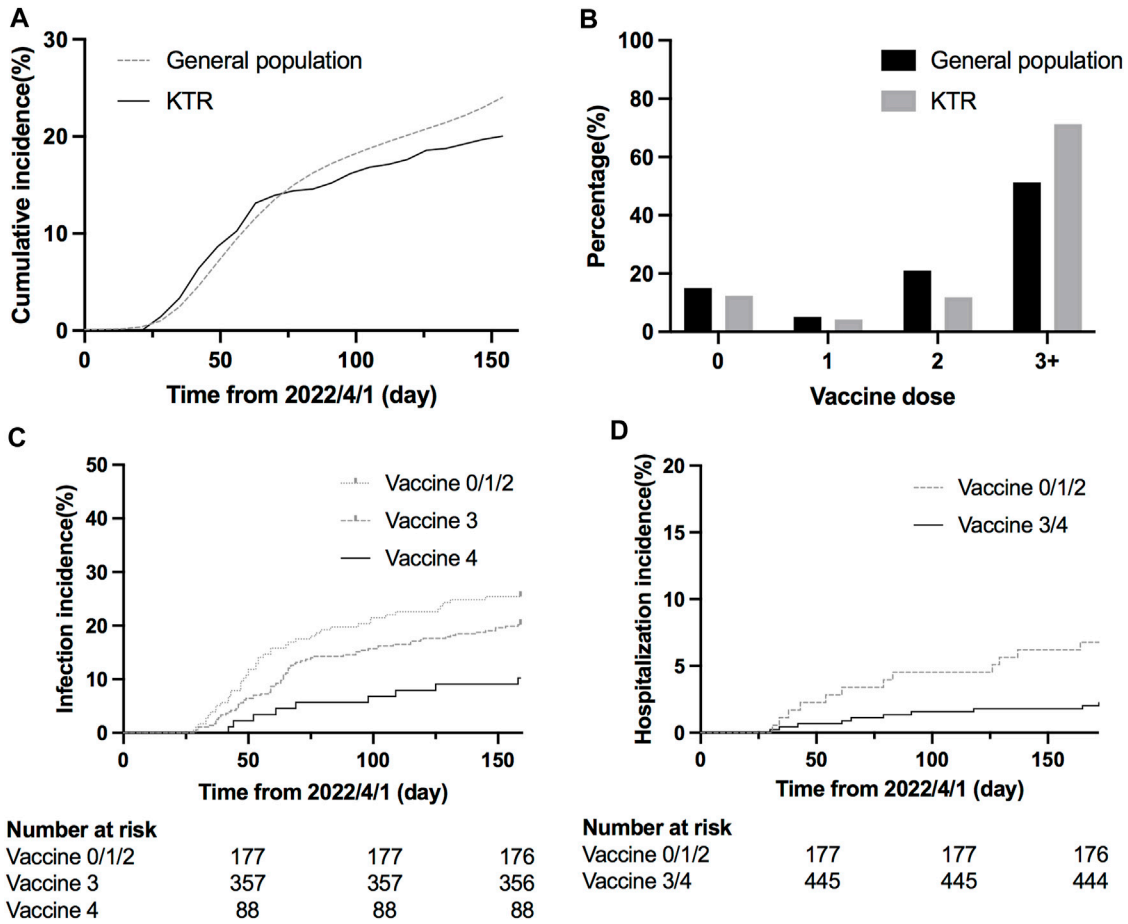


FIGURE 2 | (A) Cumulative COVID-19 incidence since the COVID-19 outbreak (SMR = 0.80, 95% CI 0.66–0.95). **(B)** Percentage of KTRs and general population according to different vaccination doses before the outbreak **(C)** Cumulative COVID-19 incidence according to different doses (hazard ratio 0.48, 95% CI 0.28–0.81, p = 0.0289 for dose 4 vs. dose 3; hazard ratio 0.36, 95% CI 0.21–0.63, p = 0.0036 for dose 4 vs. dose 0/1/2; p = 0.1352 for dose 3 vs. dose 0/1/2). **(D)** Cumulative COVID-19 hospitalization incidence according to different doses (hazard ratio 0.32, 95% CI 0.13–0.82, p = 0.0055 for dose 3/4 vs. dose 0/1/2).

TABLE 1 | Patient characteristics.

	All	Vaccine 0	Vaccine 1	Vaccine 2	Vaccine 3	Vaccine 4	*p*-value
	N = 622	*n* = 77	*n* = 26	*n* = 74	*n* = 357	*n* = 88	
Male (%)	48.7	44.2	38.5	39.2	53.8	42.0	0.0467
Age (years)	53.61 ± 13.69	56.21 ± 14.18	50.85 ± 17.00	53.38 ± 14.46	52.83 ± 13.37	55.43 ± 12.68	0.1608
Living related transplant (%)	51.1	46.8	50.0	51.4	53.2	46.6	0.7228
Transplant duration in months	137.02 ± 101.6	155.37 ± 83.22	136.35 ± 84.81	131.89 ± 87.88	133.30 ± 110.91	140.17 ± 92.25	0.5227
Serum creatinine (mg/dL)	1.47 ± 0.99	1.66 ± 1.24	1.48 ± 0.87	1.49 ± 1.20	1.43 ± 0.88	1.42 ± 1.06	0.0002
Tacrolimus level (ng/mL) *N* (%)	4.24 ± 1.88 (84.4%)	4.10 ± 1.63 (77.9%)	3.71 ± 1.98 (96.2%)	4.18 ± 2.10 (86.5%)	4.29 ± 1.85 (85.2%)	4.33 ± 2.00 (81.8%)	0.5876
mTOR inhibitor (%)	59.2	49.4	57.7	55.4	61.3	63.6	0.3271
MMF daily dose (g) *N* (%)	0.93 ± 0.39 (76.8%)	0.86 ± 0.46 (72.7%)	0.75 ± 0.31 (76.9%)	0.90 ± 0.40 (74.3%)	0.94 ± 0.38 (79.8%)	0.97 ± 0.38 (70.5%)	0.1368
Hypertension (%)	61.4	62.3	61.5	52.7	63.3	60.2	0.5597
Diabetes (%)	22.0	20.8	15.4	13.5	24.1	23.9	0.2958
Dyslipidemia (%)	45.7	41.6	34.6	52.7	46.2	44.3	0.4951
Hyperuricemia (%)	39.9	36.4	38.5	37.8	42.0	36.4	0.7941

MMF, mycophenolate mofetil.

TABLE 2 | Factors associated with COVID-19 (n = 622).

Variable	Univariate analysis			Cox regression		
	Odds ratio (OR)	OR 95% CI	p-value	Hazard ratio (HR)	HR 95% CI	p-value
Female	0.83	0.56–1.22	0.3368	0.90	0.61–1.32	0.5894
Age	1.00	0.98–1.01	0.6338			
Vaccine ≥3 doses	0.65	0.43–0.99	0.0441	0.59	0.40–0.88	0.0084
Transplant duration	1.00	0.99–1.00	0.0002	1.00	0.99–1.00	0.0101
Creatinine level	1.03	0.84–1.24	0.7569			
Tacrolimus	1.14	1.03–1.27	0.0111	1.07	0.95–1.18	0.2431
mTOR inhibitor use	0.74	0.50–1.09	0.1284	0.85	0.56–1.30	0.4538
MMF	1.07	0.73–1.55	0.7439			
Hypertension	1.28	0.85–1.94	0.2403	1.14	0.76–1.72	0.5335
Diabetes	1.41	0.89–2.20	0.1309	1.52	0.99–2.29	0.0504

CI, confidence interval; MMF, mycophenolate mofetil.

TABLE 3 | Factors associated with hospitalization due to COVID-19 (n = 622).

Variable	Univariate analysis			Cox regression		
	Odds ratio (OR)	OR 95% CI	p-value	Hazard ratio (HR)	HR 95% CI	p-value
Female	0.64	0.26–1.51	0.3177	0.63	0.25–1.54	0.3125
Age	1.00	0.97–1.03	0.7691			
Vaccine ≥3 doses	0.32	0.13–0.75	0.0085	0.37	0.15–0.90	0.0269
Transplant duration	1.00	1.00–1.00	0.9337			
Creatinine level	1.44	1.10–1.82	0.0034	1.23	0.91–1.56	0.1247
Tacrolimus	0.92	0.70–1.15	0.4987	0.89	0.67–1.13	0.3642
mTOR inhibitor use	0.47	0.19–1.10	0.0832	0.46	0.18–1.20	0.1154
MMF	1.19	0.53–2.73	0.6703			
Hypertension	2.93	1.08–10.24	0.0545	3.24	1.07–13.98	0.0632
Diabetes	1.05	0.34–2.70	0.9323			

CI, confidence interval; MMF, mycophenolate mofetil.

show a significant protective effect for infection (HR 0.62, 95% CI 0.23–0.71, p = 0.1123, **Figure 4D**). We further analyzed the infection risk for KTR with antibody titers lower than 1,689.3 BAU/mL (n = 106) to verify if an antibody masked the effect of the cellular response. Nevertheless, there was no difference between KTRs with and without positive IGRA results (**Figure 4E**). To identify the influence of confounding factors, we then performed Cox regression analysis for risk of infection (**Table 5**) or hospitalization (**Supplementary Table S1**). Antibody titer >1,689.3 BAU/mL was the only significant factor (HR 0.46, 95% CI 0.21–0.95, p = 0.0412) against infection but not with >3 doses of vaccine (HR 0.52, 95% CI 0.27–1.11, p = 0.0714).

DISCUSSION

Compared to previous studies conducted during the pandemic, this study demonstrated the effectiveness of multiple vaccine doses and antibody measurements before the outbreak of the Omicron variant, due to strict epidemic control in Taiwan. More than three doses of the vaccine provided significant protection for KTRs, and antibody titer of >1,689.3 BAU/mL may be a beneficial factor against SARS-CoV-2.

During the study period, vaccination was the only available method to prevent COVID-19, as monoclonal antibodies were not accessible at that time. Medications such as Ramdesivir, Paxlovid, and Monupiravir were only available for confirmed COVID-19 treatment in Taiwan. All the KTRs in this study received vaccines designed for the ancestral strain of SARS-CoV-2 as for the general population. Mean antibody titer against the spike protein increased with the sequential doses. Nevertheless, there was a tremendous interpatient variety. About 8% of KTRs still had no antibody response after more than three doses, which is different from that of the general population. Theoretically, KTRs should be more vulnerable to infection, but our result did not reveal this phenomenon, similar to the Danish report [10]. In Taiwan, home antigen tests and PCR tests in the hospital were officially recognized for SARS-CoV-2. Most people, including KTRs, had a test at home due to upper airway symptoms and then received medications by telemedicine from numerous local clinics and hospitals, a system established right after the outbreak. Under the same diagnostic criteria, for a short period, we believe that the infection rate reflected the real-world status. Hence, one of the possible explanations is that KTRs may take more protective measures, but still had similar results as others. Besides, multiple mutations in the spike protein resulted in antibody evasion and higher transmission ability by the Omicron variant [22, 23], which may further attenuate the different vaccine effects between KTR and the general population.

TABLE 4 | Patient characteristics of KTRs with measurement of immune responses after vaccination.

Variables	Vaccine 0,1,2 *n* = 69	Vaccine 3,4 *n* = 112	*p*-value
Male (%)	44.9	41.1	0.6444
Age (year)	52.22 ± 12.95	54.93 ± 11.83	0.1505
Living related transplant (%)	43.5	42.0	0.6399
Transplant duration in months	132.77 ± 102.94	112.61 ± 84.19	0.1529
Serum creatinine (mg/dL)	1.43 ± 1.04	1.22 ± 0.43	0.0612
Tacrolimus level (ng/mL)	4.43 ± 1.29	4.79 ± 1.64	0.1802
mTOR inhibitor (%)	49.3	57.1	0.3573
MMF daily dose (g)	0.68 ± 0.53	0.66 ± 0.46	0.8567
Steroid daily dose (mg)	3.75 ± 3.03	3.42 ± 2.32	0.4107
Hypertension (%)	63.8	66.1	0.7510
Diabetes (%)	20.3	22.3	0.8529
Dyslipidemia (%)	44.9	50.9	0.4485
Hyperuricemia (%)	46.4	36.6	0.2142

KTR, kidney transplant recipient; MMF, mycophenolate mofetil.

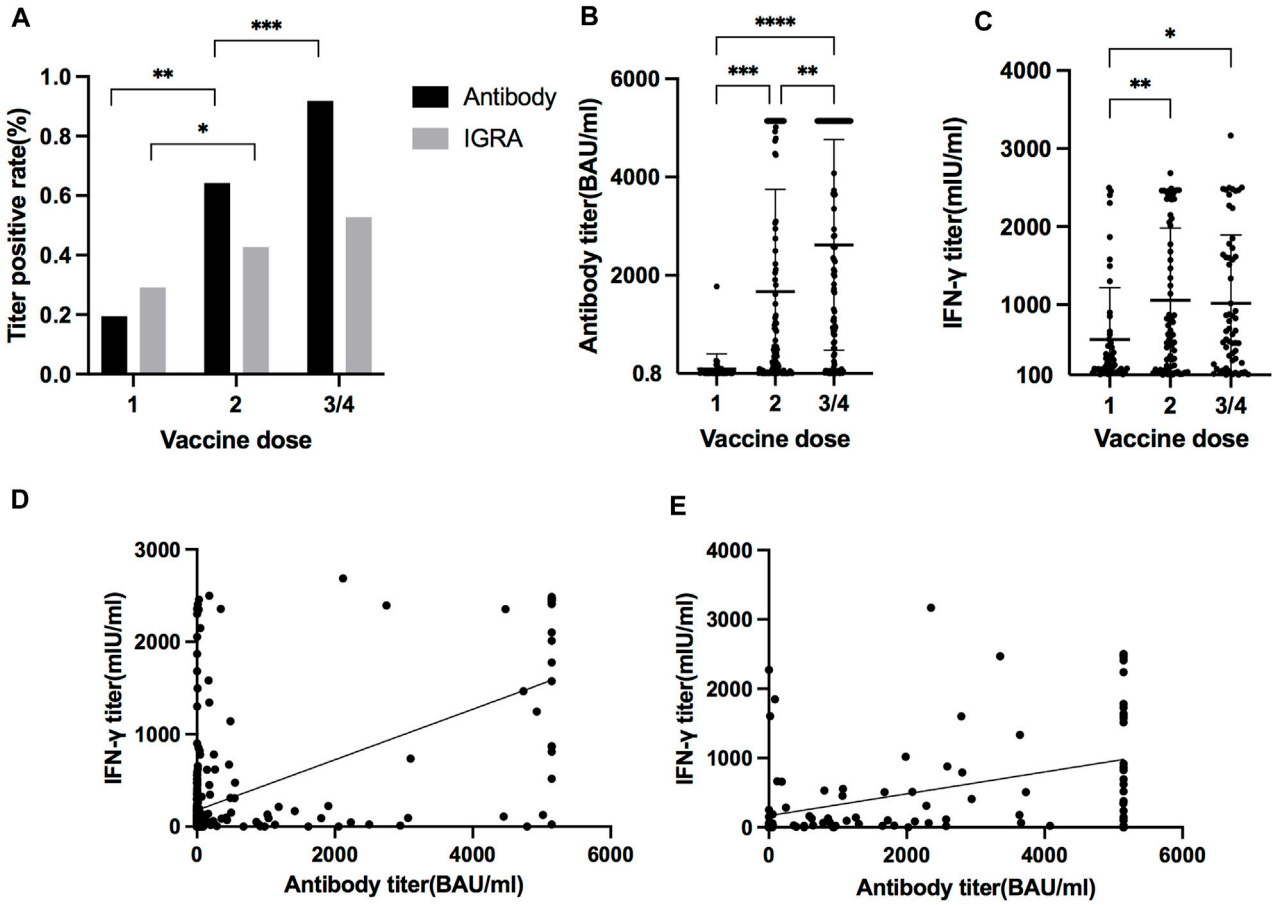


FIGURE 3 | (A) Positive antibody and IGRA measurements rate after different vaccination doses. (*p = 0.0124, **p < 0.0001, ***p < 0.0001) **(B)** Antibody titer after different dosages of vaccination. (**p = 0.0017, ***p = 0.0002, ****p < 0.0001) **(C)** IFN-γ titer after different dosages of vaccination. (*p = 0.0113, **p = 0.0035) **(D)** Correlation between antibody titer and IFN-γ titer after first and second dose of vaccines (slope 0.27, 95% CI 0.23–0.32, r^2 0.33, p < 0.0001). **(E)** Correlation between antibody titer and IFN-γ titer after third and fourth dose of vaccines (slope 0.16, 95% CI 0.096–0.22, r^2 0.18, p < 0.0001).

Meanwhile, more vaccine doses still showed protection effects in this study. There was a lower risk for infection and hospitalization for KTRs with three or more doses, especially four. Regarding mortality, there were only two cases in our cohort, making it difficult to draw a conclusion. Of the two patients who died, one had received two doses of vaccination, and the other had received three doses. Neither of them had antibody measurements, so vaccine effectiveness could not be confirmed.

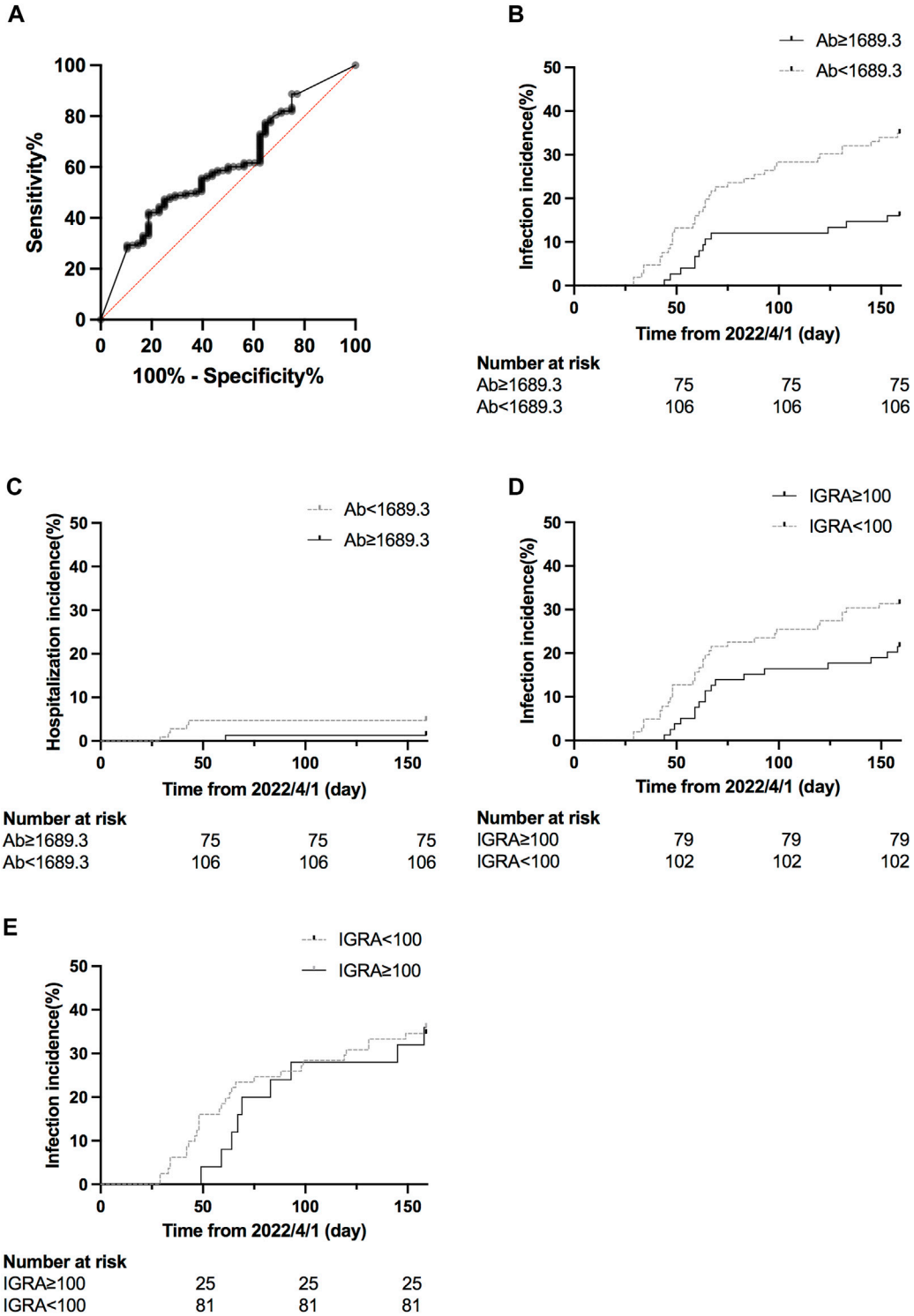


FIGURE 4 | (A) ROC curve of infection status and antibody titer. The area under the curve was 0.61, 95% CI 0.52–0.61, $p = 0.0271$. **(B)** Cumulative COVID-19 incidence for antibody titer ≥1,689.3 BAU/mL vs. antibody titer <1,689.3 BAU/mL (hazard ratio 0.41, 95% CI 0.23–0.71, $p = 0.0049$). **(C)** Cumulative hospitalization incidence for antibody titer ≥1,689.3 BAU/mL vs antibody titer <1,689.3 BAU/mL (hazard ratio 0.28, 95% CI 0.05–1.40, $p = 0.2083$). **(D)** Cumulative COVID-19 incidence for IFN-γ titer ≥100 U/mL vs < 100 U/mL (hazard ratio 0.62, 95% CI 0.23–0.71, $p = 0.1123$). **(E)** Cumulative COVID-19 incidence for IFN-γ titer ≥100 U/mL vs <100 U/mL for patients with antibody titer <1,689.3 BAU/mL (hazard ratio 0.96, 95% CI 0.46–2.03, $p = 0.9249$).

TABLE 5 | Factors associated with COVID-19 for patients with measurement of immune response after vaccination (n = 181).

Variable	Univariate analysis			Cox regression		
	Odds ratio (OR)	OR 95% CI	*p*-value	Hazard ratio (HR)	HR 95% CI	*p*-value
Female	0.98	0.51–1.92	0.9583			
Age	0.98	0.95–1.01	0.117	1.00	0.97–1.02	0.8655
Vaccine ≥3 doses	0.35	0.14–0.86	0.0200	0.52	0.27–1.11	0.0714
Transplant duration	1.00	0.99–1.00	0.0334	1.00	0.99–1.00	0.2446
Creatinine level	1.03	0.61–1.59	0.8968			
Tacrolimus	1.03	0.82–1.28	0.8018			
mTOR inhibitor use	0.67	0.35–1.30	0.2372	0.82	0.46–1.47	0.5036
MMF	0.99	0.50–1.94	0.9733			
Steroid	1.15	1.02–1.32	0.0290	1.08	0.96–1.19	0.1605
Hypertension	0.89	0.45–1.79	0.7401			
Diabetes	1.47	0.67–3.13	0.3221	1.62	0.80–3.12	0.1635
Antibody titer ≥1,689.3 BAU/mL	0.36	0.16–0.72	0.0058	0.46	0.21–0.95	0.0412
Positive IGRA	0.60	0.30–1.17	0.1409	1.29	0.63–2.55	0.4647

CI, confidence interval; MMF, mycophenolate mofetil; BAU, binding antibody unit; IGRA, interferon-γ release assay.

The literature has shown that antibodies evoked by the first-generation vaccine still affected the Omicron variant [24]. A higher titer is needed [12], which could be achieved by booster strategy. For KTRs, a meta-analysis showed a positive antibody detection rate of around 60% after the third dose, and the antibody titer also increased [1]. KTRs take various immunosuppressants that impede lymphocyte activation for antibody generation.

Moreover, the waning rate of antibodies is prominent in KTRs, even after a third dose [25]. Measurement of titer may help to identify KTRs with different risks and administer boosters to those with poor response to vaccination. For those already having a high antibody titer, the risk of side effects [26] for a booster may outweigh the limited benefit [27]. It should be noted that a high antibody titer does not equal a safe status. Our study shows that among patients with SARS-CoV-2, 49 patients had a known antibody titer, and six needed admission for further management. Most hospitalized patients (5/6) had antibodies <1,689.3 BAU/mL, but one patient had an antibody titer higher than 3,000 U/mL. For SOTR, that high antibody titer after booster did not represent equivalent neutralization capacity for the Omicron variant [28]. Hence, the result of antibody measurement should be interpreted with caution. However, it still has a more significant role in KRTs than in the general population for the risk stratification to decide between boosters. In this study, we also examined the result of IGRA as a cellular response to vaccination. Compared to antibody titer, IFN-γ level of cellular response assay did not increase significantly with the boosters after the third dose both based on the percentage of positive results and IFN-γ titers. All the KTRs were on immunosuppressants targeting mainly the T cells, hence, the response was suppressed [29]. In addition, it has been reported in the immunocompetent general population [30, 31] that T cell response could not be augmented by repeated boosters despite detectable SARS-CoV-2 specific T cell population after initial doses. Unlike virus infection, vaccination with booster doses did not provoke equivalent IFN-γ and IL-10 expression memory T cells. It is postulated that viral infections on the pulmonary site persist longer and stronger than intramuscular vaccination; they induce a robust inflammatory cytokine release, which enhances a more durable T-lymphocyte response [32] and generates more tissue-resident memory T cells [33]. In addition, we used circulating lymphocytes for the IFN-γ release assay, and the result may not reflect the response of local memory T cells evoked by boosters.

Although T cell response correlates positively to antibody response (**Figures 3D, E**), we did not find the effect of a positive IFN-γ response to SARS-CoV-2 in the prevention of infection, as reported in dialysis patients [34]. It has been shown that T cell response is crucial when humoral immunity is impaired [35]. Nevertheless, under strong antibody response, the possible secondary role of T cells could be masked [36]. Our subgroup analysis for KTRs with low or absent antibody titer did not show a protective effect against infection by a positive cellular response. We speculated that T cell response might be slower than antibody response, which could neutralize the virus at the first encounter. The cellular response might be more important for disease severity, which this small study with low admission requirements and rare mortality could not reveal.

There were several limitations in this study. First, under a pandemic status with limited availability of vaccines, KTRs received vaccines based on different platforms. We had previously shown that KTRs had weaker responses to all types of vaccines compared to the responses in general population, and the immunogenicity varied among the vaccine platforms [4]. In Taiwan, most people, including KTRs, received homologous vaccines for the first two doses. They could choose either an mRNA or protein subunit vaccine for the third dose as a personal preference. It is difficult to identify the effect of different vaccines, but we found that most (approximately 90%) KTRs would have detectable antibody titers after the fourth dose. The effect of multiple boosters was robust regardless of vaccine type. Second, we retrospectively reviewed the infection risk during an outbreak

caused mainly by the Omicron strain BA.2 [19, 20], which might not represent a general protective effect against other strains. It was well known that the Omicron strain had more immune evasion than previous strains of the first generation vaccines. This study still showed a significant effect of vaccine doses, and further observation on different variants is needed. Third, our study did not perform an antibody neutralization test, and it is difficult to correlate directly between the protection effect and antibody measurement by anti-spike protein assay. We admitted the importance of the neuralization test according to different virus strains. However, the equipment and expense requirements may become a limitation in many medical institutions. Developing new economic tests for different virus variants might be necessary for more precise measurement.

In conclusion, this study showed a protective effect against SARS-CoV-2 according to vaccine doses and laboratory measurements in KTRs. Despite impaired immune function, KTR still had increasing responses after repeated vaccination. After the third dose, the protection effect became prominent but varied among patients. Measurement of antibodies could be helpful to determine individual risk and the need for further boosters. These findings provide evidence for a specific vaccination strategy in KTRs, who require more boosters than the general population to achieve an adequate antibody titer that may be necessary in a pandemic.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the NTUH (202106046RINA). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-CC and M-KH participated in research design, writing of the paper, and data analysis. Y-JH, M-JL, S-WW, M-HL, H-SH, Y-CL, Y-TH, Y-FL, M-KT, and C-YL were involved in the performance of the research. 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.2023.11196/full#supplementary-material

Supplementary Figure S1 | All vaccine combinations received by all KTRs (n = 622) with the number and percentage of COVID-19 cases of each group.

Supplementary Figure S2 | All vaccine combinations received by KTRs with antibody and IGRA titer (n = 181) with the number and percentage of COVID-19 cases of each group.

Supplementary Figure S3 | (A) Comparison of COVID-19 rate between different vaccine doses. 31%, 30%, 21%, 10% for vaccine doses 1/2/3/4. * $p = 0.0220$, ** $p = 0.0023$, *** $p = 0.0235$ **(B)** Comparison of hospitalization rate between groups of different vaccine doses. 8%, 11%, 3%, 1% for vaccine doses 1/2/3/4. * $p = 0.0035$, ** $p = 0.0119$.

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ORIGINAL RESEARCH
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Increasing Kidney-Exchange Options Within the Existing Living Donor Pool With CIAT: A Pilot Implementation Study

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Computerized integration of alternative transplantation programs (CIAT) is a kidney-exchange program that allows AB0- and/or HLA-incompatible allocation to difficult-to-match patients, thereby increasing their chances. Altruistic donors make this available for waiting list patients as well. Strict criteria were defined for selected highly-immunized (sHI) and long waiting (LW) candidates. For LW patients AB0i allocation was allowed. sHI patients were given priority and AB0i and/or CDC cross-match negative HLAi allocations were allowed. A local pilot was established between 2017 and 2022. CIAT results were assessed against all other transplant programs available. In the period studied there were 131 incompatible couples; CIAT transplanted the highest number of couples (35%), compared to the other programs. There were 55 sHI patients; CIAT transplanted as many sHI patients as the Acceptable Mismatch program (18%); Other programs contributed less. There were 69 LW patients; 53% received deceased donor transplantations, 20% were transplanted via CIAT. In total, 72 CIAT transplants were performed: 66 compatible, 5 AB0i and 1 both AB0i and HLAi. CIAT increased opportunities for difficult-to-match patients, not by increasing pool size, but through prioritization and allowing AB0i and "low risk" HLAi allocation. CIAT is a powerful addition to the limited number of programs available for difficult-to-match patients.

Keywords: kidney-exchange, AB0-desenzitisation, HLA-desenzitisation transplantation, computerised allocation, highly immunised

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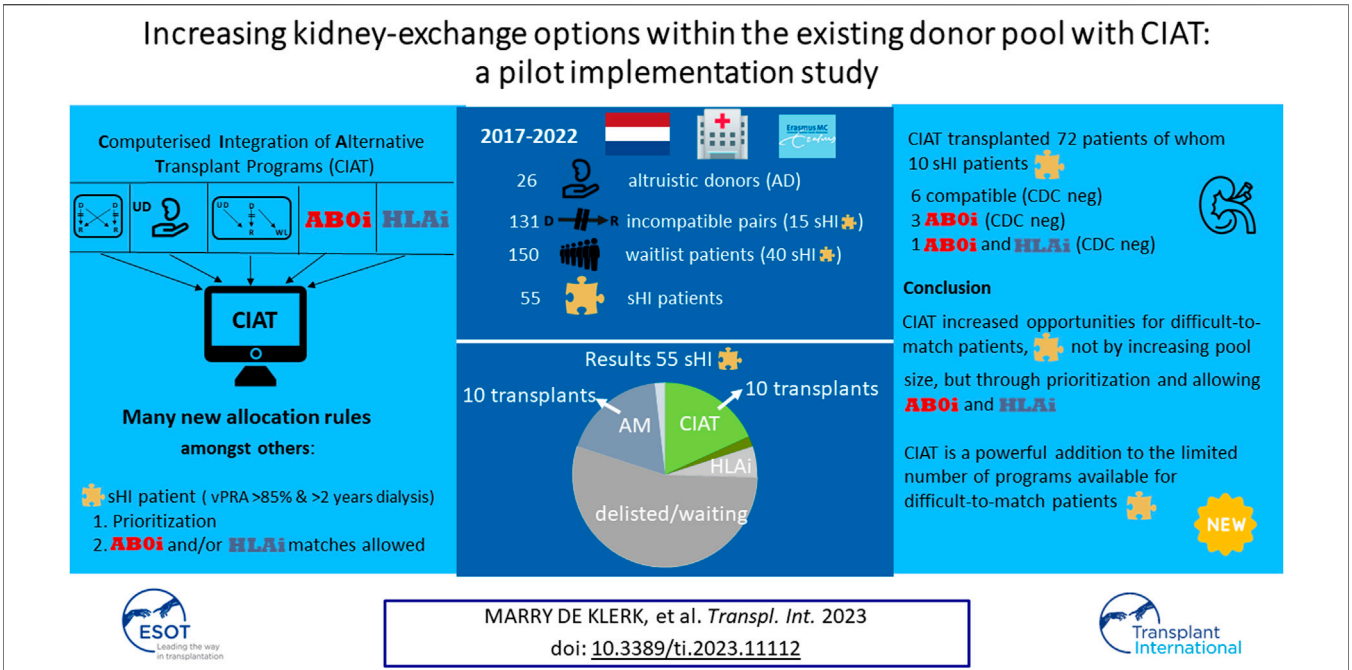
Abbreviations: AB0c, AB0 blood type compatible; AB0i, AB0 blood type incompatible; AD, altruistic donor (altruistic living donor not directed to a specific recipient); AM, acceptable mismatch program from Eurotransplant; CDC XM, complement dependent cytotoxicity cross match; CIAT, computerised integration of alternative transplantation programs; cPRA, calculated panel reactive antibody; DSA, donor specific antibody; ET, Eurotransplant; FCXM, flow cytometry cross match; HI, highly immunised; HLAi, HLA incompatible: positive cross-match; KAS, kidney allocation system; KEP, kidney exchange program; LSA, LABScreen® Single Antigen One Lambda; LW, long waiting; MFI, mean fluorescence intensity; sHI, selected highly immunised; vPRA, virtual panel reactive antibody.



GRAPHICAL ABSTRACT |

INTRODUCTION

A number of alternative, living donor kidney transplantation programs have been developed for incompatible pairs: Kidney exchange program (KEP), altruistic donor transplantation, domino donation, AB0-incompatible transplantation (AB0i) and HLA-incompatible transplantation (HLAi) [1–11]. In the Netherlands, the national KEP is the only computer based and nationally operating alternative living donor transplantation program. All other programs function locally.

In the Netherlands, current practice is for incompatible couple recipients to also participate in the deceased donor Eurotransplant waiting list. All incompatible couples are allowed in KEP. After a number of unsuccessful KEP runs, AB0i and/or HLAi couples may opt for desensitization against their intended donor, dependent on anti-AB0-titer and/or donor specific antibody (DSA) level (**Figure 1**).

Regardless of the presence of a potential living donor, immunized patients can opt for the Eurotransplant Acceptable Mismatch deceased donor program (AM program) after 2 years on dialysis, when vPRA is above 85% (**Figure 1**). Acceptance in the AM program depends on immunologic criteria [12]. Despite all these programs, many long waiting (LW) and highly immunized patients (HI) do not find a match and accumulate on the waiting list.

Computerized Integration of Alternative Transplantation (CIAT) programs were developed to optimize the kidney exchange program [13]. There are many new options in CIAT compared to current donor-exchange programs [14]. The most eye-catching innovations are integration of altruistic donation, privileges for long waiting (LW) patients, and privileges and priority for a selection of highly immunized (sHI) patients (**Figure 1**). LW is defined as more than 2 years on dialysis (independent of vPRA): in CIAT, as a privilege, AB0i allocation is allowed for them, provided that AB0 blood type titers are not too high (In our center <1:512, but there are large differences between laboratories). This privilege is introduced because outcomes for living donor AB0i kidney transplantation are superior to waiting for an AB0-compatible deceased donor transplantation [15]. sHI is defined as vPRA ≥ 85% and >2 years unsuccessful participation in the AM program or vPRA ≥ 85% and >2 years dialysis, but is declined for AM on immunologic grounds (**Figure 1**). The threshold of 2 years AM participation is based on the rapidly decreasing transplant rates with AM after that time point [12]. For highly immunized patients not eligible for AM transplantation, chances are even lower due to lack of priority allocation. All sHI patients are LW patients but when LW patients are upgraded to sHI patients, the LW qualification is no longer used for them. Of course, they keep the privilege to receive an AB0i match (**Figure 1**).

In CIAT, the chances of sHI patients are increased by giving priority. Matching of sHI patients is the very first step in the CIAT allocation algorithm. Besides, their chances are increased by privileges: acceptance of HLA-incompatible matches are allowed through delisting of HLA-unacceptables with relatively low MFI, unless they are repeated mismatches. This allows "low risk" HLAi allocation to sHI patients. The first aim in the algorithm is a compatible match, then AB0i, then HLAi, subsequently both AB0i and HLAi combinations are aimed at. Transplantation of (highly immunized) patients gives them a

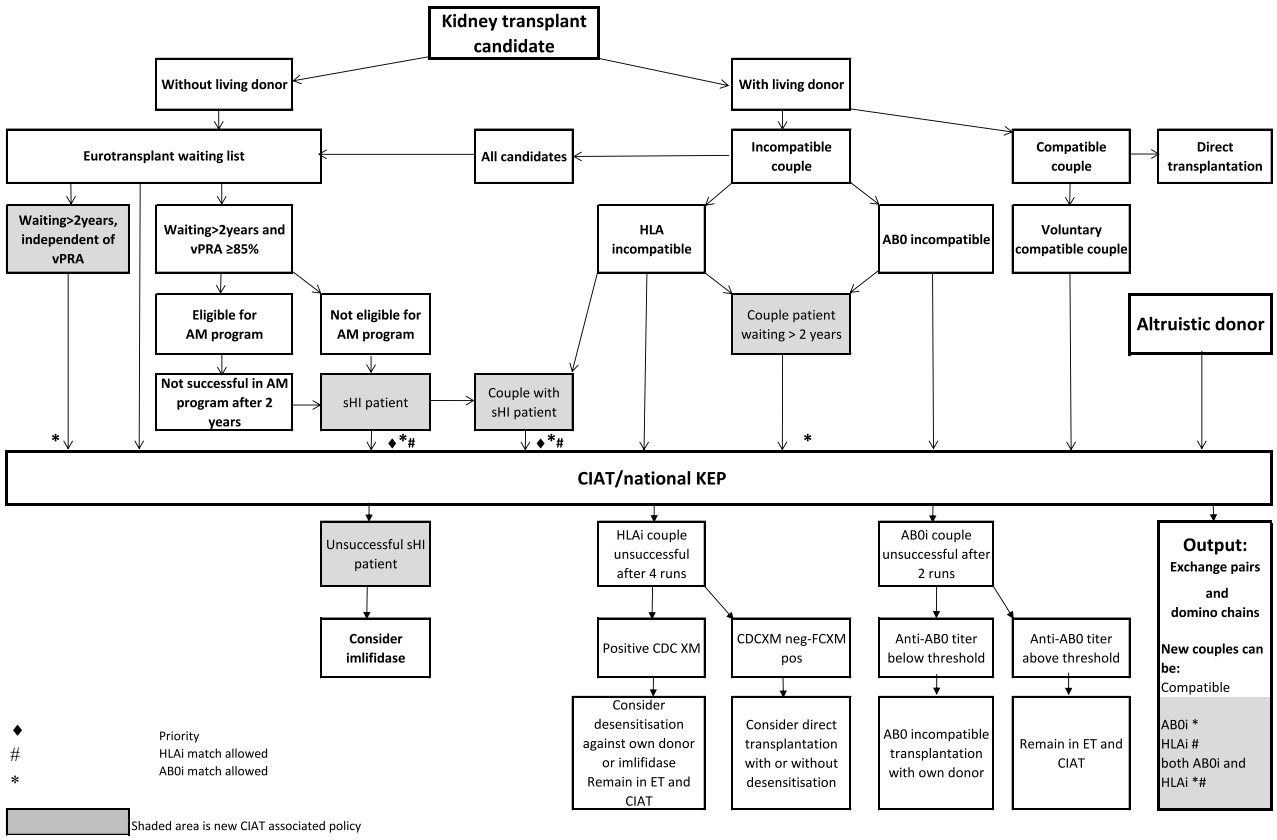


FIGURE 1 | Flowchart to show possibilities to participate in various transplantation programs, for potential transplant candidates with and without a potential living donor.

survival benefit compared to continuing dialysis [16]. In CIAT, a CDC negative cross-match is mandatory to continue with the transplant in order to prevent breakup of chains because of positive cross-matches. Because altruistic donation is better integrated, waiting list patients can also receive a living donor kidney at the end of a domino chain and privileges, and respectively priority and privileges may also hold for LW and sHI waiting list patients (**Figure 1**). In the future, when CIAT becomes the national program, the wishes regarding the donors' donation center can be taken into account. If donors decide to donate in their own center, the domino donors can still participate nationwide.

Currently, worldwide practice is to look for compatible matches first and subsequently, when unsuccessful, accepting incompatible matches requiring desensitization against their intended donor. CIAT is a step in between: it looks for the best CDC negative (in-) compatible match for difficult-to-transplant patients. E.g., CIAT may find a compatible, or AB0i, or CDC-negative HLAi, or both AB0i and HLAi match for a recipient that has a CDC-positive cross-match with the intended donor. CIAT increases chances, not by increasing the available living donor pool size, but by increasing options within the pool. E.g., allowing AB0-incompatible allocation in KEP for a blood type 0 recipient with low anti A and B titers more than doubles the potential donor pool from 47% (only blood type 0) to 100% (all AB0 blood types). Delisting a highly immunized patient's low-MFI titer unacceptable HLA-A2 allows an HLA specificity that occurs in 30% of the population. The increase in chance depends on the composition of all the patients' unacceptables being delisted. The more low-titer unacceptables can be delisted, the larger the potential pool and the increase in chance.

In our simulation study we compared results of the national KEP in 2015 and 2016 with those of a CIAT simulation using the same participant input.

Results were very promising [13]. The simulation showed increased match numbers, both overall and in difficult-to-match patients when using CIAT. CIAT found 8 matches for difficult-to-match sHI patients compared to only 1 in reality. In addition, more AB0 compatible (AB0c) matches were found for AB0i couples, while the total number of transplantations was not hampered. Prioritizing difficult-to-match patients improves their chances without affecting the chances of regular patients.

The current study describes the results of CIAT since its implementation in clinical practice over 5 years. The CIAT algorithm was used in one pioneering center, alongside the

national KEP and all other alternative transplantation programs, to gain real-life experience. The research question concerns the contribution of CIAT allocation to the total number of transplants, as well as to transplants in long-waiting and highly immunized patients in real-life.

PATIENTS AND METHODS

Patient Data and Ethics

Written informed consent to use their data for research on kidney transplantation was asked and was obtained at the moment patients present for kidney transplantation. The data for this study were retrospectively retrieved from patient files. According to the Dutch law, this study was exempt from approval from an ethics board. Patients and data were treated in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

CIAT

A local pilot was established in our center between 1st January 2017 until 1st January 2022 to gain logistic experience, to test the algorithm and to optimize the program. Observation was until 1st September 2022. All incompatible couples, compatible couples, and altruistic donors from our center that opted for an alternative donor transplantation program in the period studied, as well as the complete local deceased donor waiting list were included in the pilot.

The additional transplant options of CIAT were tested in the presence of the standard (competing) national and local, deceased and living, donor kidney transplantation programs. There were no exclusions for participation in any program: in the period studied patients participated in all programs available for them. CIAT results were assessed against standard available transplant programs.

CIAT: Identification and Handling of sHI and LW Candidates

Participation in CIAT as an LW or sHI patient was discussed and decided by a standing committee. Patients were evaluated on their medical condition in order to determine if there were contra-indications for AB0-desenzitisation. Eligibility criteria for CIAT for sHI or LW patients have been described before and in the introduction [13].

CIAT: Match Runs

In this pilot, CIAT operated locally with 4 runs per year, in between national KEP runs. So, in the study period, couples participated every 6 weeks in a match run (taking turns participating in CIAT or national KEP). AB0i, HLAi, and combined AB0i/HLAi couples, as well as (small numbers of) compatible couples and altruistic donors participated in these runs. CIAT can result in both short, closed cycles and open (domino) chains. Closed cycles were formed by 2 or more couples. In domino chains, an altruistic donor started a chain with 1 or more couples, and the donor of the last couple (domino donor) donated to a CIAT selected patient on the waiting list. This might be an sHI or LW waiting list patient. Of the HLA incompatible allocations, only those with a CDC negative cross-match proceeded to transplantation, because desensitization was not allowed in combination with a CIAT match.

During the study period, patients and couples participated in CIAT while also participating in all other available programs.

Other Alternative Transplantation Programs Available in the Period Studied

Alternative programs are not integrated, as patients participate in all programs separately. The process of finding a match amongst all these programs starts with the search for compatible matches via KEP. When unsuccessful the other programs are tried (**Figure 1**).

National KEP is the only nationally organized alternative living donor transplantation program: All 7 Dutch transplant centers participate. In national KEP about 3 times as many couples participate per run compared to local CIAT runs. This National KEP runs 4 times per year. AB0i, HLAi, and both AB0i and HLAi couples, as well as small numbers of compatible couples and altruistic donors participate in KEP. Compatible matches in short, closed cycles and open domino-chains are aimed for. In case of a domino paired procedure, the last domino donor is assigned to the transplant center of the altruistic donor. This center selects a waiting list recipient [14]. Current national KEP and CIAT have the same position in **Figure 1**, but CIAT adds options (shaded areas).

The domino paired donation program starts with an altruistic donor. Together with incompatible couples an open chain is accomplished with the last donor (the domino donor) donating to the waiting list [8]. In the Netherlands this program primarily operates locally.

The AB0-incompatible transplant program is available for AB0i couples that meet the inclusion criteria: anti-AB0 blood type IgG titers 1: 512 and lower [15]. Before proceeding with an AB0i transplant, couples are advised to participate 2 times in KEP.

The HLA-incompatible program is a desensitization program for difficult-to-match, highly sensitized patients with an HLAi living donor [17]. Couples are eligible after unsuccessful participation in the KEP and AM program.

The Eurotransplant Acceptable Mismatch program (AM) for deceased donor transplantation is available since 1989 for highly sensitized patients with vPRA $\geq$ 85% who are at least 2 years on dialysis [12, 18]. Inclusion depends on immunologic criteria (**Figure 1**).

RESULTS

Between January 2017 and January 2022, 946 transplantations have been performed in our center, and 483 with a deceased donor. There were 463 living donor transplantations, of which 338 were direct living donor transplantations and 125 were alternative program transplantations (27% of living donor transplantations). Participants in alternative transplantation programs were: 26 altruistic donors, 131 couples (70 AB0i,

53 HLAi (some of them also AB0i) and 8 compatible pairs). 69 LW and 55 sHI patients participated (**Tables 1**, **2**). Sixteen of these 55 sHI patients had been declined for the AM program. There were 15/55 sHI and 13/69 LW candidates with a potential living donor that participated as a couple. Thus, in total 28/131 couples had a difficult-to-match recipient. On average 150 waiting list patients were included per CIAT run.

Transplantations via All Available Programs

131 incompatible couples participated (**Figure 2A**). 46 (35%) were transplanted via CIAT. 27 (21%) received a direct kidney transplantation with another, direct, compatible donor or after AB0 and/or HLA desensitization. 23 (18%) were removed from the waiting list or still waiting, 16 (12%) received a deceased donor kidney, and 19 (14%) were transplanted via national KEP.

There were *55 sHI patients* (**Figure 2B**), 30 patients (55%) were not transplanted. Ten patients were transplanted via CIAT (18%), while another 10 (18%) received an AM deceased donor kidney, one patient received a HLAi deceased donor kidney after Imlifidase desensitization (2%). There were 3 (5%) direct living donor transplantations via HLA desensitization and 1 (2%) via national KEP.

There were *69 LW patients* (**Figure 2C**) of whom 14 (20%) were transplanted with a living donor kidney via CIAT; 14 (20%) were removed from the waiting list or still waiting; 36 (52%) received a deceased donor kidney (including AM), and two patients received a compatible living donor kidney via direct donation and three via national KEP (7%).

Transplantations via CIAT

In total 72 transplantations have been performed via CIAT (**Table 3**). The majority was transplanted in an open cycle starting with an altruistic donor.

In total, 46/131 couples were matched via CIAT: 30/70 AB0-incompatible couples (43%), and 10/53 HLA-incompatible couples (19%); 5 in a closed chain and 5 in an open cycle starting with an altruistic donor. Six of the eight compatible pairs were matched. All matches resulted in donation and transplantation. Two compatible pairs were not matched after one CIAT run, and they decided on direct donation and transplantation.

From the 72 patients transplanted via CIAT, 14 were LW patients, of whom 2 patients received an AB0i transplant (**Table 1**). Ten were sHI patients, of whom nine received an HLA compatible transplant, and three were AB0i. One sHI patient received an AB0i and HLAi transplant (**Table 2**). This latter patient was transplanted with a donor kidney against whom he had low titer HLA unacceptables. CDC cross match was negative. **Tables 1**, **2** shows details and characteristics of the patients transplanted or not in the various programs.

Table 4 shows characteristics and long-term outcomes of 10 CIAT transplanted sHI patients. It is a relatively young population with high vPRA and long dialysis time. Most of them received a retransplant. For 2 with a potential living donor, CIAT found a closed chain. Only 3 sHI patients received their kidney directly from the altruistic donor, all others were in a chain. Observation time is between 6 months and more than 5 years. One patient had a never functioning graft because of recipient comorbidity and rejection. Another failed after 6 months because of CMV reactivation, BK nephropathy and rejection. Transplant function of the other patients is acceptable to good (**Table 4**).

Ten other sHI patients received a kidney via the AM program, observation was between 0 and 4 years. Two kidneys failed 0.3 and 3 years after transplantation. One patient died 0.2 years after transplantation. eGFR of the remaining seven functioning kidneys is between 29 and 99 mL/min/1.73 m^2.

DISCUSSION

In the present study the implementation of additional allocation rules in a kidney exchange program was tested in one center. We compared the performance of CIAT with that of all other local and national, living and deceased donor transplantation programs available for these patients. In our 5-year pilot, CIAT allocation resulted in high numbers of transplantations in HI patients and incompatible couples in comparison to all other programs. Local CIAT even outperformed national KEP on numbers transplanted. CIAT is a major addition to the limited number of existing programs that enable kidney transplantation in difficult-to-match patients. There are no publications on a program like CIAT that integrate KEP, altruistic donation, AB0- and/or HLA-incompatible allocation and transplantation while giving priority and privileges to difficult-to-match patients. The strength of CIAT is that rules and regulations guarantee increased chances given to all these selected patients. CIAT is a new and promising program that is currently being adapted to national requirements in order to replace National Dutch KEP.

In our recent CIAT simulation we showed that, compared to the old situation, the adaptations to KEP led to 8 times more transplantations in HI patients while the total number of transplantations performed was not hampered [13]. Furthermore, far more matches were found for AB0i couples.

In the present pilot only 1 both AB0i and HLAi match was found with a negative CDC cross-match. Presently only CDC negative matches proceed to transplantation to prevent last minute declines because of positive CDC cross-match. Including a patient with a CDC positive cross-match in a chain or cycle is felt to be too complicated because unsuccessful desensitization may lead to disintegration of the complete chain or cycle. One HLAi combination with positive CDC cross-match has been declined in the period studied.

Accumulation of difficult-to-match patients on the waiting list is a universal problem [19–22]. There have been several efforts to solve this problem:

First: In Europe, the AM deceased donor program was introduced in 1989 for difficult-to-match patients, and is based on the positive identification of acceptable antigens [23]. This approach has led to significantly decreased waiting times for highly sensitized patients in the Eurotransplant region [12]. Until now the AM program was the most important program to transplant HI patients. Our pilot shows that even local CIAT

TABLE 1 | Characteristics of LW transplanted according to different programs or still waiting.

	LW patients	LW patients matched in CIAT	LW patient matched in other living donor program	LW patient matched in deceased donor program	LW patient not transplanted
Number	69	14	5	36	14
With a living donor (yes)	13	5	4	1	3
AM program (yes)	11	0	1	3	7
vPRA% (median, range)	22 (0–100)	4 (0–74)	58 (0–100)	4 (0–98)	99 (5–100)
Dialysis vintage (median, range)	3.6 (2–20.8)	3.3 (2–6)	3.1 (2.8–3.2)	3.5 (2.5–9.3)	5 (3.0–20.8)
Bloodgroup: 0	35	6 (2 AB0i)	1	21	7
A	8	5	0	1	2
B	26	3	4	14	5
AB	0	0	0	0	0

TABLE 2 | Characteristics of sHI transplanted according to different programs or still waiting.

	sHI patients	sHI patients matched in CIAT	sHI patient matched in other living donor program	sHI patient matched in deceased donor program	sHI patient not transplanted
Number	55	10	4	11	30
With a living donor (yes)	15	2	4	2	7
AM program (yes)	39	4	3	11	23
vPRA% (median, range)	99 (85–100)	97 (85–100)	100 (94–100)	99 (88–100)	99.5 (91–100)
Dialysis vintage (median, range)	5 (1.8–23.9)	4.2 (2.1–8.8)	7.5 (3.7–17)	4.1 (2.8–12.2)	5.5 (2.7–23.9)
Bloodgroup: 0	23	3 (1 AB0i, 1 HLAi and AB0i)	1	7	12
A	18	5 (1 AB0i)	2	2	9
B	8	1 (1 AB0i)	1	1	5
AB	6	1	0	1	4

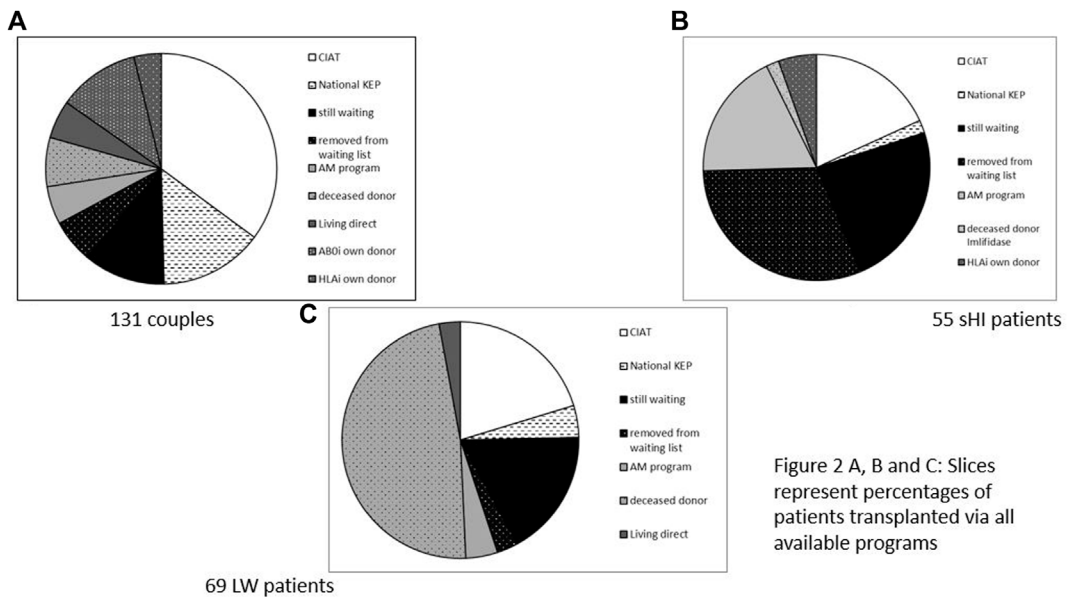
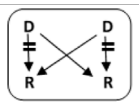
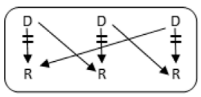
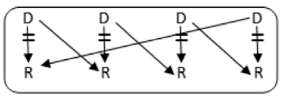
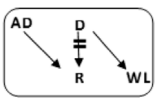
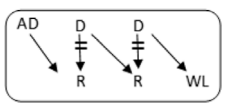
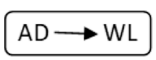


FIGURE 2 | (A–C) Slices represent percentages of patients transplanted via all available programs. **(A)** 131 Couples. **(B)** 55 sHI patients. **(C)** 69 LW patients.

can transplant a comparable number of sHI patients. A national CIAT program with a larger pool will likely result in higher transplant rates. In the United States, in 2013 a successful new Kidney Allocation System (KAS) was introduced with priority for highly sensitized patients in the regular deceased donor kidney allocation system [24, 25].

TABLE 3 | CIAT match and transplant results in the pilot period.

2017–2022				
Number	**Cycle/chain**	**Pair**	**Waiting list patient**	**Transplant**
6		12		12
2		6		6
1		4		4
12		12	12	24
6		12	6	18
8			8	8
Total		46	26	72

AD, anonymous/altruistic donor.

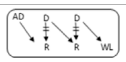
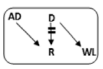
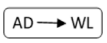
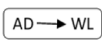
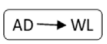
Second: In the direct living donor kidney transplantation population, blood type A and AB recipients are far more easily transplanted compared to blood type B and 0 recipients. The chances of finding a direct living donor for highly sensitized patients are low [21]. This led to the introduction of the aforementioned KEP program, but also to AB0-incompatible and HLA-incompatible transplantation programs. KEP is the backbone of living donor kidney transplantation for incompatible couples and various adaptations to the basic program have been performed and simulated in order to improve the success rate of the program for all participants, and for those difficult-to-match.

Third: Participation of compatible pairs in KEP was studied in simulations and in reality and appeared to improve the chances of difficult-to-match patients by enlarging the pool and adding blood type 0 donors [26, 27]. However, ethical issues like the definition of benefit for the compatible pair needs to be faced [28]. In spite of that, in our present study, compatible couples were successfully included.

Fourth: Another way to improve KEP results might be to expand the pool by international collaboration [29–31]. For collaboration a mutual, international protocol and agreement on the language of instruction are mandatory. Different countries, however, hold different laws and regulations. A simulation showed that, when countries are allowed to have different constraints and goals with regard to the cycles and chains, this may lead to a large discrepancy between the number of participating couples compared to the number of successful matches per country [32]. Another problem is that couples willing to participate in an international program likely will be those very difficult to match. This is reflected by the study by Valentin et al. were 71% of participating patients had vPRA > 80% [30]. If we consider the above-mentioned complexity that international KEP is confronted with, there are still many hurdles and barriers to be taken [33].

However, difficult-to-match patients from one country may benefit from participation to donation programs in another country with a slightly different HLA-pool. In a simulation

TABLE 4 | Characteristics and long-term outcome of 10 via CIAT transplanted sHI patients. Patient 10 received an AB0i and HLAi transplant with a negative CDC cross-match.

Patient nr	Age	Gender	vPRA	Time on dialysis (years)	Previous kidney transplants	Potential living donor	AB0 combination D->R	Type chain	HLAmm	Time after transplant (years)	Rejection therapy	eGFR mL/min/1.73 m^2
1	73	M	98	2.7	1	n	A->A		1-1-0	5.1	N	39
2	39	F	100	8.8	3	y	***B->A***		1-1-0	0.0	Y	NFG
3	46	F	85	2.7	1	n	A->A		1-2-2	4.3	N	40
4	48	F	99	4.9	0	n	***A->O***		0-1-2	3.4	N	31
5	72	F	91	6.7	0	n	0->0		2-1-1	2.5	N	87
6	25	F	96	4.6	1	y	***A->B***		2-2-0	2.4	Y	70
7	53	M	100	2.1	5	n	0->A		1-1-0	2.8	N	76
8	63	F	92	2.4	0	n	A->AB		1-1-1	2.6	N	35
9	32	F	99	3.8	2	n	A->A		1-1-1	0.8	N	46
10	40	M	94	8.6	1	n	***A->0***		1-1-2	0.6	Y	Failed

Bold-italic values are the blood type incompatible combinations between donor and recipient.

Mumford et al. showed that highly sensitized patients have modestly increased chances of a match in a different European deceased donor pool [34]. Individual participation of very difficult-to-match patients in foreign living or deceased donation programs could be successful and less complicated.

Finally: As we demonstrated in our current pilot study, an easier way to improve access to transplantation for difficult-to-match candidates is to make adjustments to the current KEPs. The adaptations we studied were priority and privileges for sHI and privileges for LW candidates. This increases options within the same donor pool. In our recent simulation and in the present pilot we demonstrated the benefit for difficult-to-match patients with this approach [13]. Adaptations to other existing KEPs in simulations suggest better results when AB0 incompatible matching is allowed [35, 36]. Real-life AB0-incompatible matching in a kidney-exchange program was allowed in a small-scale Australian study and showed promising results [37]. Integration of KEP and desensitization programs has been attempted temporarily and on a small scale by [38]. CIAT is the first program that combines the possibilities and benefits of different alternative transplantation programs in a kidney exchange program. In the present study, we showed that adaptations to a regular KEP program indeed lead to higher transplantation rates for difficult-to-match patients. However, not only the algorithm is responsible for this success: Rules, regulations, and agreements concerning priority and privileges for selected patients are indispensable. Just by giving priority, 6 sHI patients received a completely compatible match. By combining priority and allowing AB0i and HLAi allocation for sHI patients, a larger part of the potential donor pool becomes available which further increases their chances: another four received an incompatible transplant. In the period studied, CIAT found as many matches for sHI patients as the AM program: both 10 transplantations. CIAT enables transplantation of difficult-to-match patients, even in small pools. We performed 72 transplantations via CIAT, of whom only six patients were allocated an incompatible transplant: four sHI and two LW patients. All others were completely compatible matches.

In conclusion: In spite of current programs that aim at reducing inequality in transplant numbers for difficult-to-match patients, sHI and LW candidates still accumulate on the waiting list. Modifying the algorithm and prioritizing the sHI patients, while allowing AB0-and/or HLA-incompatible allocation, resulted in increased transplant numbers in this population. The participation of altruistic donors is essential as "fire starters" and to enable the participation of waiting list patients. Easy-to-match incompatible pairs and compatible pairs are essential for success because, in order to complete a puzzle, both the difficult and the easy pieces are indispensable. CIAT is a new and welcome addition to existing programs in matching difficult-to-match kidney transplant candidates.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data are available on request. Requests to access these datasets should be directed to Marry de Klerk, marry.deklerk@erasmusmc.nl.

ETHICS STATEMENT

Written informed consent to use their data for research on renal transplantation was asked and has been obtained at the moment patients present for kidney transplantation. The data for this study were retrospectively retrieved from patient files. According to the Dutch law, this study was exempt from approval from an ethics board. Patients and data were treated in accordance with the Declaration of Helsinki and the Declaration of Istanbul.

AUTHOR CONTRIBUTIONS

MdK: participated in research design, in the performance of the research, in data analysis, and in the writing of the paper. JK-vG: participated in the performance of the research, in data analysis, and in the writing of the paper. DR: participated in research design and in the writing of the paper. MB: participated in research design and in the writing of the paper. AdW: participated in the writing of the paper. MR: participated in the writing of the paper. JvW: participated in the writing of the paper. MK: participated in research design and in the writing of the paper. KG: participated in research design, built the program, participated in the writing of the paper. JR: participated in research design, in the performance of the research, in data analysis, and in the writing of the paper. 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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Exploring Staff Attitudes Towards Unspecified Kidney Donors in the United Kingdom: Results From the BOUnD Study

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Unspecified kidney donation (UKD) has made substantial contributions to the UK living donor programme. Nevertheless, some transplant professionals are uncomfortable with these individuals undergoing surgery. This study aimed to qualitatively explore the attitudes of UK healthcare professionals towards UKD. An opportunistic sample was recruited through the Barriers and Outcomes in Unspecified Donation (BOUnD) study covering six UK transplant centres: three high volume and three low volume centres. Interview transcripts were analysed using inductive thematic analysis. The study provided comprehensive coverage of the UK transplant community, involving 59 transplant professionals. We identified five themes: staff's conception of the ethics of UKD; presence of the known recipient in the donor-recipient dyad; need for better management of patient expectations; managing visceral reactions about the "typical" unspecified kidney donor; complex attitudes toward a promising new practice. This is the first in-depth qualitative study of attitudes of transplant professionals towards UKD. The data uncovered findings with strong clinical implications for the UKD programme, including the need for a uniform approach towards younger candidates that is adhered to by all transplant centres, the need to equally extend the rigorous assessment to both specified and unspecified donors, and a new approach to managing donor expectations.

Keywords: living kidney donation, unspecified donation, unspecified kidney donor, non-directed, altruistic donation

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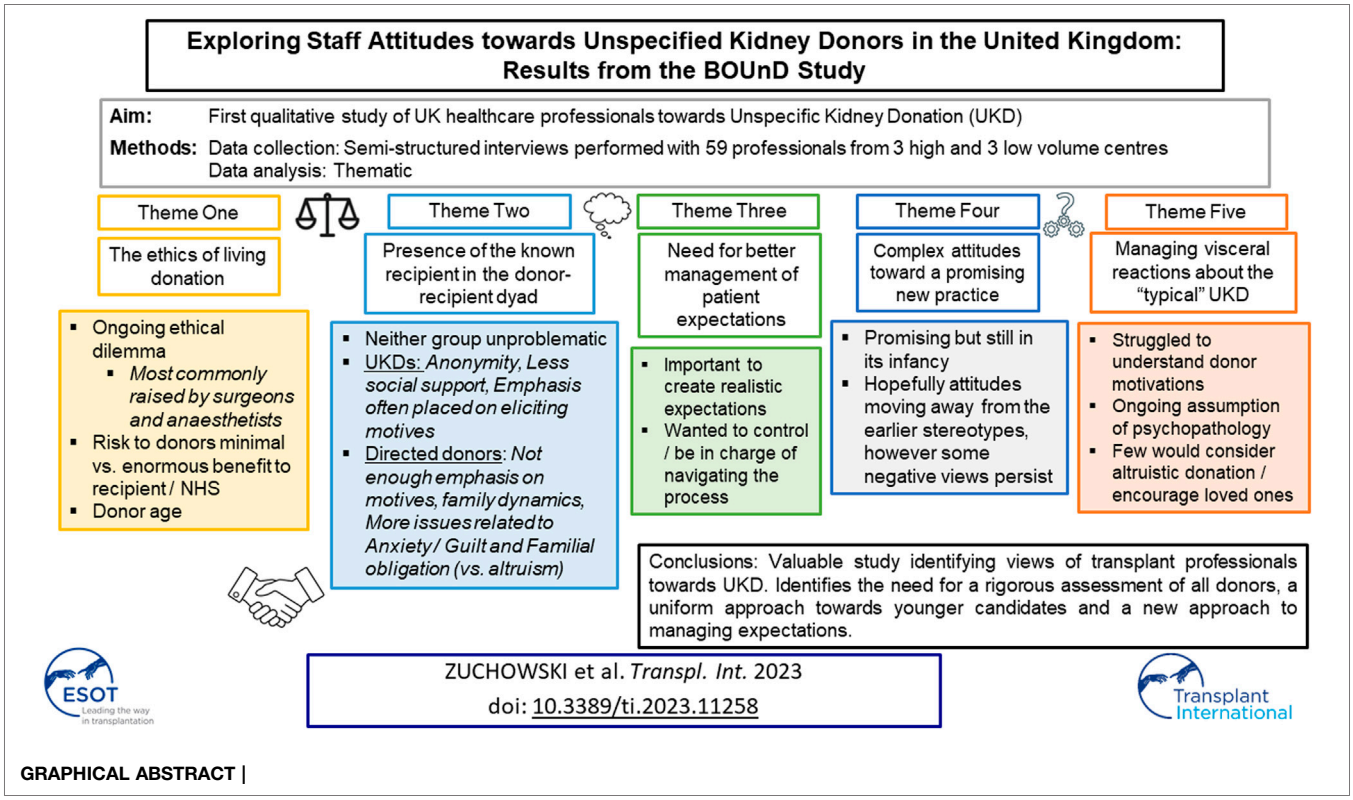
Abbreviations: BOUnD, Barriers and Outcomes in Unspecified Donation; HTA, human tissue authority; LKD, living kidney donation; NHS, National Health Service; SKD, specified kidney donation; SKDrs, specified kidney donors; UKD, unspecified kidney donation; UKDrs, unspecified kidney donors; UKLKSS, UK Living Kidney Sharing Scheme; UK, United Kingdom.



GRAPHICAL ABSTRACT |

INTRODUCTION

Living kidney donation (LKD) is the gold standard treatment for End Stage Kidney Disease [1]. LKD benefits the recipient, who experiences an improved quality and duration of life, and reduces pressure on waiting lists. Kidney transplantation in general reduces the economic burden of renal replacement therapies, thereby allowing healthcare resources to be redistributed more efficiently [2]. In the United Kingdom (UK) there are two pathways to LKD: specified kidney donation (SKD) to a recipient known to the donor, and unspecified kidney donation (UKD) from an unknown donor to an anonymous recipient [3]. Unspecified kidney donation accounts for around 7%–9% of the UK living kidney donor programme and has made a significant contribution, both directly and as part of the UK Living Kidney Sharing Scheme (UKLKSS). Unspecified Kidney Donors (UKDrs) are used within the UKLKSS to trigger a chain of transplants (called "altruistic donor chains") between 2 or more incompatible donor–recipient pairs. The remaining organ from the donor at the end of the chain is then allocated to a recipient on the national transplant list [1].

Despite this, some transplant professionals feel uncomfortable caring for these individuals, mainly due to concerns that wishing to donate is a manifestation of an underlying psychopathology [4, 5]. Consequently, a mandatory and rigorous psychological assessment is undertaken in all UKDrs [6]. Such an assessment is optional for specified kidney donors (SKDrs) and is at the discretion of the individual case or transplant centre. The programme also remains controversial because there is a general lack of data on outcomes and other aspects due to its relative novelty [1, 7, 8]. Concerns have been raised about whether the UKD programme in its current form represents an optimal use of NHS resources. This concern is based on anecdotal reports that UKDrs receive more meticulous and lengthy screening than other Living Kidney Donors, thus creating additional healthcare costs. UKD raises a number of ethical concerns for medical professionals, primarily the dilemmas around subjecting a healthy individual with no connection to the recipient to a serious operation. For these reasons, some healthcare professionals may have concerns that could influence the messages that they convey to potential donors.

A qualitative study exploring the experiences of UKDrs suggested that some participants were distressed and confused by discouragement from healthcare professionals, and the study highlighted the desirability for consistent messaging from staff members [9]. Participants also reported feeling distressed by the rigorous mental health assessment, believing that their motivations and overall sanity were being judged [9]. One study has explored transplant physicians' views on the nature of altruism in UKDrs and questioned whether it existed [10]. We therefore wished to explore the attitudes of healthcare professionals in the UK towards unspecified kidney donation, as well as to investigate whether there were barriers to donation.

To our knowledge, this is the first study to provide an in-depth exploration of the attitudes of UK transplant professionals

towards UKDrs, and forms part of the Barriers and Outcomes in Unspecified Donation (BOUnD) study, which is exploring the barriers to UKDrs in the United Kingdom [8]. A qualitative study was performed to determine potential issues that are not necessarily apparent in questionnaire-based research. The aim of this study was to investigate the broader views and experiences of the UK professional transplant community towards UKD, and explore to differences between centres, and different members of the multidisciplinary team.

PATIENTS AND METHODS

Participants and Setting

The participants in this study were recruited as part of the BOUnD study [8]. Funded by the National Institute for Health Research (NIHR), staff and patients were recruited from all 23 UK transplant centres. To explore the attitudes of UK transplant professionals in more depth, a sub-study recruited staff from six UK transplant centres: three high volume centres and three low volume centres. Centres were defined as high or low volume based on UKD numbers at these centres in 2016/17 [11]. Analysis of national data demonstrated that approximately 50% of UKDrs donated at five of the 23 transplant centres. Centres were grouped according to numbers of UKDrs and those with the highest and lowest total numbers were approached. Using opportunistic sampling, representatives of staff groups involved in the UKD programme were recruited, including but not limited to, transplant co-ordinators, nursing staff, nephrologists, clinical psychologists, and surgeons.

Interview Topic Guide

Semi-structured interviews were conducted according to a topic guide. This was developed based existing literature on the topic and staff focus grouped performed as part of BOUnD. The interview topic guide covered:

1) Terminological preferences for UKDrs
2) Staff perceptions of UKDrs and thoughts on their specific motivations
3) Staff perceptions of their own work with UKDrs
4) Perceptions of the transplant professionals working with UKDrs and how treatment differed to SKDrs
5) Opportunity to reflect and provide suggestions for developing the programme.

Telephone and in-person interviews were conducted by two researchers (authors 8 and 9).

Qualitative Analysis

All interviews were audio-recorded and transcribed verbatim. The interviews were anonymised, and full transcripts were circulated to members of the study team (authors 1, 7, 8, 9, 12). The data was analysed using NVivo 11 Plus software.

An inductive thematic analysis of the data was conducted. This methodology was chosen because it is data-driven in nature and not linked to any pre-existing theoretical model [12]. It is considered suitable when investigating a diverse data set that is expected to reflect a broad range of attitudes towards the research questions [12]. The analysis involved multiple consecutive readings of the transcripts in order to become familiar with the data and to identify and code themes and categories and highlight relevant patterns across the data set [13, 14]. The next step was to analyse the codes and consider how these could be grouped thematically to encompass a range of ideas around a common topic [15]. This grouping of codes into themes and sub-themes was the product of repeated discussion between the coder (MZ) and the research team (HM, SN, JC). The analysis conformed to the COREQ (Consolidated criteria for reporting qualitative research) checklist [16]. In order to ensure reliability and eliminate preconceptions about the data set, the analysis was conducted blind.

RESULTS

59 interviews were conducted between April and November 2016. Thirty were from high volume centres and 29 from low volume centres. The average interview length was 32 min (Range: 10–76 min; SD = 15.33).

Participant Characteristics

The study provided broad coverage of the UK transplant community. The majority of participants were women (57%), and the most frequent professional roles were transplant coordinators (20%), and nursing staff (17%) (**Figure 1**).

Staff Attitudes Towards UKD

Five major themes emerged from the data [1]: staff's conception of the ethics of UKD [2]; presence of the known recipient in the donor-recipient dyad [3]; need for better management of patient expectations [1]; managing visceral reactions about the "typical" UKD and implications for treatment and [4] complex attitudes toward a promising new practice. Each theme and corresponding sub-theme(s) are discussed in detail below (**Figure 2**). **Table 1** provides supporting quotations.

Theme 1: Staff's Conception of the Ethics of UKD

Many staff expressed the view that UKD is ethically unproblematic. They had an overriding awareness of, and commitment to, ethical principles and their role within transplantation and living donation, and for the most part felt that UKD fell within those ethical parameters. However, the data remained heterogenous on this topic, resulting in the following sub-themes.

Duty of Non-Maleficence: The Paradox of Inflicting Injury on Healthy Individuals for a Positive Purpose

It was apparent that whilst some participants perceived operating on healthy individuals as an ethical problem, others did not. This was most commonly raised by surgeons, although many did not think it was a decisive reason against UKD. This was mainly due to recognition that the benefits of UKD outweighed the harms, providing the donors were fully informed, aware of the risks, and

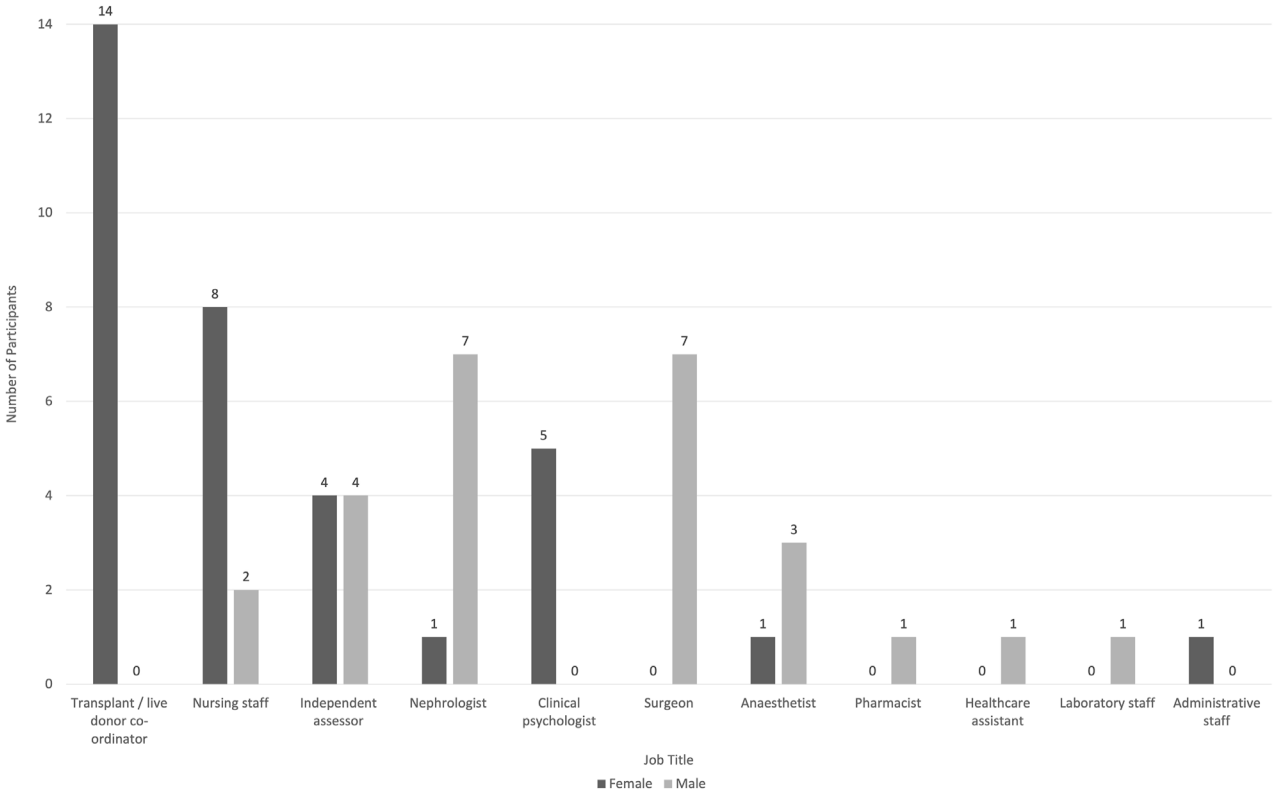


FIGURE 1 | Participant characteristics.

that they had sufficient capacity to consent. Some regarded the concern as outdated. Many doctors tended to express a sense of awareness of the paradoxical nature of their actions, i.e., the dilemmas of a healthy individual undergoing unnecessary surgery, albeit for a greater good. Many healthcare professionals did not think that their ethical reservations influenced potential donors.

Balancing Risk to Donors and Benefit to Recipients

Whilst staff members acknowledged the risks of donation, they were commonly weighed against the benefits, which they felt clearly favoured UKD because of the benefit to recipients and other aspects of the healthcare system. They emphasised the overriding benefit of avoiding dialysis and freeing up dialysis facilities for new patients, and to start a new life. Across all centres the prevailing attitude was that as long as people were psychologically and physically fit to be donors, the risks to the donor was minimal in comparison to the benefit to the recipient.

Ethical Concerns Surrounding Minimum Age Limits for Donors

Many staff expressed reservations about encouraging UKD amongst young individuals; referring to people in their mid-twenties. Concerns were related to their ability to provide informed consent and that they may not fully grasp how the risks could affect them later in life. Some participants brought up concerns for women specifically, due to potential implications around pregnancy that perhaps may not have been considered by younger women. Some related the decision to their own children. Others felt uncomfortable discriminating on the basis of age, with some centres having a minimum age restriction and others not. Some did not think that age should affect suitability whilst others were very strict with this criterion. Concern was also expressed that younger people might be more susceptible to media messaging and therefore more easily influenced and impulsive in their decision-making process.

Theme 2: Presence of the Known Recipient in the Donor-Recipient Dyad

Many participants expressed the view that a major factor influencing their attitudes was the presence of a known vs. unknown recipient. Some staff members said that the donor-recipient relationship in some cases made the donation process more difficult for the staff due to presence of complex family dynamics. They commented that, in some respects, UKD was more straightforward because of the absence of a relationship between donor and recipient. However, there was a notable lack of consensus on this issue. For some staff, UKD presented more difficulties than SKD due to issues such as UKDrs struggling with the requirement for anonymity from the unknown recipient or lack of support network for UKDrs. Overall, however, there was a greater perception that SKD was more emotionally complex due to emotional and physical proximity between the donor and the recipient, and

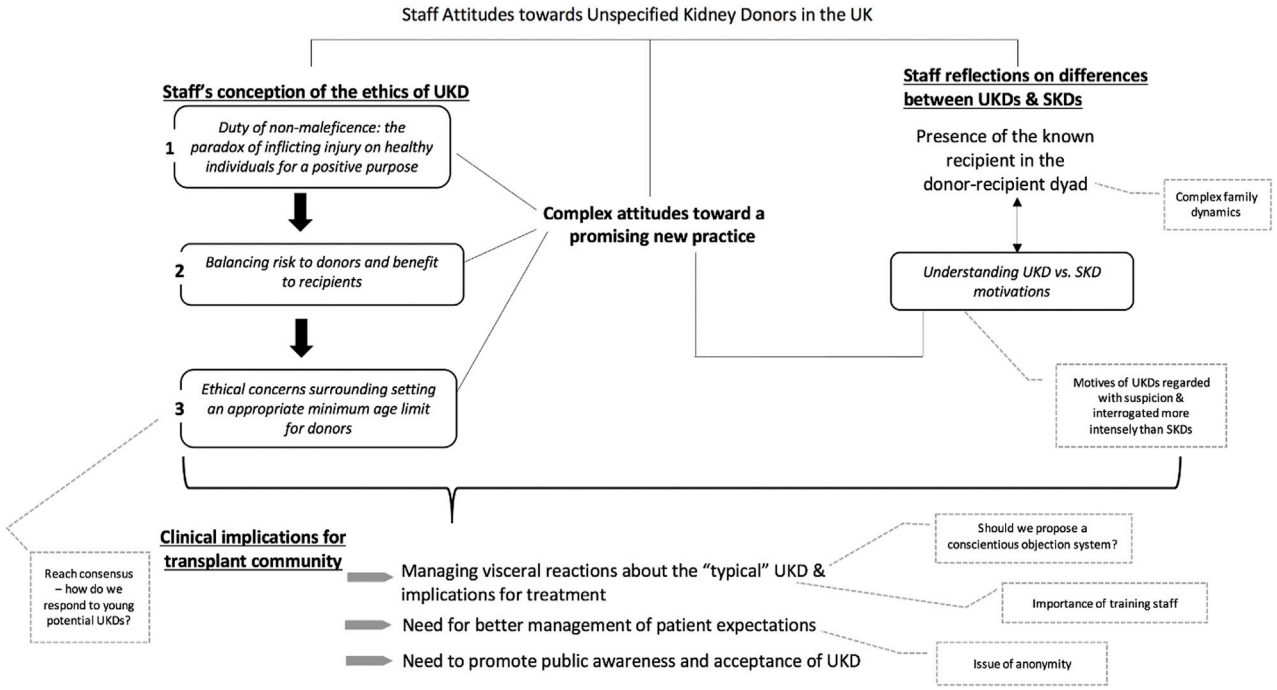


FIGURE 2 | Thematic diagram.

therefore associated with issues such as anxiety, guilt and familial obligation (as opposed to altruism).

Understanding UKD vs. SKD Motivations

The role of altruism as a motivator for UKD was questioned by some participants. The emphasis often placed on the mandatory psychological assessment by professionals was considered to be important not only to elicit a UKDrs' psychological state, but to further clarify their motivation to donate. Some found UKDrs' motivations to be complex or unclear as, at times, it was difficult to know if candidates were purely selfless or self-interested. Some staff noted that less attention was paid to the motivations of SKDrs, and the potentially complex family dynamics and psychological impact on both the donor and recipient.

Theme 3: Need for Better Management of Patient Expectations

Many professionals emphasised the importance of creating realistic expectations for the UKD process: the rigorous psychological assessment, the risks associated with the operation and recovery and the potential emotional consequences post-donation. Anonymity was raised as an issue, especially with regard to the negative emotions that may be experienced should there be no acknowledgment from the recipient and the need to prepare UKDrs for this, as it may present more of an emotional challenge for donors than anticipated. It was also stressed that donors should be informed of these issues from the very beginning of the process. Overall, UKDrs were thought to underestimate surgical risks and wanted to maintain control of the process and be in charge of navigating it.

Theme 4: Managing Visceral Reactions About the "Typical" UKD and Implications for Treatment

Many participants admitted that they struggled with understanding why UKDrs come forward. Despite their roles facilitating living donation, some said that they would not themselves consider donating as a UKD or encourage family members to do so. Some participants reported that they did not think that their personal opinions influenced self-withdrawal. UKDrs were referred to by some as being a mentally unstable group.

Theme 5: Complex Attitudes Toward a Promising New Practice

UKD was generally regarded as still being in its infancy and that peoples' attitudes may change once more people donate and transplant professionals have more experience. Some transplant professionals said that there was a need for the transplant community to understand where UKDrs fitted in within living donation. Comments could be reasonably interpreted as suggesting that UKDrs were not as highly valued as SKDrs.

Those working in lower volume centres, who consequentially had less experience, felt that they were unable to make specific generalisations about how they perceive UKDrs as an overall group. Across all centres participants tended to acknowledge that

TABLE 1 | Themes with corresponding subthemes and quotations (H—denotes high volume transplant centre, L—denotes low volume transplant centres).

Theme/Subtheme	Quotes
1. Staff's conception of the ethics of UKD	"As long [as they do that and] the process is informed, I don't think it raises any additional ethical issues over and above that."—H "I am a big believer in if you want to do something and you have got the capacity to consent to it, that you should be allowed to do it, and to that point I even find my role a little bit difficult because I think . . . who am I to suggest that this person might not be able to do something they want to do?"—L "I don't see it any different from somebody donating blood in the sense of . . . once you have stepped over that point, then I can't see what the difference would be. . . That would be my simplistic answer."—L "It's ethical as long as there's been a full psychological, maybe psychiatric assessment of that person and I think for me that is the biggest, because there are some very lovely people out there that just want to benefit mankind and so if they go in there and they have no psychological or psychiatric drivers, then I think it's a very magnanimous thing to do"—H "You can live quite happily with one kidney as long as everything is all right. I haven't got any ethical problems with it at all. If someone wants to do that, why not?"—H
1a. Duty of non-maleficence: the paradox of inflicting injury on healthy individuals for a positive purpose	"I think it's always the stress, like you say, of operating . . . more for the surgeons, of operating on someone who is completely well, you know, and in some ways it's easier just to deal with an emergency when someone is bleeding."—H "I am not sure whether we should actually ethically be doing this, because doctors and I guess any healthcare professional is supposed to do no harm and these people are specifically . . . we are allowing them to put themselves in harm's way and not even, you know, to benefit themselves or their family"—H "It comes down to 'doing no harm,' and my understanding is that the Hippocratic Oath doesn't have that as part of it; it's something that came much later in medicine . . . I am a nephrologist, but I have seen a lot of harm to a lot of patients over the years—unintentional, drugs being prescribed, wrong doses given, infections not being dealt with properly, symptoms not being listened to . . . so the world is clearly not perfect. And I think most patients I look at, say people who come forward, understand that something for nothing doesn't occur in the real world. They realise there is risk, most of the people I have come across are willing to take much more risks than others and I don't think it's a misunderstanding of risk, I think it's actually doctors working professionally acting on behalf of the people who tend to be more risk-averse than those individuals I think."—H "Although medical ethics to do no harm, you know, is a bit old hat I think now, 50 years on, I am sure from a recipient's point of view, the fact that altruistic donation is now permissible within the law, makes an enormous difference for them because it's just, you know, an extra chance, one opportunity."—H
1b. Balancing risk to donors and benefit to recipient	"I don't have any problem with the ethics because you are doing something which is a small risk to do quite a large good . . . I think it is a question of looking at how much good you are doing in somebody who has got a normal personality and is not mentally ill."—L "we have a 7,000 patients waiting, so if we can get, you know, some altruistic donors, that will have a great impact on our waiting list."—H "I think that the consensus view is that it is appropriate for an individual to perform an act like this for the grander good, principally for the good of another individual. As I say I think the overarching ethic of that is quite appropriate and I can work with that."—L "we are reducing the number of people who are on the waiting list. If we get more altruistic donors to donate their kidneys . . . hopefully people will be getting their organs quicker and we will reduce the number of people on dialysis"—H "what we have forgotten is actually that not only are people going away with new kidneys and a brand new life, but also you are freeing up dialysis for other people so it's almost a double whammy. You have got people starting a brand new life without the shackles of dialysis, be that at home or in a unit, they go off to start this new life and then you have also got these free areas for people to come and start dialysing"—H "you see people you know coming off dialysis and getting a fantastic gift so that's very beneficial."—L "it's more rewarding because of course you are maximising transplant opportunities for other people in the pool by the fact that maybe two or three people through the paired scheme, they get transplanted"—H
1c. Ethical concerns surrounding minimum age limit for donors	"I think my concern is with youngsters they can be 18 to 30 and I know it's a broad age group and it's a broad age range but they're still developing and maturing and it's ensuring that they understand what they are doing, it's not just a good idea or a nice thing to do. Have they thought through implications."—L

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TABLE 1 | (*Continued*) Themes with corresponding subthemes and quotations (H—denotes high volume transplant centre, L—denotes low volume transplant centres).

Theme/Subtheme	Quotes
	"For me, the biggest dilemma I had, we were approached by an 18 year old which felt uncomfortable. It was a female so of course then there's the additional of . . . of course she didn't want a family, the minute you are 18 you don't think further than tomorrow, so she hadn't thought about babies and the implications of having a single kidney around pregnancy and things like that . . . She had got obviously a long road ahead of her with potentially just a single kidney. So I was really uncomfortable with it, although the protocols said it was fine to carry on."—L "We all have concerns about the youngsters coming forward. Some of them . . . have had some sort of mental health issues, organise a psychiatric review and I think because they have all dealt with a lot more than I have, and they have seen a lot more, I think I am gathering my experience so they compare to the others in the team, but there is a lot of concern about the young ones coming through and their motivation."—L "I'm worrying about 10 years time, 20 years time—all the young ladies that come through that haven't had families, you know, how are you going to. . .? Are we going to facilitate damage to them? Is it going to be something, for example, when they have a baby they can't then"—H "We have seen people, for example, donors who are very young, a 23 years old coming forward as an altruistic donor, for me I am a bit conservative. What do you know at 23? You are only 23, what do you know about life? . . . I have got a 27 years old son and I know what a boy of 27 . . . and coming forward at 23 and 'You say you want to be an altruistic donor?' That's early, I would say 'Please it can wait'?"—H "Quite often other members of the team in particular will say 'I don't feel very comfortable about this, they are only 19 or they are only 21' . . . but I don't share that view . . . young people make decisions, sometimes those decisions may not be wise decisions . . . you are young you can still get drunk, drive a car, crash it, get pregnant, have a tattoo, all sorts of things which I might not agree with but you're still legally entitled to do it and I think an altruistic donor is entitled to make a decision even if they are only 18 or 19. I don't think it's for me to say 'Oh you are too young, you don't know what you are talking about' so I don't share the anxiety of the other team members."—H
2. Presence of the known recipient in the donor-recipient dyad	"There is an issue isn't there, if you have donated a kidney and that kidney doesn't work for whatever reason, like if a husband donates it to . . . or a mum to a child, and it didn't work, the guilt that you would feel for that not working. But if it's an altruistic you wouldn't really necessarily know what had happened and how that was going on and whether the control from you"—H "The relationship is part of the meaning. It can be that the relationship . . . well it's making them feel guilty about . . . had made them feel forced to do it, but actually what we found out is they don't want to do it at all . . . so there's different processes that happen, no, it's not straightforward, it's not . . . It can be, often less straightforward than the altruistics"—L "I think they have got no vested interest really, emotional interest in the recipient's wellbeing. They have given their kidney and I suppose as soon as they have given their kidney they feel as though they are in the right, their job has ended. You know, it's not as if they had to take care. They don't provide a carer role or a supportive role to the recipient so from that point of view they are different I suppose."—H "some of these altruistic donors afterwards, they do expect something back from the recipient, so when they don't hear something back from that recipient, even just to say 'Oh OK, we are OK', they find that very difficult"—H "that is a question of you can never say to them 'I did this for you, so you have got to do this for me later' because there is a pressure/coercion bit that can happen post-op. . .. we always assume that it's going to be offered up front at the beginning but it might be that the donor later says 'Well I did that for you and you are not being really helpful and fair now,' you know, 'mum has left you 60% of the inheritance and I am only getting 20% because the other 20% is going somewhere else, how about upping mine by sharing?' 'Well why did I do that?' 'Well, look what I did for you'—you can hear it can't you?"—L
2a. Understanding UKD vs. SKD motivations	"But my personal feelings on altruistic is it doesn't really exist. I think everyone gets something out of it, I don't honestly believe they are doing it just for the good of others, and even if they are quiet about it and they're not standing on a soap box going 'Look at me, I have donated a kidney,' they must internally get some sort of validation or some purpose from doing that."—H "I think altruistic by strict definition to me is a truly selfless act and I think there are very few things in life that are truly selfless acts and I don't think altruistic donation is one of them"—L "I have . . . reservations about the human's ability to be truly altruistic and whether we are facilitating some form of process by which there's either cathartic process or some form of other process going on that we are facilitating in the name of altruism"—H

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TABLE 1 | (*Continued*) Themes with corresponding subthemes and quotations (H—denotes high volume transplant centre, L—denotes low volume transplant centres).

Theme/Subtheme	Quotes
	"I think the ethics are very difficult aren't they, because again it's touching on what is true altruism, what really is the endpoint for what people hope to get out of donating a kidney? As I said before we have been stung here within the last year and we got some very bad press from somebody who you can argue therefore isn't altruistic, so you could say are we using the right word when we call it altruistic donation? If we didn't use that word then the ethical issues may not be quite as large."—H "The differences, we over-cook the altruistic, we do, we do, particular on the psychological side"—H "They do have a psychological assessment . . . and I think that's really important to get that right because if they have got an ulterior motive or if they are going into it not necessarily 100% sure of what they are actually doing, then that could potentially lead to problems"—H "They don't automatically get a psychological assessment if they are a live donor pair, whereas an altruistic we always ask for them to be assessed."—L "I think people also think people do it on alternative motives, I don't think people can quite believe that somebody would just do it altruistically."—L "More the issue around directed donation is an element of . . . if you think there's an element of coercion. So occasionally you will see a family group come in and I remember this recently . . . the brother said 'I don't know why I am here, my sister told me to come, I am sorry but I really don't want to do this' so that's more the get-out in the directed side, someone who has come along because they don't feel they can say no."—L
3. Need for better management of patient expectations	"Because that relationship isn't there for the non-directed altruistic donor, it's absolutely essential that they have a full understanding of the risks so I do spend a lot more time with them talking about risk . . . Whereas with the directed donor, if it's a complex paediatric case or someone with a medical problem where the disease might recur, I will give much more tailored information and say 'Look, this is a really high risk transplant for this recipient, you need to be aware of x, y, z' and then we will have a discussion around that with the directeds whereas we can't do that with the undirecteds."—H "The downside I think is managing . . . I think it's more about managing the post-op and managing people's expectations and as I say, I think not having, not seeing . . . they are often really keen and ask all the time how the recipient is doing, because they are often not in this hospital they don't get that feedback"—L "their expectations will probably be one of the sort of difficult things to manage . . . it's so important we give them as much information as we possibly can. It might be worth just asking the sort of individual as part of their process, what did they expect as part of the outcome of this? Would they expect the recipient to be in touch? You know, I think establishing what their expectations would be."—L "Well I think directed donors, they have someone specific in mind so they have got that motivation, they know that person whereas from an altruistic they don't know that person . . . I think they have to have those expectations clearly put out at the beginning. They need to be prepared a lot more I think."—H "the challenges are . . . actually making them accept the risk because they are just like 'Oh it's fine. If you are telling me I can do it, I can do it so that's fine,' 'but it still comes with risk'—that's what you find. Whereas it's not an emotive 'but it doesn't matter if something happens to me, I want my loved one fine'—they haven't got that loved-one pull like I keep saying but you . . . 'Are you really listening? You know, ultimately you could die under anaesthetic' 'Yeah, yeah, yeah that's fine' and I just think. . . . 'well don't just say 'Yeah, yeah it's fine, are you really listening, are you understanding that point you know, you don't have to do this?'"—L
4. Managing visceral reactions about the "typical" UKD and implications for treatment	"I am sure they have their own reasons for making that decision, but personally I find that it's a very difficult decision to understand . . . I think the only thing is, I still struggle with why anybody would want to do it. I wouldn't!"—L "Because you think if you want to help people you could go and volunteer at a soup kitchen or, you know, once a week instead of drastically being operated on and having an organ removed."—H "I have never heard of anybody being put off by an abrupt doctor or, you know, a rude psychiatrist or something, I have never heard of anything like that."—H "At what point are we facilitating some form of pathological behaviour"—H "I think as a group it's easy to look at them and think they are all strange, with some hints of, you know, mad behaviour."—H "There are some who have pathological traits to them and it's those that I am trying to ensure don't give"—L "I think you are more likely to get a pathological personality offering it than you would . . . statistically, than somebody giving it to their spouse for various reasons."—L

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TABLE 1 | (*Continued*) Themes with corresponding subthemes and quotations (H—denotes high volume transplant centre, L—denotes low volume transplant centres).

Theme/Subtheme	Quotes
	"they are not very easy to work with because they have unrealistic expectations, they think that OK, I am here to give you an organ and because I am a special donor you have to treat me specially, and we do treat all our donors specially because they're all special people"—H "Some of them have proven to be mentally unstable"—L "I do think a lot of people think people are mad and I think that people think why would you do that?"—L "I do sometimes wonder if we should even be doing it . . . when we first started I think, I think maybe some healthcare professionals (myself included) felt it was a dubious decision mainly in that anyone that came forward to do it could be considered slightly mad."—H "I just think we have all, individually, had experiences of altruistic donors being slightly mentally unstable or not predictable or . . . I would say needy afterwards actually, and I think a lot of them have proven to be attention-seeking, self-publicity seeking and are rather daunted by their lack of attention or lack of emotion given to them afterwards"—L
5. Complex attitudes toward a promising new practice	"I think we . . . as a transplantation community need to think about where altruistic donors fit in as well. I think there is a conception that these altruistic donors don't necessarily go to the fittest of recipients because they are altruistic donors and I think there is a danger that we could see them as a second-rate donor compared to directed donors perhaps . . . I think as a transplant community I don't think we have quite worked out where altruistic donors fit in, that they could be directed towards patients who weren't necessarily a last resort really, that somebody may actually get more benefit from these kidneys."—L "I think we are all a bit . . . I think altruistic donation as a viable source of organs is still actually very much in its infancy, and we do such small numbers, and I think people's opinions of it will change if we continue to increase in the numbers"—L "I think there has been a change actually because I can remember when . . . the first time I did an altruistic donation, it's quite a few years ago now and I remember . . . one of the coordinators saying 'It's a bit odd, she has offered, this woman has offered, I don't know why. Why would anybody offer a kidney? It's such a big thing, you know'. . .And then as the years have gone by, I suppose 3 or 4 or 5 years ago now, they have said . . . now, it's 'Oh we have got a altruistic one and there's been a change, you know.'"—H "So whenever there is a new programme people are understandably slightly cautious so it's partly a temporal issue, it's a fairly new thing, it's only been going for what, 6/7 years I guess? That's partly it, so from an infrastructure and legal perspective"—H
5a. Need to promote public awareness and acceptance of UKD	"Letting people know about it . . . I think everybody I have spoken to pretty much has heard about it on the radio or TV, or has known somebody who has had a kidney problem so has investigated it. I don't know how you would know about it otherwise, but I know people I talk to don't have any idea that it's something you can do."—L "As with everything, get out there and education. The more people know or see some good results . . . well the problem is your anonymity with the recipient and things like that, but there's nothing like good story stuff to make people think that that's perhaps that's something they could do. Education, I mean things like the advertising on telly."—L "Well I mean the only thing would be a publicity campaign. I mean I think you could do . . . if you got it on national telly after Emmerdale or Coronation Street, you know, then . . . that's what you need, you need a big publicity programme because actually if you had . . . I can't remember the statistics but you could actually solve the waiting list dilemma completely if you had 1,000 altruistic donors a year rather than 100."—L "I think a lot of it is to do with promoting living donations continuously and also the different aspects, the different options we offer in that direct donation obviously and then there's the paired pool sharing scheme so providing more awareness to the public that way, and I am sure there are other ways as well."—H "we should utilise our . . . the ones, the individuals that have basically donated, it would be nice to utilise them a little bit more in campaigning. 'Actually we can do this, these individuals have done it, and they are doing very, very well' and their stories I think would be more beneficial to the media and the public to see that actually you can do this great deed and still live a normal life as well"—L

living donor kidneys were the best option for someone on the waiting list (when compared to deceased donor kidneys), and that UKD was a promising and growing avenue for live donor transplantation. There was some impression that attitudes were moving away from the earlier stereotypes of UKDrs being driven by pathological motives, although these views persisted and were still quite commonly held.

Need to Promote Public Awareness and Acceptance of UKD

Almost all the staff members who stated that they were broadly in favour of the UKD programme suggested the need to find better ways to promote it amongst the public.

They expressed the view that this would both increase numbers and ensure that future potential donors fully understood the process before offering to donate, therefore

reducing dropout rate and conserving resources. Many staff members referred to the effectiveness of utilising past donors in public awareness campaigns, as well as publicising the experience of both donors and recipients.

DISCUSSION

This qualitative interview study explores the views and experiences of UKD participants drawn from the professional transplant community in the United Kingdom. It provides an in-depth analysis of 59 interviews, currently representing the largest qualitative study investigating transplant professionals' attitudes toward UKD. The main findings are that many participants expressed reservations about proceeding with younger potential donors and favoured specifying a minimum age limit that is higher than the current legal minimum (18 years old). Additionally, many staff expressed concerns about the psychological stability of UKDrs and found their motivations to be complex or unclear. Many staff raised the need to manage UKD expectations particularly around communication with recipients. Finally, the results demonstrate that some healthcare professionals did not think that their personal opinions influenced voluntary self-withdrawal by UKD candidates.

Over the past decade, transplant professionals have criticised the ethics surrounding LKD [17–19], primarily due to the obligation of the principle of non-maleficence. The present study probed the ethical concerns that medical staff must balance when considering all aspects of LKD. Most participants, whilst they still may not be completely comfortable with UKD, recognised that the potential benefits outweighed potential harms, and acknowledged that UKDrs undergo a rigorous assessment process, including a thorough psychological assessment. It was noted that amongst the various roles covered in this study, it was predominantly surgeons who raised the ethical concern of operating on healthy individuals most frequently. We speculate that this is because they are ultimately responsible for the physical act and are answerable should complications occur.

Another ethical consideration was related to donor age; specifically the concern that younger donors may not fully understand the longer-term implications of their decision to donate. Previous research has explored whether minors and young adults should be legally permitted to qualify as donor candidates [20–23]. Using qualitative methodology, Thys et al. (2019) found three reasons for a cautionary view of living donation by minors and young adults, which were all echoed in the present study: concern about the long-term medical and psychosocial risks of donating a kidney at a young age, younger donors' capacity to make informed decisions, perhaps related to their developmental stage and the possibility of younger individuals' greater susceptibility to familial pressure. Similarly, the present study highlighted the ethical dilemmas surrounding age of donation in UKDrs specifically. One emergent concern, specifically for young women, was the possibility of complications related to pregnancy [24, 25]. Our findings suggested there is an inconsistency between transplant centres in the approach taken to younger candidates. As things stand, younger potential donors who are turned down on the grounds of their age by one centre could present to another centre for a different outcome. A national consensus on a minimum age limit or alternatively transparent regional variation would be preferable. Transplant units should publicly clarify what their local policy is both for staff members and potential donors.

One critical issue that emerged from the data was the complexity around the role of altruism within UKD, and why staff placed an overwhelming emphasis on it when discussing UKDrs' motivations. In the UK, all LKD candidates must undergo evaluation by an Independent Assessor on behalf of the Human Tissue Authority (HTA) in order for the donation to be legally approved. The HTA refer to "altruism" as a means of distinguishing between types of donor, rather than it being a prerequisite for UKD. Our findings indicate that staff attach more weight to the concept of "altruism" than the minimum standard applied by the HTA. In fact, almost all staff reported that they referred to UKDrs as "altruistic donors" even though some donors prefer the term "unspecified" [26]. We question how important it is that donors are motivated by "pure altruism," as opposed to what might be seen as less selfless reasons. For example, staff members cited a range of motivations they had seen, including war veterans giving back if they have taken a life in the past, individuals atoning for bad behaviour, relationship with renal failure patients, or people seeking religious "credits." Whilst none of these can be characterised as strictly altruistic we argue that likewise they cannot be put in the same category as receiving material or financial benefit. Previous discussions in the transplant literature demonstrate inconsistency in the way the principle of altruism is applied to living donation [27, 28]. Saunders (2012), for example, argued that while rejecting certain questionable motivations, it is short-sighted to place overriding emphasis on altruism as the guiding principle. He suggested that solidaristic donation—motivated by feelings of social or group-focused solidarity—seems to encompass altruism as well as other acceptable motivations [28]. The present study supported the argument that a broader definition of acceptable motivations is appropriate and would perhaps open the door to a larger pool of donors.

There is an apparent assumption held by many staff members that SKDrs choose to donate purely out of love and loyalty to their loved one or family member. Conversely, the motives of UKDrs are regarded with suspicion and interrogated more intensely by some members of the medical team. Whilst we acknowledge that SKDrs may derive more benefit than UKDrs due to their personal connection with the recipient, we question whether the more critical approach towards UKDrs motivations by the medical team is justified or logical. Many staff members, whilst acknowledging the importance of the rigorous assessment of UKD, noted that the same standards were not always applied to SKDrs and questioned whether they should be, due to potential issues such as guilt, family obligation, manipulation or reciprocity. Some authors have even suggested restraint of the LKD programme because of the possible social and familial tensions it may provoke [29]. To date there has been very

little research on the complex family dynamics of LKD but what little literature does exist demonstrates that feelings of obligation, psychological distress and social-familial alienation following donations are very real [30, 31]. There is an argument to be made that assessment of SKDrs should be brought up to the same rigorous standards to that of UKDrs.

The traditional mindset, documented in previous literature [32, 33], that UKDrs are driven by a form of psychopathology, was also suggested by the current study. Our study demonstrated that there is still a lot of negativity towards UKD, and thus the need to educate individuals towards a more open-minded mentality towards all living kidney donors. Whilst there is not a strong body of evidence affirming the psychological wellbeing of UKDrs, neither is there evidence of an underlying psychopathology. Previous research demonstrates that UKDrs have positive outcomes [34] and equivalent psychological outcomes to SKDrs [9, 35]. Motives are honourable, however the evidence to date for the personal benefit of UKD is mixed [36], and studies reporting benefits are mainly retrospective [37]. The BOUnD study will hopefully help to fill this gap in the literature [8]. We feel strongly that further training amongst staff is necessary to develop a consistent and affirmative approach to UKDrs at all centres. A concerted effort to increase healthcare professionals' awareness of the value of UKDrs, and to address their concerns, would greatly strengthen the overall programme.

A previous study investigating the experiences of completed, medically and self-withdrawn donors [38], found that some potential UKDrs who self-withdrew from the programme reported that they did so because of their impression that some healthcare staff were against them subjecting themselves to surgery. However, in this study, staff members did not perceive that their personal opinions were a factor in self-withdrawal. The clinical implication of this disconnect would be to ensure that the staff's private opinions do not affect their treatment of donors or influence the way they communicate with them. It is important for staff members to present a consistent and unbiased position even if they have personal reservations about UKD. Should professionals strongly object to UKD, it may be advisable to consider whether professionals should be allowed to conscientiously object to being involved. Such a system would allow healthcare professionals to choose to opt out from the practice if it goes against their personal beliefs and values.

Many staff members expressed the view that donor expectations needed to be managed, specifically when it comes to the issue of anonymity. There is however a larger discussion amongst UKD programmes globally around whether or not the condition of anonymity should be revisited [10, 26, 39, 40]. In one of the few qualitative studies of physicians' attitudes towards UKD, Fortin et al. (2008) found considerable opposition to lifting the strict requirement for anonymity [41]. This is in line with the current study, which found that some staff acknowledged that some UKDrs struggled with the requirement for anonymity, principally due to a strong psychological need for connection with the recipient. This correlates with a paper by Pronk et al which identified that some UKDrs remained troubled by and curious about the lack of contact with their recipient many years after their donation [42]. Future studies need to probe this issue both from the perspective of the recipient as well as the donor to determine if there is a mutual reciprocal benefit that challenges the current rules around anonymity. It should be noted that for a donor, the ability to know the outcome of the donation does not contradict the principle of altruism. Rather, knowledge of the outcome may relate to the need for closure.

Strengths and Limitations

The strengths of this study lie in the number of interviews which allowed for data saturation. It is acknowledged that the data were collected 7 years ago from only six centres, and that transplant professionals' perspectives could have evolved since. However, a significant shift in either positive or negative views or opinions does not appear apparent within the academic or clinical environment.

Many participants, particularly those working in low volume transplant centres, acknowledged that they had only minimal clinical experience working with UKDrs. Consequently, these interviews were much shorter than those conducted in higher volume centres, however the overall impressions were similar. Additionally, opportunistic sampling is a limitation which should be addressed in future research. However, the sample in our study was still representative of the transplant community. Finally, we were not able to adjust for interviewees' exposure. Despite these limitations, this is the first qualitative study to assess the approach of transplant professionals towards UKDrs in depth and as such offers valuable insights.

This paper is applicable to other areas of transplantation, and indeed the wider healthcare setting, by acknowledging the relationship between professionals' views and the impact of their subconscious communication to patients. Participants in this study were explicitly asked whether they felt they unduly influenced UKDrs during their interactions with them and reported that they did not. However, a study conducted simultaneously within a group of donors and withdrawn donor candidates who would have been cared for by some of these same individuals reported differently. Healthcare professionals ought to be mindful of how their views may negatively influence patients in the clinical environment as they may not be fully aware of their impact.

Conclusion

This study provides valuable insight into the practice of UKD and has identified key areas which need addressing. There needs to be clarity on the age limit policy for each transplant centre, a discussion around the necessity of formal psychological assessment for all living kidney donors, and a new approach to managing UKDrs' expectations, particularly around anonymity. Specific suggestions are to enhance training and improve consistency between all members of the multidisciplinary teams across all UK transplant centres. Implementing these findings will strengthen the practices towards LKD, improve the donation experience for everyone involved, and result in an increased acceptance of unspecified donation as a key element in the kidney transplant programme.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Berkshire Research Ethics Committee (Ref 15/SC/0637). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MZ: data curation, formal analysis, methodology, resources, software, validation, visualization, writing—original draft, writing—review and editing. NM: conceptualization, funding acquisition, methodology, project administration, supervision, validation, visualization, writing—review and editing. HD: writing—review and editing. PG: investigation, writing—review and editing. SN: methodology, supervision, writing—review and editing. JC: methodology, supervision, writing—review and editing. TA: investigation, writing—review and editing. AC: investigation, writing—review and editing. LW: investigation, writing—review and editing. PM: writing—review and editing. HM: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, supervision, validation, visualization, writing—review and editing. 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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Serum Levels of Adropin Improve the Predictability of MELD and Child-Pugh Score in Cirrhosis: Results of Proof-of-Concept Clinical Trial

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Adropin is a peptide that was suggested to have a role in cirrhosis. The present study aimed to determine the ability to use serum adropin levels to improve their prediction accuracy as an adjunct to the current scores. In a single-center, proof-of-concept study, serum adropin levels were determined in thirty-three cirrhotic patients. The data were analyzed in correlation with Child-Pugh and MELD-Na scores, laboratory parameters, and mortality. Adropin levels were higher among cirrhotic patients that died within 180 days (1,325.7 ng/dL vs. 870.3 ng/dL, $p = 0.024$) and inversely correlated to the time until death ($r^2 = 0.74$). The correlation of adropin serum levels with mortality was better than MELD or Child-Pough scores ($r^2 = 0.32$ and 0.38, respectively). Higher adropin levels correlated with creatinine ($r^2 = 0.79$. $p < 0.01$). Patients with diabetes mellitus and cardiovascular diseases had elevated adropin levels. Integrating adropin levels with the Child-Pugh and MELD scores improved their correlation with the time of death (correlation coefficient: 0.91 vs. 0.38 and 0.67 vs. 0.32). The data of this feasibility study suggest that combining serum adropin with the Child-Pugh score and MELD-Na score improves the prediction of mortality in cirrhosis and can serve as a measure for assessing kidney dysfunction in these patients

Keywords: cirrhosis, MELD, Child-Pugh score (CPS), adropin, fibrosis

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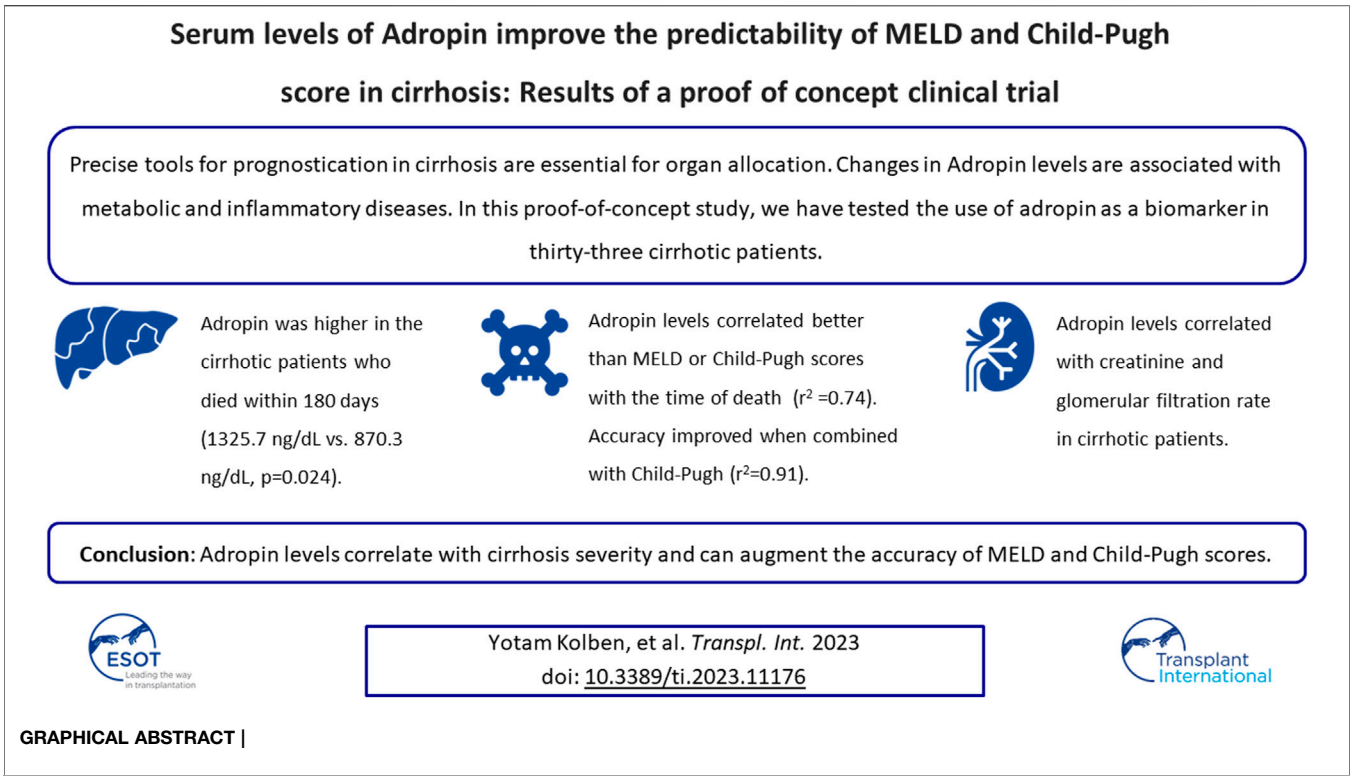
INTRODUCTION

Cirrhosis is a progressive fibrosing nodular condition that disrupts the entire typical architecture of the liver [1]. More than 160 million people worldwide had cirrhosis in 2017, and more than 0.8 million patients with cirrhosis die yearly [2]. Liver transplantation is currently the only curative therapy available. Prioritizing liver allocation in non-acute liver failure is based on

Abbreviations: AUC, area under the curve; CPS, Child-Pugh score; DM, diabetes mellitus; DILI, drug-induced liver injury; HCC, hepatocellular carcinoma; INR, international normalized ratio; IRB, institutional review board; MELD, model for end-stage liver disease; MELD-Na, Na-model for end-stage liver disease; NAFLD, nonalcoholic fatty liver disease; NASH, GFR nonalcoholic steatohepatitis glomerular filtration rate.



GRAPHICAL ABSTRACT |

mortality prediction scores. Precise organ allocation is crucial in the face of organ shortage, which accounts for a large proportion of wait-list mortality [3]. Accurate prognostication is essential for coordinating patients' expectations, assessing therapeutic risk-benefit balance, and more.

The Child-Pugh score (CPS) classification and the Model for end-stage liver disease (MELD) score are commonly used models for predicting mortality in cirrhosis [4]. However, these models have several drawbacks. Ascites and encephalopathy included in the CPS classification are subjective and may be variable according to the physician's judgment and the use of diuretics and lactulose. The international normalized ratio (INR) does not sufficiently reflect coagulopathy and liver function and is variable throughout different laboratories [4]. Although adding sodium to MELD enhances its performance [5], improving the currently available methods for assessing the degree of severity and predicting prognosis in chronic liver disease remains an unmet need [6–12].

Adropin is a 76-amino-acids-secreted peptide encoded by the Enho gene and is conserved among humans, mice, and rats [13]. The physiological role of Adropin in the liver is unknown. High levels of Adropin correlated with a low incidence of type 2 diabetes mellitus (DM), elevated HDL cholesterol, lower BMI, LDL cholesterol, triglyceride levels, and blood pressure [14–16]. Preliminary data suggested that serum levels of Adropin may be related to the degree of disease severity in cirrhotic humans [17].

The present single-center proof-of-concept study aimed to determine the potential of using serum levels of adropin as a prognostic biomarker in patients with chronic liver disease and to determine its use as an adjunct to MELD and CPS for improving their performance in predicting mortality.

PATIENTS AND METHODS

Ethical Considerations

This single-center prospective, observational study was approved by the Institutional Review Board (IRB) (Hadassah medical center 0634-19-HMO, NCT04660409). Participants signed informed consent during enrollment as defined by the local IRB.

Study Population

Adult subjects (18–80 years) with chronic liver disease of all etiologies were enrolled. The main exclusion criteria were evidence of other acute severe disease or any acute medical condition within 48 h of blood tests. Controls were adults without known liver disease.

Serum Adropin Concentration and Clinical Data

A single blood test for serum levels of adropin was obtained. Serum was collected using a serum separator tube and centrifuged for 20 min at 1,000 × g. Samples were stored in aliquots at −80°C.

TABLE 1 | Patients characteristics.

Characteristic	Etiology		Total (*N* = 33)
	NASH (*N* = 16)	Other (*N* = 17)	
Adropin levels–ng/dL	973.1 ± 136.1	934.3 ± 89.7	953.1 ± 79.3
Female sex–no. (%)	5 (31.3)	11 (64.7)	16 (48.5)
Age–yr	67.9 ± 2.4	67.0 ± 2.6	67.7 ± 1.8
Etiology[a]–no. (%)			
NASH	16 (100)	0 (0.0)	16 (48.5)
AIH	2 (12.5)	6 (35.3)	8 (24.2)
HBV	0 (0)	5 (29.4)	5 (15.2)
HCV	1 (6.3)	3 (17.6)	4 (12.1)
Biliary cirrhosis[b]	1 (6.3)	1 (5.9)	2 (6.1)
DILI	1 (6.3)	1 (5.9)	2 (6.1)
Other[c]	1 (6.3)	4 (23.5)	5 (15.2)
Hepatocellular Carcinoma–no. (%)	5 (31.3)	4 (23.5)	9 (27.3)
Child-Pugh group–no. (%)			
A	1 (6.3)	5 (29.4)	6 (18.2)
B	12 (75.0)	8 (47.1)	20 (60.6)
C	3 (18.8)	4 (23.5)	7 (21.2)
MELD-Na score–mean	18.1 ± 1.5	14.1 ± 1.4	16.0 ± 1.24
Sodium–mmol/L (136–145)	133.2 ± 1.0	133.8 ± 0.9	133.5 ± 0.7
Bilirubin–μmol/L (5–21)	40.7 ± 7.9	70.2 ± 38.0	55.9 ± 19.8
INR (0.9–1.2)	1.34 ± 0.05	1.26 ± 0.03	1.3 ± 0.03
Creatinine–μmol/L (62–115)	116.9 ± 17.1	103.1 ± 12.4	109.8 ± 10.4
Albumin–gr/L (32–48)	31.7 ± 1.7	31.3 ± 1.4	31.5 ± 1.1

Abbreviations: AIH, autoimmune hepatitis; DILI, drug-induced liver injury; HBV, hepatitis B virus; HCV, hepatitis C virus; INR, international normalized ratio; MELD, a model for end-stage liver disease; NASH, nonalcoholic steatohepatitis.

[a]*For eight patients, cirrhosis was attributed to more than one diagnosis.*

[b]*Including one patient with primary sclerosing cholangitis and one with primary biliary cirrhosis.*

[c]*Including patients with alcoholic hepatitis (who also had NASH, diagnosis), cardiac cirrhosis, sickle cell disease (one each), and two cryptogenic cirrhosis.*

Adropin levels were determined using a sandwich enzyme immunoassay kit for adropin (Cloud Clone Corp., Katy, TX, United States) according to the manufacturer's procedure. The optical absorbance was measured spectrophotometrically at 450 nm.

Subjects' clinical and laboratory data were generated from the patient's medical records.

Statistical Analysis

Comparing adropin serum levels between cirrhotic and non-cirrhotic patients was carried out using the non-parametric Mann-Whitney test. The same test was used to compare different scoring systems trying to predict mortality. ROC analysis was used when the score was significantly different between groups. The Kruskal-Wallis non-parametric test was applied to compare Adropin levels between three independent groups. The non-parametric tests were used due to the small sample size and the non-normal distribution of adropin levels in some subgroups compared. The Pearson correlation coefficient was calculated to assess the strength of the linear association between adropin levels and other quantitative variables. All tests applied were two-tailed, and a *p*-value of 0.05 or less was considered statistically significant.

RESULTS

Patient Characteristics

Clinical and laboratory data were obtained from 33 cirrhotic patients (**Table 1**). Sixteen (48.5%) of the patients were females, with a higher female rate among patients without nonalcoholic steatohepatitis (NASH, 64.7% vs. 31.3%). The common etiology was NASH, assigned to 16 (48.5%) patients. It was followed by hepatitis B and hepatitis C with five (15.2%) and four (12.1%) cases each. Eight patients were assigned with more than one diagnosis.

The average MELD-sodium (MELD-Na) score was 16.0, corresponding with most patients (60.6%) in Child-Pough group B. The severity was marginally higher in patients with NASH cirrhosis, albeit not reaching significance (MELD-Na 18.1 vs. 14.1, $p = 0.39$). Hepatocellular carcinoma (HCC) was diagnosed in 27.3%. Five-of the HCC patients had NASH, and four with viral hepatitis.

The laboratory values are described in **Table 1**. Patients with NASH cirrhosis had insignificantly lower bilirubin levels (40.7 μmol/L vs. 75.1 μmol/L, $p = 0.47$) and higher creatinine levels (116.9 μmol/L vs. 103.1 μmol/L, $p = 0.51$).

Serum Adropin Levels in Patients With Chronic Liver Disease

Serum adropin levels were higher among patients with chronic liver disease relative to controls, albeit not reaching statistical significance (953.1 ng/dL vs. 735.0 ng/dL, $p = 0.37$).

Among the patients with chronic liver disease, subjects with NASH and viral-mediated liver disease had similar serum levels of adropin, 973.1 ng/dL and 946.4 ng/dL, respectively. Lower serum Adropin levels were noted among patients with

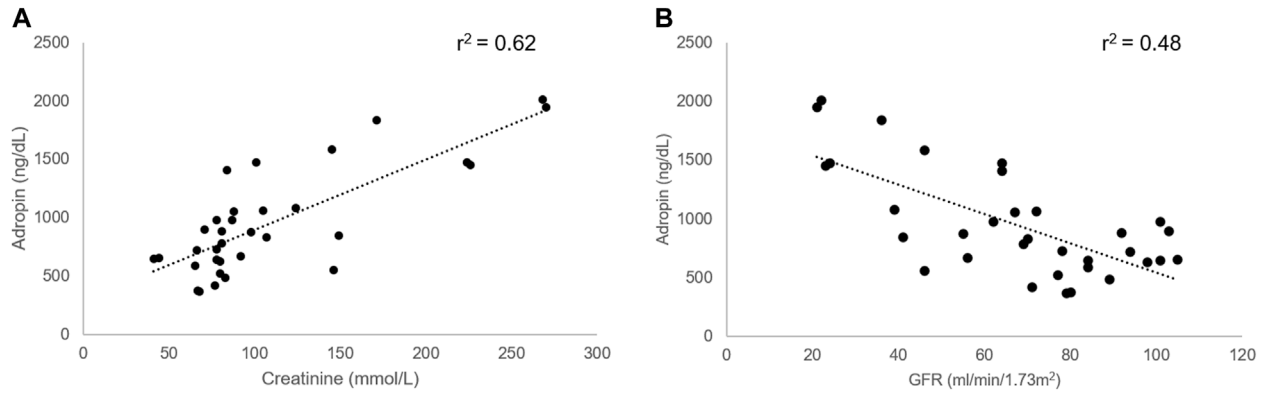


FIGURE 1 | Correlation between adropin and kidney function in cirrhotic patients. A higher level of adropin correlated with concomitant higher creatinine levels **(A)** and lower GFR **(B)** in cirrhotic patients. Abbreviations: GFR, glomerular filtration rate.

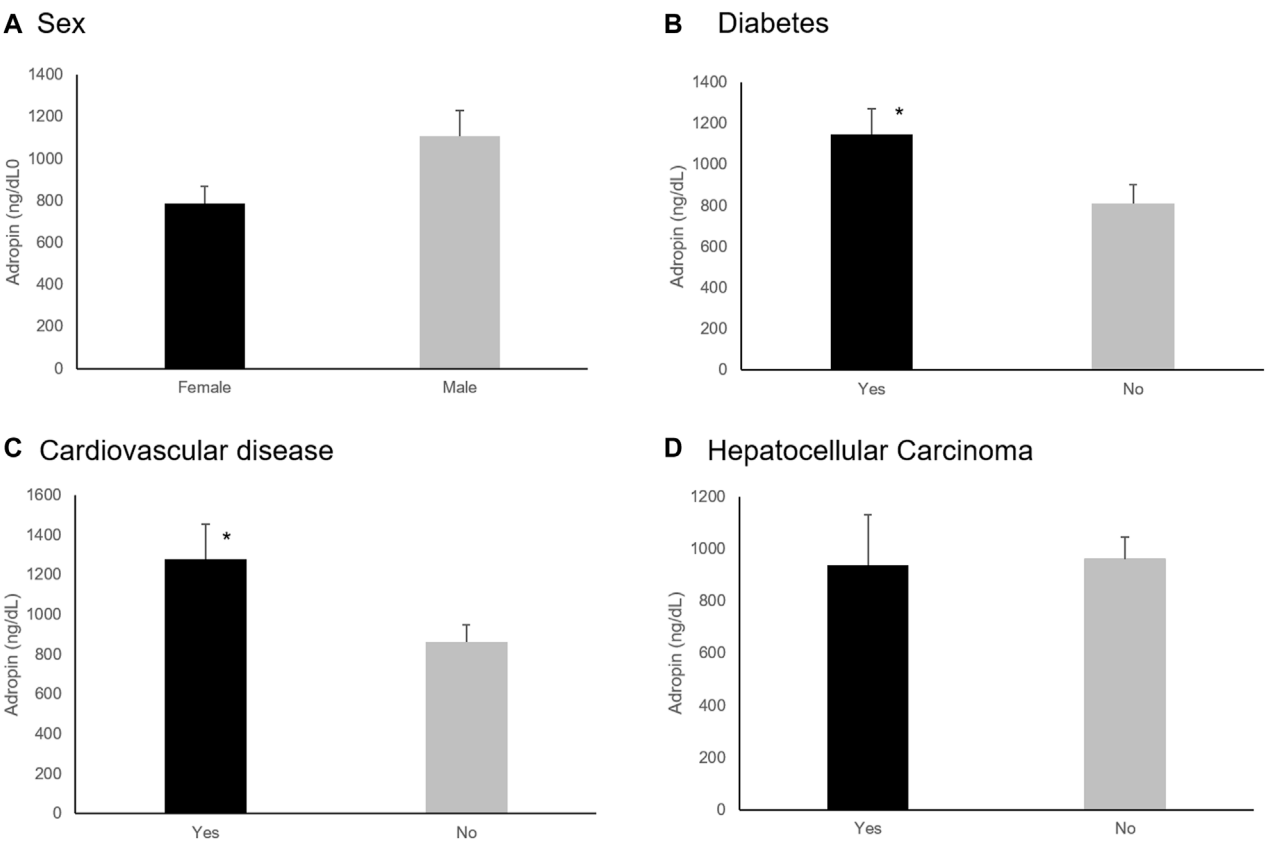


FIGURE 2 | Effect of sex and underlying medical conditions on Adropin levels. **(A)** Females had lower levels of Adropin ($p = 0.056$). **(B)** Diabetes Mellitus and **(C)** cardiovascular disease diagnosis increased adropin levels significantly ($p = 0.035$ and $p = 0.031$, respectively). **(D)** the presence of Hepatocellular carcinoma did not affect adropin levels. *$p < 0.05$.

autoimmune hepatitis relative to all other diagnoses (818.9 ng/dL vs. 996.0 ng/dL, $p = 0.38$). Patients with chronic liver diseases not attributed to the above causes had non-significantly higher adropin levels (1,112.6 ng/dL). It included patients diagnosed with biliary cirrhosis, DILI, cardiac cirrhosis, sickle cell disease, and cryptogenic cirrhosis. Finally, adropin levels were not affected by the development of HCC (938.2 ng/dL vs. 958.7 ng/dL, $p = 0.54$).

Adropin levels were higher in males with chronic liver disease (1,109.8 ng/dL vs. 786.6 ng/dL, $p = 0.056$), and including the

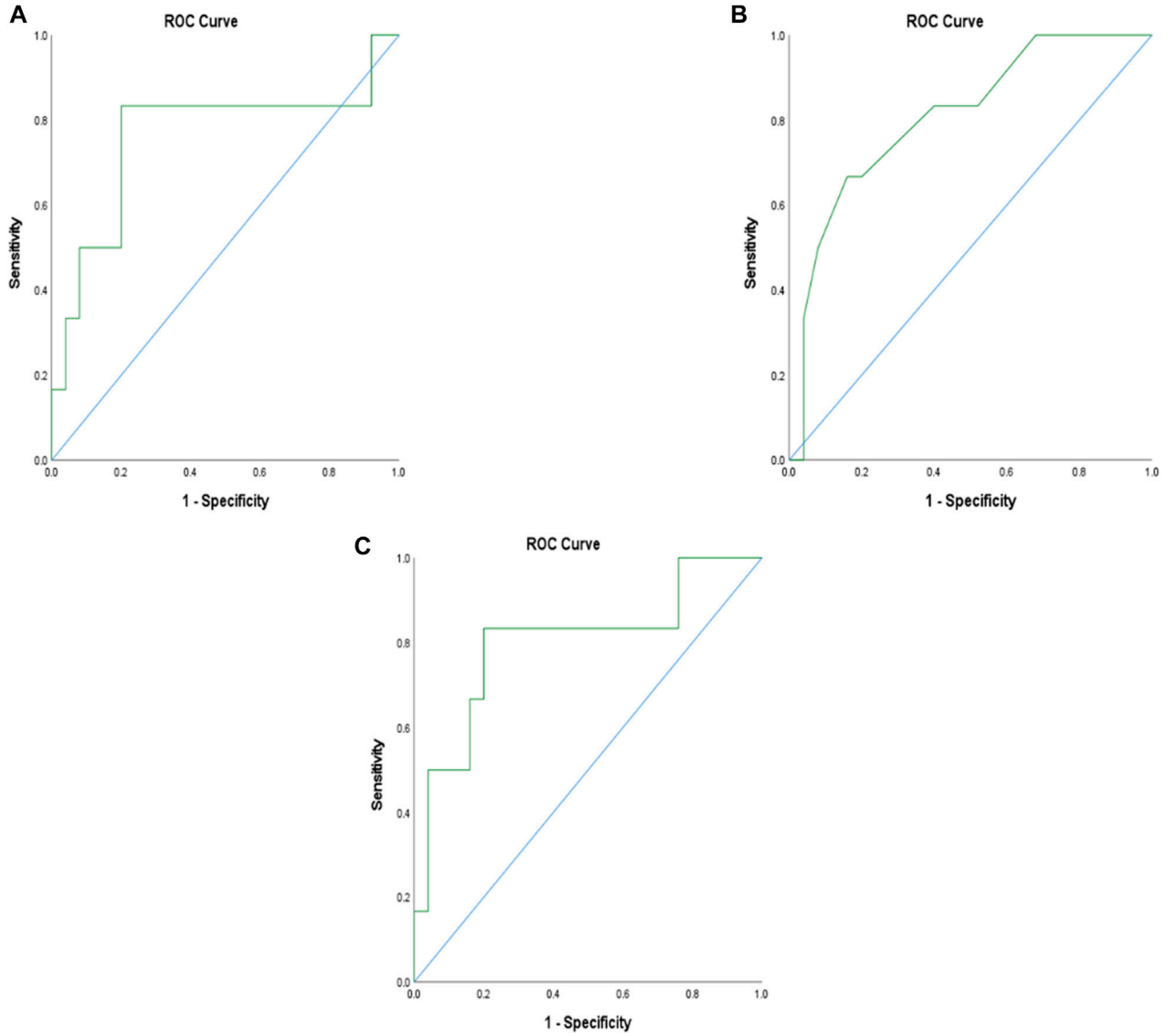


FIGURE 3 | ROC analysis of adropin, MELD-Na, and adropin with Child-Pugh integration score to predict mortality within 180 days. **(A)** ROC analysis of adropin; AUC = 0.76, **(B)**, ROC analysis of MELD-Na; AUC = 0.8 **(C)** ROC analysis of the integration of adropin and Child-Pugh score; AUC = 0.80. Abbreviations: AUC, the area under the curve; MELD, a model for end-stage liver disease.

healthy controls, the sex difference widened (1,067.3 ng/dL vs. 779.2 ng/dL, $p = 0.049$).

Correlation of Serum Adropin Levels and Kidney Functions

An association between adropin levels and serum creatinine levels was documented. Patients with higher creatinine levels had higher adropin levels ($r^2 = 0.62$, $p < 0.001$). The glomerular filtration rate (GFR) was calculated according to the 2021 CKD-EPI equation. Calculated GFR also correlated with adropin levels ($r^2 = 0.48$, $p = 0.032$, **Figure 1**).

Correlation of Serum Adropin Levels and AFP

A negative correlation trend was noted with alpha-fetoprotein (AFP, $r^2 = -0.34$, $p = 0.2$).

No correlation was found with age, bilirubin, INR, sodium levels, or albumin.

Correlation of Serum Adropin Levels With Concomitant Disease

Underlying medical conditions impacted the adropin levels regardless of the etiology of chronic liver disease. Patients with

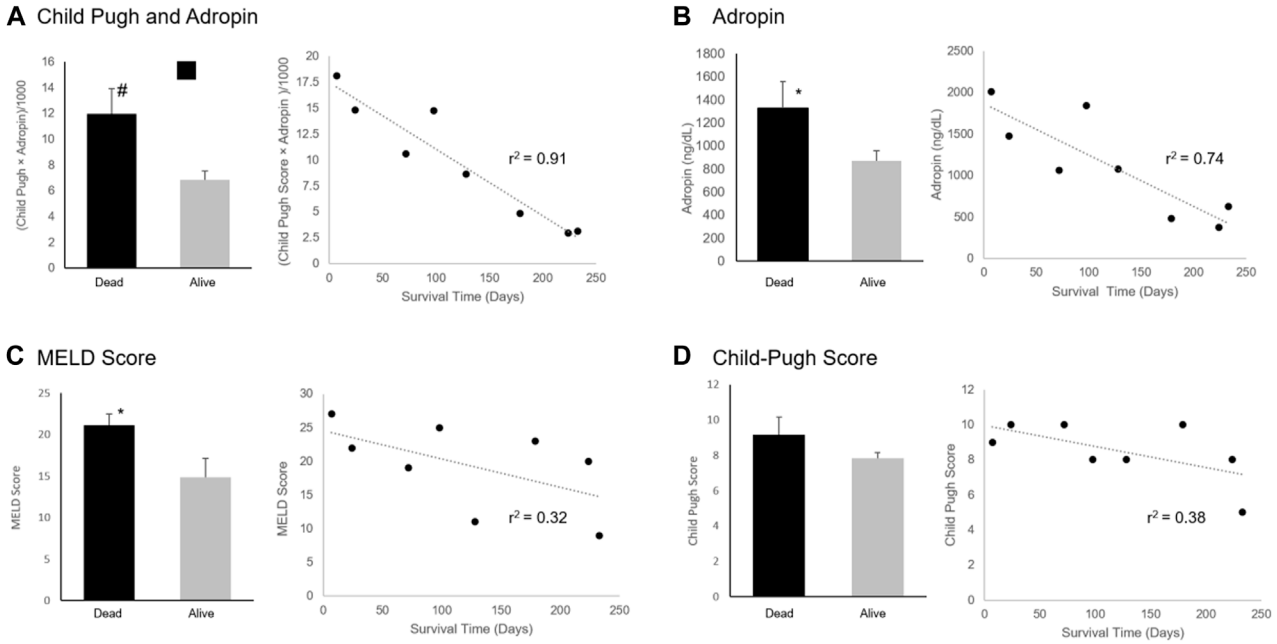


FIGURE 4 | Adropin, MELD Score, and Child-Pugh Score Association with Mortality. Difference between surviving and mortality after 180 days and correlation with time of death of **(A)** An integration of adropin and Child-Pugh score, **(B)** Adropin levels, **(C)** MELD-Na score, and **(D)** Child-Pugh score. Abbreviations: MELD, a model for end-stage liver disease. #$p < 0.01$, *$p < 0.05$.

DM had significantly higher Adropin levels (1,148.0 ng/dL vs. 810.5 ng/dL, $p = 0.035$). A similar effect was noted among patients with cardiovascular diseases (1,279.5 ng/dL vs. 865.2 ng/dL, $p = 0.031$). No effect was noted in patients with hypertension and hyperlipidemia (**Figure 2**).

Serum Levels of Adropin Predict Mortality in Patients With Chronic Liver Disease and Improve the MELD-Na and CPS Predictability

Eight patients (24.2%) died between 7 and 233 days following enrollment in the study. Adropin levels were higher among the six patients that died within 180 days (1,325.7 ng/dL vs. 870.3 ng/dL, $p = 0.024$). The area under the curve (AUC) in ROC analysis for adropin to predict mortality within 180 days was 0.76. When implementing adropin levels of 1,058 ng/dl as a cut-off point, sensitivity and specificity for death during the following 180 days were 83% and 80%, respectively. MELD-Na, but not CPS, was also significantly higher in patients who died 6 months after being tested (21.2 vs. 14.9, $p = 0.043$) with an AUC of 0.8. An integration of adropin levels with the CPS ($Child - Pugh\,score \times Adropin/1000$) increased 180 days mortality differentiation, with higher values predicting mortality ($\frac{CPS \times Adropin}{1000}$, no units, 11.95 vs. 6.87, $p = 0.006$), with an AUC of 0.8 (**Figure 3**). When implementing a cut-off point of 8.54 (no units), the sensitivity and specificity of predicting death within the next 180 days were 83% and 80%, respectively, identical to adropin alone.

Adropin levels were inversely correlated to the time until death ($r^2 = 0.74$) (**Figure 4**). Adropin levels correlated better than MELD-Na or CPS with patients' survival time ($r^2 = 0.32$ and 0.38, respectively). An integration of adropin levels with the CPS ($Child - Pugh\,score \times Adropin/1000$) had excellent correlation with the time of death ($r^2 = 0.91$).

An integration of Adropin levels and MELD-Na ($MELD - Na\,score \times Adropin/1000$) showed a trend for improved prediction of the 180 days mortality (23.64 in deceased vs. 7.17 in alive, $p = 0.082$). Correlation with the time of death was not as precise ($r^2 = 0.67$) as the combination with CPS (**Figure 4**).

DISCUSSION

This feasibility single-center trial showed that serum levels of Adropin predicted mortality better than the often-used MELD-Na and CPS. Moreover, adding Adropin levels to the Child-Pugh and MELD-Na scores improved mortality prediction. Serum levels of adropin levels positively correlated with poor prognostic factors such as mortality and kidney injury.

Serum adropin levels decreased and negatively correlated with liver injury in NASH mice. Knockout of Adropin significantly exacerbated hepatic steatosis, inflammatory responses, and fibrosis in mice. Administration of Adropin bioactive peptides slowed NASH progression in mice [18]. In humans, in a study with 99 patients with alcoholic cirrhosis, serum Adropin levels correlated positively with disease severity [17]. Higher Adropin levels were found in hepatocytes of patients with chronic hepatitis

with a higher degree of fibrosis [19]. This data correlates with our findings. Interestingly, elevated Adropin levels were found in patients with systemic sclerosis, suggesting a role for Adropin in the fibrosis process, which may be different from its role in other metabolic processes [20].

Previous studies demonstrated that higher adropin levels correlated with a better metabolic profile [14, 21]—the present study associated higher levels with DM and cardiovascular diseases. The difference may be explained by the potential effect of cirrhosis on adropin levels.

The correlation between adropin and creatinine was significant. Previous studies regarding adropin and kidney function focused mainly on diabetic nephropathy and showed a negative correlation between adropin and nephropathy progression [22, 23]. Hepatorenal syndrome is a common and severe complication of cirrhosis, with limited treatment options. Much of its pathophysiology beyond circulatory dysfunction is yet to be defined [24]. The present study's data suggest that adropin may play a role in this pathologic process.

This study is limited by the relatively small number of patients and being a single-center study. The significance of the data supports extensive studies for determining the potential role of adropin as a biomarker and therapeutic target in these patients. In addition, the exact cause of death was unknown, preventing us from inferring about adropin relation to cirrhosis-related deaths. However, as cirrhosis is a significant driver of morbidity and mortality, an association with all-cause mortality is essential for medical decision-making.

In summary, elevated serum adropin levels are a poor prognostic factor in patients with chronic liver disease independent of the etiology. Adropin was superior to the standard prognostic models, CPS and MELD-Na, in predicting mortality and correlated with decreased renal function in cirrhotic patients. It can serve as a variable for improving the prediction performance of current scores. Larger cohorts are expected to shed light on the potential use of adropin as an additional biomarker to diagnose better and predict the prognosis of chronic liver disease and as a potential new therapeutic target in cirrhosis and hepatorenal syndrome.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hadassah Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YK, ArK, AsK, and YuI: conducted the study, analyzed the data, and wrote the manuscript. SW-Z, conducted the tests and wrote the manuscript. ME, regulated the patients. YaI wrote the mansucript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

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ORIGINAL RESEARCH
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Results of Lung Transplantation for Cystic Fibrosis With Selected Donors Over 65 Years Old

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Lung transplantation is limited by the shortage of suitable donors. Many programs have begun to use extended criteria donors. Donors over 65 years old are rarely reported, especially for young cystic fibrosis recipients. This monocentric study was conducted for cystic fibrosis recipients from January 2005 to December 2019, comparing two cohorts according to lung donor age (<65 years or ≥65 years). The primary objective was to assess the survival rate at 3 years using a Cox multivariable model. Of the 356 lung recipients, 326 had donors under 65 years, and 30 had donors over 65 years. Donors' characteristics did not differ significantly in terms of sex, time on mechanical ventilation before retrieval, and partial pressure of arterial oxygen/fraction of inspired oxygen ratio. There were no significant differences in post-operative mechanical ventilation duration and incidence of grade 3 primary graft dysfunction between the two groups. At 1, 3, and 5 years, the percentage of predicted forced expiratory volume in 1 s (p = 0.767) and survival rate did not differ between groups (p = 0.924). The use of lungs from donors over 65 years for cystic fibrosis recipients allows extension of the donor pool without compromising results. Longer follow-up is needed to assess the long-term effects of this practice.

Keywords: lung transplantation, elderly donors, cystic-fibrosis, lung procurment, lung aging

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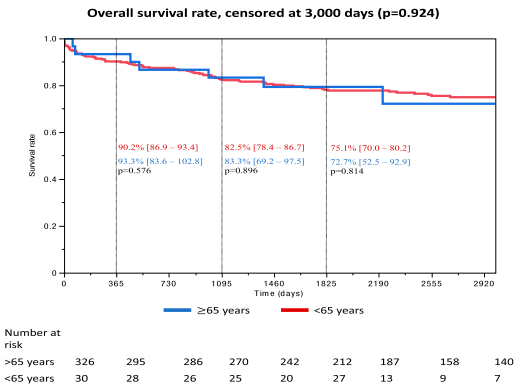
INTRODUCTION

The number of lung transplants has increased steadily since the first transplant in 1986. While optimal donor criteria were [1] have been defined, the need to broaden them has gradually emerged with marginal donors [2–4]. Over time, donation methods have evolved, including donation after circulatory death and *ex vivo* lung perfusion (EVLP) strategies. This makes it

Abbreviations: BLT, bilateral lung transplantation; CF, cystic fibrosis; CLAD, chronic lung allograft dysfunction; ECMO, extracorporeal membrane oxygenation; EVLP, *ex vivo* lung perfusion; HELT, high emergency lung transplant; ICU, intensive care unit; IPF, idiopathic pulmonary fibrosis; LOS, length of stay; LT, lung transplantation; MV, mechanical ventilation; OS, overall survival; PGD, primary graft dysfunction, TLC, total lung capacity.

Results of lung transplantation for Cystic fibrosis with selected donors over 65 years old

- Elderly donors provide an opportunity to enlarge the donor pool. Donors over 65 years old are rarely reported especially for young cystic fibrosis recipients.
- A monocentric retrospective study compared donors <65years vs >65 years in a an homogeneous, young cystic fibrosis recipient population.
- 326 patients had donors under 65 years, and 30 had donors over 65 years.
- At 1, 3 and 5 years, the percentage of predicted forced expiratory volume in 1 second (p=0.767) and survival rate did not differ between groups (p=0.924).
- The use of lungs from donors over 65 years for cystic fibrosis recipients allows a donor pool extension without compromising results.



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GRAPHICAL ABSTRACT |

possible to requalify certain grafts. However, the consequences of certain intrinsic selection criteria, such as age, remain uncertain.

Intuitively, the use of "young" grafts should be preferred for all recipients. However, data on lung aging are scarce and their consequences in transplantation are little known. Over the years, the donor age barrier has been gradually pushed, and the use of older grafts became a necessity.

At the beginning of the modern era of LT, it was considered that the ideal donor's age should be <55 years [1]. This was based on retrospective analysis on the UNOS registry that showed a negative association between donor age and extended graft ischemic time, particularly in donors aged >55 years where ischemic time usually exceeded 6 h [5–7]. Howeverover time, LT indications were widened progressively, and optimal donors no longer corresponded to the emerging needs. For this reason, the boundaries of donor age, as well as other criteria, have been progressively modified.

The broadening of the age limit appears logical for diseases affecting older groups of recipients such as Idiopathic pulmonary fibrosis (IPF) and emphysema. However, for young recipients the question is crucial, as one of the pitfalls of allocation priority rules is that optimal transplants go to most urgent cases; often elderly patients, when some stable young patients are possibly offered grafts with expanded criteria.

It is essential to evaluate this practice in order to know the outcomes after LT with older donors. In our monocentric experience, we wanted to evaluate the effect of graft age in cystic fibrosis by comparing donors >65 and <65. We studied both survival and functional evolution.

PATIENTS AND METHODS

To assess the effect of donor age on outcomes after lung transplantation, a retrospective analysis of all bilateral lung transplants performed for cystic fibrosis was conducted in our center between January 2005 and December 2019.

Re-transplants were excluded. Two cohorts were defined according to donor age, one group with donors aged <65 years and the other group with donors aged ≥65.

Primary objective was the comparison of survival rate at 3 years between the two groups. Additionally, we conducted a secondary analysis, shown in a **Supplementary Material**, for survival rate at 5 years (**Supplementary File S1**). Secondary endpoints included the occurrence of grade III Primary Graft Dysfunction (PGD3) [8] at 24, 48, and 72 h following LT, the initial duration of mechanical ventilation (MV), the initial length of stay in the intensive care unit (ICU LOS), overall hospital length of stay (hospital LOS), the occurrence of graft neoplasm and Chronic lung allograft dysfunction (CLAD) onset at 3 and 5 years.

Donors' Lungs Allocation, Assessment and Procurement

All lungs were offered to our center by the *Agence de biomédecine* (ABM). Once the offer was accepted, the final assessment and retrieval were conducted by our procurement team. Assessment routinely included bronchoscopy, and macroscopic evaluation of the lung. Emphysematous lungs with bullae or rarefied parenchyma were rejected. *Ex vivo* lung perfusion with the Toronto technique [9] was used to evaluate and optimize marginal lungs. We retrospectively used the donor score [10] to assess the quality of

the graft (range, 0–18; based on age, history of smoking, P/F Ratio, chest radiographs, and bronchoscopic findings).

Demographic data of donors and recipients were retrospectively recorded. Post-transplant follow-up parameters included lung function parameters at 1st; 2nd, 3rd as well as 5th postoperative year, such as forced expiratory volume in 1 s (FEV1) and ratio of FEV1/forced vital capacity (FVC). Predicted FEV1 was calculated for each recipient using the formula FEV1 = race × [(0.0395 × height) - (0.025 × age) - 2.6]. Since all recipients in the analyzed cohorts were Caucasian, "race" was substituted by "1" in the formula. The measured FEV1 was then expressed as the percentage predicted FEV1, and as a ratio to best post-operative FEV1 in order to assess intra-patient function evolution according last ISHLT consensus on CLAD [11]. Predicted total lung capacity (TLC) was calculated for each donor and recipient using the formula TLC = (height × 7.992)—7.081 for men and TLC = (height × 6.602)—5.791 for women.

Our surgical protocol for lung transplantation in CF consists of a sequential double lung transplant through a double anterolateral thoracotomy sparing the sternum [12]. Peripheral Veno-Arterial Extra Corporeal Membrane Oxygenation (ECMO) was initiated through femoral cannulation when intraoperative support was required. Post-operative ECMO was only used if PaO_2/FiO_2 < 100 mmHg or hemodynamic impairment [13].

Bronchial complications are described when major interventional treatment was necessary.

From an immunological point of view, regarding cellular rejection, we evaluated the A-score. A-score is calculated at specific time-points by adding the A-grades (perivascular mononuclear cell infiltrate graded A0–A4) of all transbronchial biopsies (TBB) performed up to the time-point, and dividing by the number of TBBs. Biopsies which were unable to be evaluated and given a grade of "Ax" are excluded from the calculation [14].

Statistical Analysis

Continuous variables are presented as median and (25th–75th percentile), and were compared using a Mann Whitney non-parametric test. Categorical variables are presented as n (%) and were compared using a Chi-squared test or Fisher's exact test.

Time to death (graft survival) and CLAD onset (freedom from CLAD) were estimated using the Kaplan-Meier method and compared by log-rank test.

Cox univariable regression was used to evaluate the association between clinical or biological factors, and 3-year survival for primary objective and at 5-year survival for secondary objective. The same analyses were performed for CLAD onset. Cox multivariable models were used to assess the association between the age group (≥65 or <65 years) and survival onset or CLAD onset with adjustment for potential confounding factors. Confounding factors with a significance of $p < 0.05$ on univariable analysis were selected for multivariable analyses.

We used an adjusted-repeated-measures mixed-model testing group outcome (donor age groups) for FEV1 and FEV1/FVC ratio changes over time.

Propensity score matching was performed with ratio 2:1 for control group as sensitivity analysis (**Supplementary File S2**).

For all analyses, $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SAS software (version 9.4; SAS Institute, Carry, NC).

RESULTS

Between January 2005 and December 2019, 772 lung transplants were performed at our center. Among them, 392 were (BLTs) for CF. We classified this population by donor age forming two groups. A total of 355 BLTs were performed with donors aged <65 years, and 37 with donors aged ≥65 years. Thirty-six patients were excluded due to a follow-up time under 3 years. Therefore, the analysis included 356 patients (326 with a donor aged <65 years, and 30 with a donor aged ≥65 years) (**Figure 1**).

Characteristics of donors and recipients are reported in **Table 1**. Donors had a median age of 45 (34–54) in <65 years group, and 68 (66–70) in ≥65 years group, $p < 0.001$. The elderly group included fewer smokers ($p < 0.001$), and higher Oto score [10] ($p = 0.001$) despite taking age into account. There was no difference in sex, P/F ratio, tracheal aspiration quality and MV duration before retrieval of the donor lungs.

Recipients of donor lungs ≥65 years of age were significantly older than those receiving donor organs <65 years ($p = 0.026$). CMV mismatch tends to be more significant in ≥65 years than <65 years ($p = 0.096$). There was no difference observed in terms of waiting time on the list ($p = 0.779$), TLC ratio ($p = 0.798$), use of EVLP ($p = 0.459$) or need for high emergency lung transplant allocation ($p = 0.544$).

The elderly group showed higher total ischemia time in minutes than the younger group ($p = 0.029$). There was no difference between immediate post-operative extubation rate, primary graft dysfunction, length of MV duration, ICU or hospital stay. Interestingly, there were more bronchial complications in the younger donor group ($p = 0.009$).

Survival rates according to donor age are reported in **Figure 2**. No significant difference was observed between the two groups during the follow up censored at 3,000 days ($p = 0.924$), at 1 year (<65 years group: 90.2%, 95% CI [86.9–93.4] vs. 93.3% [83.6–102.8], $p = 0.576$), 3 years (<65 years group: 82.5% [78.4–86.7] vs. 83.3% [69.2–97.5], $p = 0.896$) and 5 years (N = 303, <65 years group: 75.1% [70.0–80.2] vs. 72.7% [52.5–92.9], $p = 0.814$).

In univariable analysis, donor age was not associated with survival rate at 3 years (HR = 0.94 [0.38–2.35], $p = 0.896$) and remained nonsignificant after adjustment for confounding factors (adjusted HR = 0.82 [0.13–5.11], $p = 0.836$) (**Table 2**). The same results were observed for survival rate at 5 years (N = 303, univariable HR = 1.11 [0.48–2.54], $p = 0.814$, and adjusted HR = 1.43 [0.48–4.25], $p = 0.517$) (**Supplementary File S1**).

CLAD occurrence is reported in **Figure 3** and did not differ with donor age during the follow up censored at 3,000 days ($p = 0.175$). In univariable analysis, donor age was not associated with CLAD occurrence at 3 years (for group ≥65 years, HR = 0.23 [0.03–1.65], $p = 0.143$) and remained nonsignificant after adjustment for confounding factors (for group ≥65 years,

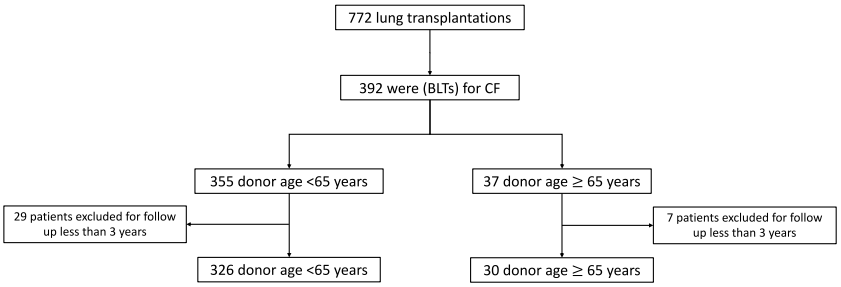


FIGURE 1 | Flow chart.

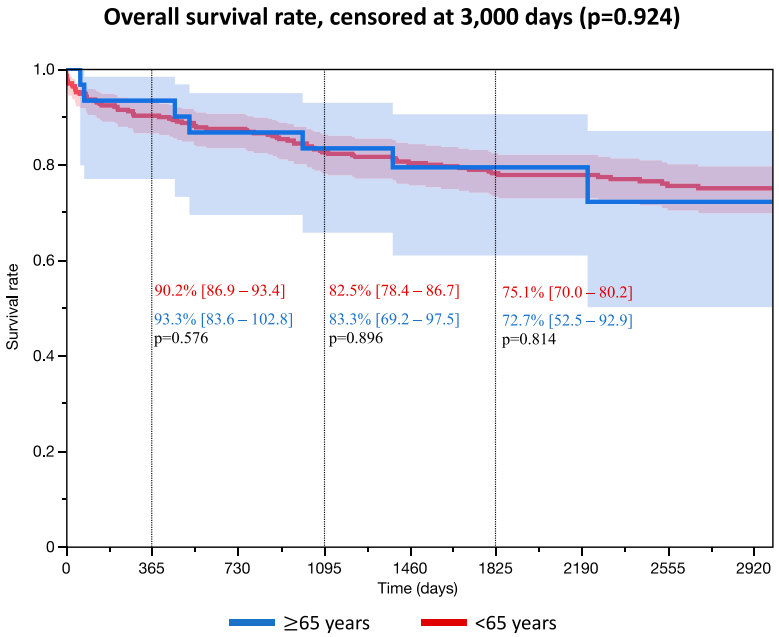
TABLE 1 | Characteristics of the study population.

Variables	<65 years (N = 326)	>65 years (N = 30)	*p*-Value
Donor age in years	45 (34–54)	68 (66–70)	<0.001
Donor sex (female)	130 (39.9)	16 (53.5)	0.155
Mechanical ventilation duration in days	2 (1–3)	1 (1–2)	0.551
Pao_2/Fio_2 at offer	375 (324–455)	385 (325–448)	0.817
Smoking history	137 (42.0)	3 (10.0)	<0.001
Tracheal aspiration quality			0.482
Clean	173 (54.8)	18 (63.7)	
Dirty	124 (39.2)	8 (29.6)	
Bloody	19 (6.0)	1 (3.7)	
Oto score	7 (4–9)	8 (7–10)	0.001
Recipient age in years	28.1 (23.9–33.8)	30.9 (25.7–40.9)	0.026
Recipient sex female	176 (54.0)	20 (66.7)	0.177
HELT	51 (15.6)	6 (20.0)	0.544
Time on waiting list	21 (7–58)	23 (7–57)	0.779
TLC ratio	1.07 (0.89–1.32)	1.09 (0.95–1.26)	0.798
Lobar transplant	27 (8.3)	0 (0.0)	0.026
CMV mismatch d+/r−	89 (27.3)	4 (1.3)	0.096
EVLP	35 (10.7)	2 (6.7)	0.459
Intraoperative ECMO	135 (41.4)	7 (23.3)	0.046
Post-operative ECMO	77 (23.6)	6 (20.0)	0.648
OT extubation	117 (35.9)	11 (36.7)	0.932
Tracheostomy	36 (18.4)	7 (24.1)	0.316
Duration of mechanical ventilation	2 (0–6)	1 (0–14)	0.836
Intensive care stay in days	6 (4–11)	9 (4.5–16.5)	0.161
Total hospital stay in days	28 (22–40)	30 (23.5–43)	0.736
PGD 3 at hours			
H24	81 (25.1)	6 (20.0)	0.277
H48	82 (25.4)	6 (20.0)	0.261
H72	58 (18.0)	5 (16.7)	0.546
Bronchial complications	78 (27.1)	1 (4.8)	0.009
Total ischemia time in minutes (N = 318)	368 (315–426)	400 (362–470)	0.029
Graft neoplasm	4 (1.23)	1 (3.33)	0.348
A score 1 year	0.111 (0–0.286)	0 (0–0.200)	0.095
A score 3 years	0.111 (0–0.250)	0 (0–0.208)	0.150
A score 5 years	0.105 (0–0.250)	0 (0–0.222)	0.149

Continuous data are presented as median (25th–75th percentile) and dichotomous data as n and percentage.

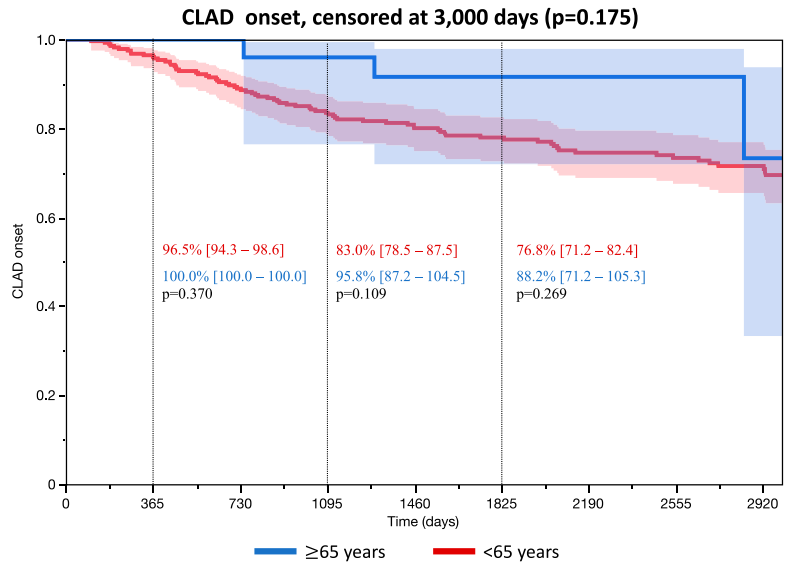
adjusted HR = 0.89 [0.11–7.03], $p = 0.913$) (**Table 3**). The same results were observed for CLAD occurrence at 5 years (N = 303, univariable HR = 0.46 [0.11–1.90], $p = 0.284$, and adjusted HR = 1.27 [0.28–5.82], $p = 0.763$) (**Supplementary File S1**).

The percentage of predicted FEV1 values were calculated to normalize the measured FEV1 and also expressed as a ratio to best post-operative FEV1 in order to assess intra-patient functional evolution. Mixed-model for repeated-measures of FEV1 during follow-up demonstrated no significant interaction between time and donor age group (p for interaction = 0.767 for predicted FEV1, p for interaction 0.344 for ratio to best post-operative FEV1). The same results were observed for obstructive impairment of lung function with FEV1/FVC (p for interaction = 0.369) (**Figure 4**).



Number at risk									
>65 years	326	295	286	270	242	212	187	158	140
<65 years	30	28	26	25	20	27	13	9	7

FIGURE 2 | Kaplan Meier survival estimates *each proportion of survival rate was reported on the number of patients with follow up at the time of 1, 3, and 5 years.



Number at risk									
>65 years	284	272	248	226	200	173	146	126	106
<65 years	26	26	26	25	18	16	10	7	5

FIGURE 3 | Occurrence of CLAD *each proportion of CLAD onset was reported on the number of patients with follow up at the time of 1, 3, and 5 years.

TABLE 2 | Cox univariable and multivariable analyses for survival rate at 3 years.

Variables	Univariable HR (95% CI)	*p*-Value	Adjusted HR (95% CI)	*p*-Value
Donor age ≥65 years	0.94 [0.38–2.35]	0.896	1.14 [0.13–10.19]	0.908
Donor sex (female)	0.99 [0.60–1.65]	0.989		
Mechanical ventilation duration in days	1.00 [0.93–1.05]	0.969		
Pao_2/Fio_2 at offer	1.00 [0.99–1.01]	0.772		
Smoking history	1.20 [0.73–1.99]	0.472		
Tracheal aspiration quality		0.205		
Clean	Ref.			
Dirty	0.88 [0.52–1.48]	0.636		
Bloody	0.24 [0.03–1.78]	0.164		
Oto score	0.96 [0.88–1.05]	0.375		
Recipient age in years	**0.96 [0.92–0.99]**	**0.009**	0.81 [0.69–0.95]	0.017
Recipient sex female	1.15 [0.69–1.90]	0.589		
HELT	**2.02 [1.14–3.56]**	**0.016**	9.75 [1.52–22.15]	0.014
Time on waiting list	1.00 [0.99–1.01]	0.649		
TLC ratio	**2.08 [1.01–3.88]**	**0.049**	0.69 [0.02–18.50]	0.866
Lobar transplant	**2.92 [1.48–5.76]**	**0.002**	1.04 [0.42–4.83]	0.976
CMV mismatch d+/r−	1.14 [0.66–1.97]	0.647		
EVLP	0.57 [0.21–1.56]	0.272		
Intraoperative ECMO	1.56 [0.95–2.56]	0.082		
Post-operative ECMO	**1.76 [1.03–2.99]**	**0.038**	0.02 [0.01–0.61]	0.023
OT extubation	0.82 [0.48–1.40]	0.476		
Tracheostomy	1.80 [0.88–3.71]	0.107		
Duration of mechanical ventilation	**1.01 [1.00–1.02]**	**0.028**	1.01 [1.00–1.02]	0.018
Intensive care stay in days	**1.03 [1.01–1.05]**	**<0.001**	1.07 [0.99–1.17]	0.059
Total hospital stay in days	1.00 [0.99–1.01]	0.051		
PGD 3 at hours				
H24	2.00 [1.19–3.39]	0.010		
H48	1.97 [1.16–3.32]	0.011		
H72	**3.30 [1.95–5.58]**	**<0.001**	17.15 [1.61–35.54]	0.019
Bronchial complications	**2.02 [1.15–3.55]**	**0.014**	5.47 [1.40–21.43]	0.011
A score 1 year	2.76 [0.70–9.58]	0.142		
A score 3 years	**6.17 [1.53–21.96]**	**0.007**	11.11 [0.48–25.74]	0.133
A score 5 years	6.88 [1.66–25.33]	0.005		
Graft neoplasm	—	0.999		
Total ischemia time in minutes	1.00 [0.99–1.01]	0.159		

Bold values represents p<0.05.

Sensitivity Analysis

When applying a propensity score match, allocating a matching ratio of 2:1 for the number of control patients, we found similar results. For survival rate at 3 years, univariable HR = 1.46 [0.46–4.61], p = 0.516, and after adjustment for covariates, adjusted HR = 0.91 [0.26–3.16], p = 0.880. For CLAD onset at 3 years, univariable HR = 0.70 [0.07–6.68], p = 0.746 and after adjustment for covariates an adjusted HR = 0.46 [0.10–2.18], p = 0.428 (**Supplementary File S2**).

DISCUSSION

In our single center retrospective study, a young cohort of 392 BLTs for CF was studied over a 15-year period. Grafts from donors aged 65 years or older accounted for 9.4% of the transplant volume, and resulted in no differences in outcomes compared to grafts from younger donors in our principal analysis at 3 years and in the secondary analysis at 5 years. These encouraging results generally reassure our practice and lead us to continue to accept lung graft offers from donors aged ≥65 years.

Additionally, in our study, we demonstrated no differences between the two groups in deterioration of lung function over time. Regarding susceptibility to cellular rejection, the older graft did not appear to modify the occurrence of events as estimated by A-score. Thus, there does not appear to be a difference in terms of the occurrence of chronic lung allograft dysfunction (CLAD). Another interesting point is that there is no difference in cancer occurrence in the graft, although the even rate is too low to provide sufficient statistical power in this analysis. This could be assessed in the future with a longer follow-up time.

The Historical Point of View

Various experiences have been reported in the literature [15–17]. On one hand, in a 2007 retrospective study, De Perrot et al showed that the use of donors of >60 years of age was associated with lower 10-year survival [15]. These results were supported by Baldwin et al who reported their experience in 2015 [16]. On the other hand, in 2015, Sommer et al reported encouraging results with their retrospective study of donors aged >70 years in a cohort of COPD and restrictive patients. Interestingly, they found no survival difference but observed poorer lung

TABLE 3 | Cox univariable and multivariate analyses for CLAD onset at 3 years.

Variables	Univariable HR (95% CI)	*p*-Value	Adjusted HR (95% CI)	*p*-Value
Donor age ≥65 years	0.23 [0.03–1.65]	0.143	0.89 [0.11–7.03]	0.913
Donor sex (female)	0.96 [0.54–1.72]	0.891		
Mechanical ventilation duration in days	1.02 [0.96–1.07]	0.424		
Pao_2/Fio_2 at offer	1.00 [0.99–1.01]	0.538		
Smoking history	**1.94 [1.09–3.44]**	**0.024**	1.57 [0.63–3.96]	0.329
Tracheal aspiration quality		0.778		
Clean	Ref.			
Dirty	0.88 [0.47–1.68]	0.711		
Bloody	1.31 [0.47–3.77]	0.614		
Oto score	0.95 [0.86–1.05]	0.319		
Recipient age in years	**0.89 [0.84–0.94]**	**<0.001**	0.86 [0.69–0.93]	<0.001
Recipient sex female	0.62 [0.35–1.10]	0.103		
HELT	0.68 [0.27–1.71]	0.411		
Time on waiting list	1.00 [0.99–1.01]	0.572		
TLC ratio	0.91 [0.30–1.09]	0.859		
Lobar transplant	0.72 [0.17–2.97]	0.649		
CMV mismatch d+/r−	**2.31 [1.29–4.12]**	**0.005**	1.93 [0.78–4.77]	0.153
EVLP	0.66 [0.21–2.13]	0.487		
Intraoperative ECMO	1.22 [0.68–2.19]	0.491		
Post-operative ECMO	1.27 [0.59–2.72]	0.536		
OT extubation	1.02 [0.56–1.84]	0.935		
Tracheostomy	0.40 [0.10–1.79]	0.213		
Duration of mechanical ventilation	1.00 [0.99–1.01]	0.379		
Intensive care stay in days	1.00 [0.99–1.01]	0.803		
Total hospital stay in days	**1.01 [1.00–1.02]**	**0.045**	1.01 [1.00–1.03]	0.045
PGD 3 at hours				
H24	1.07 [0.54–2.10]	0.841		
H48	1.06 [0.53–2.14]	0.866		
H72	1.08 [0.48–2.42]	0.843		
Bronchial complications	**2.14 [1.13–4.05]**	**0.020**	2.36 [0.96–5.81]	0.061
A score 1 year	**3.92 [0.97–14.09]**	**0.055**		
A score 3 years	**12.29 [3.02–24.87]**	**<0.001**	2.39 [0.25–16.97]	0.411
A score 5 years	**14.83 [3.45–37.85]**	**<0.001**		
Graft neoplasm	1.22 [0.17–8.85]	0.844		
Total ischemia time in minutes	1.00 [0.99–1.01]	0.509		

Bold values represents p<0.05.

function in restrictive recipients transplanted with older grafts [17]. Similarly, Hecker et al showed no survival differences with grafts over 65 years old [18].

Regarding these discrepancies, in a recent publication, Renard et al recommended caution with the use of elderly grafts, and preferential matching with elderly recipients [19].

Interestingly, one paper in the literature by Auråen et al [20] seems to have directly focused on the CF patient population but presents different conclusions, demonstrating a lower overall survival for donors over 55 years of age. These results were multicenteric from 5 Scandinavian centers, with a CF subgroup representing a sample size of 165 patients, which is smaller than that in our monocentric cohort.

Cystic Fibrosis in France

The constitution of a donor/recipient pair calls for multiple compromises, the parameters of which are adjusted according to the severity of the recipient's clinical condition. It goes without saying that in an emergency situation, such problems would not arise because the right graft is the one which is available to save the patient's life.

In France the high emergency lung transplantation (HELT) system gives urgent patients priority access to optimal grafts [21]. With this pool being limited, patients on standard lists are therefore sometimes offered marginal grafts, and the choice comes down to a trade-off between the different parameters. It is in this context that our team used grafts> 65 years of age in this cohort of young patients.

Since January 2020, the problem has changed, as BLTs have become increasingly rare in this patient group thanks to the marketing of new CF therapeutics [22].

Physiological Data Regarding Aging Lungs

Although data on lung aging are limited, it is generally accepted that FEV1 decreases with age. This is due to changes in lung tissues, which result in larger alveoli without damage to their walls. This reduces alveolar surface tension and causes a decrease in the lungs' elastic recoil, leading to a reduced maximum achievable flow during breathing.

Additionally, muscle performance and chest wall elasticity both decrease with age, resulting in an increased residual volume

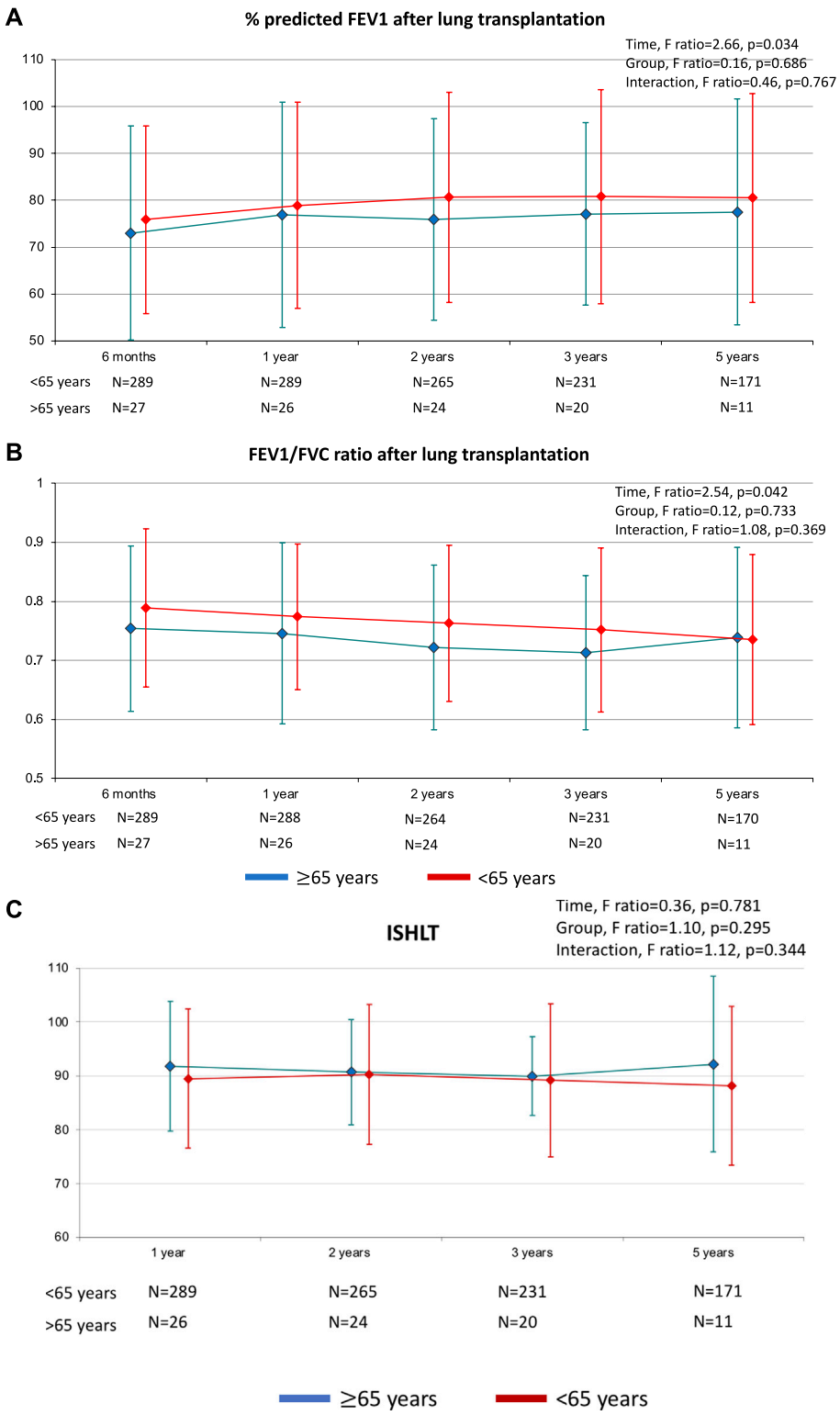


FIGURE 4 | Post-operative spirometry results. **(A)** The percentage predicted forced expiratory volume in 1 s (FEV1), defined as measured FEV1 expressed as a percentage of the predicted FEV1. **(B)** FEV1/FVC: FEV1 measured/forced vital capacity. **(C)** ISHLT: FEV1 measured/best post operative FEV1.

that counteracts any potential increase in total lung capacity (TLC) from reduced elastic recoil [23].

The consequences of the biological aspects of lung aging, such as telomere shortening, have yet to be fully understood.

Tailored Graft Selection

The determinants of lung transplant survival are numerous. It is likely that the choice of donor is important from an immunological, viral (CMV), and size matching point of view. When possible, we tend to customize the choice of "the best" graft. However, taken alone, there is no certainty about the relevance of the age criterion when it comes to survival.

In our study, we found out that the older grafts were significantly better size-matched because no lobar transplant was performed in this group, there was less CMV mismatch, and preoperative plasmapheresis.

In this context of a tailored choice, it also seems interesting to consider the indication, as seen in the Sommer study, which demonstrated poorer functional results with elderly donors in the group of IPF patients in comparison with the COPD group. In our case, CF patients are examined, and they do not appear to have more functional impairment with elderly donors. Could the loss of elasticity of the lung tissue of older donors, advanced by Miller et al [23], be an explanation for the functional results of IPF patients.

Limitations

This is a retrospective and monocentric study over an extended period during which transplant management practices have evolved. The use of older donors is more frequent in the most recent period and therefore this group has a shorter follow-up-period.

Furthermore, it is interesting to note that some poor prognostic factors at the time of organ selection, such as CMV mismatch or donor smoking history, although statistically non-significant, were more frequent in the group of younger donors. This suggests that there may be a likely allocation bias. This could be explained by a desire at the time of selection to avoid combining multiple risk factors.

A limitation of our study is that we do not present data on the presence of DSA and humoral rejection for which there were missing data for some of the cohort. Our management strategy has evolved over time and has been previously published [24].

From a statistical point of view, the groups are strongly imbalanced in terms of numbers.

Strenghts of This Study

We present a homogenous cohort of young patients transplanted for CF. Despite decreasing numbers of LT in CF thanks to the development of new treatments, we keep updating our database rigorously and as a next step, a 10-year survival could be explored.

Conclusion

Donor age alone should not be a reason to refuse a lung graft offer even in young recipients. While immediate and intermediate results do not show any significant statistical differences, long term results still need to be identified.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics review board of Foch Hospital (IRB 00012437), number of approval: 20-10-07 (10/29/2021). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors designed the clinical protocol. AR and MSt collected the data. MG, MSa, and AV performed the data analysis, and wrote the initial draft and the final manuscript. MG, JF, CPr, JD, CPi, OB, SD, DG, GT, CC, FP, ML, and AC participated in clinical care. MG, MSa, AV, and ES revising the final manuscript. NB collected data and English proofread the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2023.11180/full#supplementary-material

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LETTER TO THE EDITOR
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Acute and Severe Hypercalcemia Early After Kidney Transplantation in a Patient Previously Treated With Etelcalcetide

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Keywords: kidney transplant, calcium-sensing receptor (CASR), hyperparathyroidism, kidney transplant clinics, parathyroid

Dear Editors,

Secondary hyperparathyroidism (SHPT) is frequent in patients with chronic kidney disease, especially in those on chronic haemodialysis (HD). Etelcalcetide, an intravenously-administered direct CaSR-agonist, is widely used worldwide for SHPT treatment. Yet, little has been described so far regarding its potential post-kidney transplant (KT) impact. We previously reported acute and severe hypercalcemia in the early post-transplant course in two patients previously treated with high-dose etelcalcetide [1]. We here report another case.

A 68-year-old Caucasian male received a deceased-donor KT for kidney failure of unknown origin. He has been on HD for 4 years and treated with vitamin D analogue and etelcalcetide (15 mg/dialysis session, last dose the day before KT) for 2 years for SHPT. Pre-transplant serum calcium and iPTH values-measured the day before transplantation-were 2.30 mmol/L (2.15–2.50 mmol/L) and 50.9 pmol/L (1.6–8.5 pmol/L), respectively.

The first week post-KT was uncomplicated. Kidney function rapidly improved and calcemia remained within the normal range. On day 8, the patient presented tonic-clonic seizures associated with severe hypertension. Brain MRI was suggestive for PRES-syndrome. Laboratory tests revealed severe hypercalcemia (total serum calcium 3.25 mmol/L, contrasting with a normal value 3 days before), hypophosphatemia (0.74 mmol/L, [0.81–1.45 mmol/L]), and elevated iPTH level at 65.6 pmol/L (**Figure 1**). Tacrolimus trough level at 30 ng/mL while two previous dosages (on day 3 and day 5) were into targets (10–14 ng/mL) with an unchanged dose at 25 mg/day. Hematologic and auto-immune tests were normal and pre-KT radiologic findings showed no bone lesion.

Antiepileptic drug (levetiracetam 2.000 mg/day), cinacalcet (120 mg/day), anti-hypertensive treatment, intravenous hydration and tacrolimus posology reduction were initiated. Cervical MRI showed two parathyroid hyperplasia's foci.

Two weeks later, the neurological status of the patient improved. Kidney function continued to improve with plasma creatinine values around 106 µmol/L (53–115 µmol/L). Yet, calcemia remained constantly >2.90 mmol/L despite cinacalcet (that was poorly tolerated, causing nausea and vomiting). Therefore, we performed on day 24 a subtotal parathyroidectomy by resecting *in toto* the two parathyroid hyperplastic foci and the left superior parathyroid gland, together with a partial resection of the right inferior one. Pathological examination confirmed the diagnosis of tertiary hyperparathyroidism.

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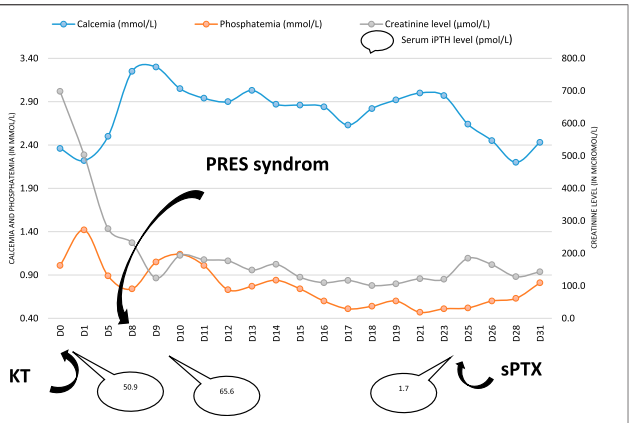

FIGURE 1 | Calcemia, phosphatemia, creatininemia and iPTH serum level evolution. Abbreviations: KT: kidney transplantation; sPTX: subtotal parathyroidectomy.

After surgery, calcium, phosphate and iPTH values returned into the normal range (**Figure 1**) and clinical symptoms resolved rapidly. A brain MRI was repeated on day 20 and was normal. The patient was discharged on day 36.

Overall, we report another case of severe and acute hypercalcemia occurring early after KT, most likely related to SHPT flare-up secondary to etelcalcetide interruption, that prompted early parathyroid surgery. In our knowledge, such severe clinical presentation has not been reported before the etelcalcetide era, even in patients treated with cinacalcet. Indeed, although pre-transplant cinacalcet treatment has been shown to potentially induce hyperparathyroidism rebound, nephrocalcinosis and secondary hypercalcemia developing usually months after KT [2, 3], hypercalcemia usually does not exceed 2.9 mmol/L and rarely requires any acute treatment-contrasting with the clinical presentation of the present case and those previously published [1].

Also the causal relationship between acute hypercalcemia and PRES-syndrome cannot be definitively proven here-as the patient presented with concurrent severe hypertension and tacrolimus overdose-it might have participated in this severe manifestation [4, 5].

In conclusion, patients treated with high-dose of etelcalcetide require close monitoring of calcium levels after transplantation. Larger studies are required to confirm our observation and assess the causal relationship between etelcalcetide and severe post KT hypercalcemia.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The patient signed informed consent for this publication.

AUTHOR CONTRIBUTIONS

MF and AD: writing of the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

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

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